MECHANISMS UNDERLYING CHANGES IN MICROVASCULAR BLOOD FLOW IN A DIABETIC RAT MODEL: RELEVANCE TO TISSUE REPAIR

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Submitted in total fulfilment of the requirements of the degree of Doctor of Philosophy

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DECLARATION

This is to certify that:

(i) The thesis comprises only my original work, completed while I was a postgraduate student in the faculty of Medicine at the University of Melbourne. This thesis contains no material that has been accepted for the award of any other degree or diploma at any other university or institution.

(ii) To the best of my knowledge and belief, the thesis contains no material previously written or published by another person except where due reference is made in the text.

(iii) This thesis is less than 10,000 words in length, exclusive of tables, figures, references and appendices.

Signed:

Maryam Bassirat
ACKNOWLEDGMENTS

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**SUMMARY**

Diabetes mellitus is a chronic syndrome affecting carbohydrate, protein, and fat metabolism. It is characterized primarily by relative or absolute insufficiency of insulin secretion (type I diabetes or IDDM) or concomitant insensitivity / resistance to the metabolic action of insulin on target tissues (Type II diabetes or NIDDM), both resulting in hyperglycaemia. Diabetes mellitus is known to induce microvascular changes and alterations to neuronal functions.

The neurovascular system comprising of unmyelinated primary afferent sensory neurones and the microvasculature innervated by these nerves play a major role in modulating inflammatory and tissue repair processes. Sensory nerve terminals respond to injury via the release of sensory neuropeptides which mediate inflammation and tissue repair. These processes are known to be altered in diabetes.

This thesis is concerned with the role of diabetes in modulating microvascular blood flow directly and indirectly via modulating sensory nerve activity and the effect of these changes on repair processes in skin of 4 weeks streptozotocin (STZ)-induced diabetic rats. The following hypotheses were examined:

1. That factors implicated in long-term diabetic vascular damage play a role in altering skin microvascular function in early diabetes.
2. That preventing the deleterious effects of these factors could improve skin microvascular blood flow and skin repair processes in early diabetes.

Specifically, the thesis examined the involvement of a number of factors including hyperglycaemia, endothelins (ETs), free radicals, and advanced glycated end-products (AGEs) in modulating skin microvascular blood flow in early diabetes. It was shown that preventing the deleterious effects of these factors, using specific inhibitors / antagonists, could improve microvascular blood flow and consequently modulate tissue repair processes in early diabetes.
Chapter 1 reviews issues related to diabetes mellitus, neurogenic inflammation, and wound repair. The function of sensory neuropeptides and their interaction with other agents (e.g. free radicals, ETs, AGEs) during inflammation is discussed in detail. The literature review reveals that investigations of early changes in microvascular blood flow in the skin is lacking and that this area needs further attention as it impacts on possible future early intervention approaches to limit long-term diabetic vascular complications.

Chapter 2 describes the general methodology including induction of diabetes with STZ (in Sprague-Dawley rats) as well as methods used to induce an inflammatory response and to measure two specific parameters of this response, namely plasma extravasation and vasodilatation (using laser Doppler flowmetry). Procedures to electrically stimulate C-fibres and to induce a full thickness skin wound are also described.

In chapter 3, the effect of diabetes on sensory nerve function, as determined by the ability of these nerves to mount an inflammatory response, was examined. One of the aims was to examine changes in inflammatory responses with time. Sensory-independent effects of diabetes on the microvasculature were examined in sensory-denervated (capsaicin-treated) rats. In addition, the hyperglycaemic effect of diabetes, independent of possible neurotoxic effects of STZ, was examined in alloxan-induced diabetic rats. The first set of results demonstrated that there is an overall reduction in the neurogenic inflammatory responses (vasodilatation and plasma extravasation) over 10 weeks of diabetes and this reduction reached a maximum 4 weeks after induction of diabetes. Using two different laser Doppler flowmeter machines, with different laser light penetration capabilities, it was shown that capillary blood flow at both superficial and deeper compartments of the skin is reduced after 4 weeks of diabetes. In addition, it was also demonstrated that post-terminal changes in microvascular function are more apparent at this early stage of diabetes. Overall, the results supported the notion that there are disturbances in endothelial cells and smooth muscle cell function in skin microvasculature in early diabetes.
In chapter 4, the role of parasympathetic nerves and local tissue factors in modulation of microvascular blood flow in early diabetes were examined. Results showed a significant reduction in microvascular responses to acetylcholine (ACh), muscarine chloride, nicotine, and bradykinin (BK) in early diabetes. Reduction in the above responses could be attributed to a number of factors that were further explored in chapters 6, 7, and 8.

In chapter 5, guanethidine-treated diabetic rats were used to examine the role of the sympathetic nerves in the modulation of microvascular blood flow in early diabetes. Results demonstrated that the contribution of sympathetic efferents in regulating microvascular blood flow is increased in early diabetes.

Chapters 6, 7, and 8 examined possible role of ETs, free radicals, and AGEs, in modulating microvascular blood flow in diabetes. This was investigated using specific inhibitors and receptor antagonists. The evidence in chapter 6 showed that ETs are involved in altering microvascular blood flow and endothelial cell permeability in 4 weeks diabetic rats. It was also shown that local pharmacological manipulation at the level of the skin microcirculation, using ET receptor antagonists (e.g. BQ-123 & BQ-788), could normalize local diabetic vascular responses with subtle differences in the relative contribution of these two receptor subtypes. Results in chapter 7 demonstrated that free radicals play a role in modulating diabetic microvascular blood flow at this early stage. In addition, evidence was provided to suggest that the inhibitory effects of ETs and free radicals on microvascular blood flow were additive. Results from chapters 6 and 7 collectively showed that ETs play a greater role than free radicals in altering endothelial cell permeability. Using different free radical scavengers, there were subtle differences in the relative contribution of different free radicals in altering microvascular blood flow. The results again showed that local pharmacological manipulation at the level of peripheral skin microvasculature could normalize diabetic vascular responses.
In chapter 8, evidence was provided that short-term exposure of control microvasculature to early (e.g. amadori) and late (AGEs) glycation products could modulate microvascular function in these rats. Furthermore, amadori products exerted a positive enhancing effect on microvascular function in early diabetes. It was proposed that in diabetic rats, amadori products could provide a respite for the microvasculature and hence, improve the inflammatory responses. When anti-RAGE IgG was used, results showed that AGE receptors are involved in inhibiting the inflammatory responses in diabetic rats. Furthermore, evidence was provided to suggest that short-term aminoguanidine (AG) treatment could improve the inflammatory responses in diabetic rats and that the duration of treatment could play an important role with reference to the extent of preventing neurovascular damage.

Chapter 9 describes the role of insulin treatment in the modulation of skin blood flow in early diabetes. Results in this chapter showed that early onset insulin treatment could have an important role in preventing early skin microvascular alterations by diabetes. This raised the possibility that adequate control of hyperglycaemia at the early stages of diabetes may reduce the long-term impact on microvascular and tissue repair processes.

The experiment in chapter 10 was devised to utilize the information gained from previous chapters in order to examine the impact of preventing the deleterious effects of a number of factors namely ETs, free radicals, AGEs and hyperglycaemia on modulating tissue repair of full thickness wounds in early diabetes. The results showed that all treatments used have contributed, to varying degrees, to different stages of wound repair and to the overall improvement in repair processes in diabetic rats. Furthermore, Pharmacological manipulations at the level of microvasculature (e.g. local injection of BQ-123 and superoxide dismutase (SOD)) and systemic treatments (e.g. insulin, AG, Tirilazad mesylate) were shown to improve repair processes in early diabetes.

Chapter 11 summarizes the overall findings of this thesis.

The thesis concludes that a number of factors that are known to cause long-term vascular complications, also contribute to early changes in skin microvascular blood flow in
diabetes. The treatment protocols used in this thesis (chapters 6, 7, 8, and 9) showed that the action of these factors is reversible at the early stages of diabetes. These same treatments were also effective in improving the repair processes in these rats. This work raised the exciting possibility that early intervention could prevent microvascular alterations and argues that further research is needed to determine the impact of early intervention on decelerating late diabetic complications.
## GLOSSARY OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Acetylcholine</td>
<td>ACh</td>
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<tr>
<td>Adenine diphosphate</td>
<td>ADP</td>
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<tr>
<td>Adenine triphosphate</td>
<td>ATP</td>
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<tr>
<td>Advanced glycated end-products</td>
<td>AGEs</td>
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<tr>
<td>American diabetes association</td>
<td>ADA</td>
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<tr>
<td>Aminoguanidine hydrochloride</td>
<td>AG</td>
</tr>
<tr>
<td>Analysis of variance</td>
<td>ANOVA</td>
</tr>
<tr>
<td>Angiotensin-converting enzyme</td>
<td>ACE</td>
</tr>
<tr>
<td>Antibody against RAGE</td>
<td>Anti-RAGE IgG</td>
</tr>
<tr>
<td>Arachidonic acid</td>
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<tr>
<td>L-arginine</td>
<td>L-Arg</td>
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<td>Arteriovenous anastomoses</td>
<td>AVA</td>
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<tr>
<td>Basal blood flow</td>
<td>BBF</td>
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<tr>
<td>Basic fibroblast growth factor</td>
<td>bFGF</td>
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<tr>
<td>Bisindolylmaleimide</td>
<td>BIM</td>
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<tr>
<td>Bovine serum albumin</td>
<td>BSA</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>BK</td>
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<tr>
<td>Calcitonin gene-related peptide</td>
<td>CGRP</td>
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<td>Central nervous system</td>
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<tr>
<td>Coleystokinin</td>
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<td>Colony stimulating factor</td>
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<td>Cyclic adenosine monophosphate</td>
<td>cAMP</td>
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<tr>
<td>Cyclic Guanosine monophosphate</td>
<td>cGMP</td>
</tr>
<tr>
<td>Cyclo-oxygenase</td>
<td>COX</td>
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<tr>
<td>Diabetes control and complications trial</td>
<td>DCCT</td>
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<tr>
<td>Diabetes mellitus</td>
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<td>Diacylglycerol</td>
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<td>Deoxyribonucleic acid</td>
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<td>Dorsal root ganglia</td>
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<td>Electrical stimulation</td>
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<td>Endothelial NOS</td>
<td>eNOS</td>
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<tr>
<td>Endothelin</td>
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<tr>
<td>Endothelin-1</td>
<td>ET-1</td>
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<tr>
<td>Endothelium-derived hyperpolarizing factor</td>
<td>EDHF</td>
</tr>
<tr>
<td>Endothelium-derived relaxing factor</td>
<td>EDRF</td>
</tr>
<tr>
<td>Endothelium-derived constrictor factor</td>
<td>EDCF</td>
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<td>Epidermal growth factor</td>
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<tr>
<td>Fasting plasma glucose</td>
<td>FPG</td>
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<tr>
<td>Fibroblast growth factor</td>
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<tr>
<td>Glomerular filtration rate</td>
<td>GFR</td>
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<tr>
<td>Glucose transporter</td>
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<tr>
<td>Glycosaminoglycan</td>
<td>GAG</td>
</tr>
<tr>
<td>Guanosine monophosphate</td>
<td>GMP</td>
</tr>
<tr>
<td>Guanosine triphosphate</td>
<td>GTP</td>
</tr>
<tr>
<td>Hertz</td>
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</tr>
<tr>
<td>Human leucocyte antigen</td>
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</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>H$_2$O$_2$</td>
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<tr>
<td>5-Hydroxytryptamine</td>
<td>5-HT</td>
</tr>
<tr>
<td>Inducible NOS</td>
<td>iNOS</td>
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International association for the study of pain (IASP)
Inositol triphosphate (IP₃)
Insulin-dependent diabetes mellitus (IDDM)
Insulin-like growth factor (IGF)
Interleukin (IL)
Intradermal (i.d.)
Intraperitoneal (i.p.)
Intravenous (i.v.)
Kilo pascal (kPa)
Laser Doppler flowmeter (LDF)
Low density lipoprotein (LDL)
Malonyldialdehyde (MDA)
MAP kinase (MAPK)
Messenger ribonucleic acid (mRNA)
Moor laser Doppler flowmeter (MLDF)
N-acetylcysteine (NAC)
Nerve growth factor (NGF)
Neurogenic inflammation (NI)
Neurokinin A (NKA)
Neurokinin-1 (NK-1)
Neuronal NOS (nNOS)
Nicotinamide adenine dinucleotide (NAD)
Nicotinamide adenine dinucleotide hydrogen (NADH)
Nicotinamide adenine dinucleotide phosphate (NADP)
Nicotinamide adenine dinucleotide hydrogen phosphate (NADPH)
Nitric oxide (NO)
Nitric oxide synthase (NOS)
N⁶-nitro-L-arginine methyl ester (L-NAME)
Non insulin-dependent diabetes mellitus (NIDDM)
Noradrenaline (NA)
Neuropeptide Y (NPY)
Oral glucose tolerance test (OGGT)
Oxidised LDL (oxLDL)
Perimed laser Doppler flowmeter (PLDF)
Peripheral vascular disease (PVD)
N-Phenacyl thiazolium bromide (PTB)
Phosphoinositol (PI)
Phospholipase A₂ (PLA₂)
Phospholipase C (PLC)
Phospholipase D (PLD)
Platelet-derived angiogenesis factor (PDAF)
Platelet-derived growth factor (PDGF)
Polymorphonuclear Leucoyte (PMN)
KCl
Prostacyclin (PGI₂)
Protein kinase C (PKC)
Reactive oxygen species (ROS)
Receptor for advanced glycated end-products (RAGE)
Sodium nitroprusside (SNP)
Soluble RAGE (sRAGE)
<table>
<thead>
<tr>
<th>Term</th>
<th>Abbreviation</th>
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<tr>
<td>Standard error of means</td>
<td>SEM</td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>s.c.</td>
</tr>
<tr>
<td>Substance P</td>
<td>SP</td>
</tr>
<tr>
<td>Substance P-like immunoreactivity</td>
<td>SPLI</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>SOD</td>
</tr>
<tr>
<td>Streptozotocin</td>
<td>STZ</td>
</tr>
<tr>
<td>Thiobarbituric acid</td>
<td>TBAR</td>
</tr>
<tr>
<td>Transforming growth factor</td>
<td>TGF</td>
</tr>
<tr>
<td>Tumour necrosis factor</td>
<td>TNF</td>
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<tr>
<td>Vascular endothelial growth factor</td>
<td>VEGF</td>
</tr>
<tr>
<td>Vasoactive intestinal contractor endothelin</td>
<td>VIC</td>
</tr>
<tr>
<td>Vasoactive intestinal peptide</td>
<td>VIP</td>
</tr>
<tr>
<td>Vasodilatation</td>
<td>VD</td>
</tr>
<tr>
<td>Volts</td>
<td>V</td>
</tr>
<tr>
<td>World health organization</td>
<td>WHO</td>
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Bassirat M and Khalil Z. The role of endothelin and superoxide anions in modulating inflammation in streptozotocin-induced diabetic rat model.

*Proceeding of the Australian Neuroscience Society (ANS)* 9:144 (Canberra, Jan 1998)


*The Australasian Society for Immunology (ASI): Society for Leukocytes and Inflammation Research (SLIR)* (Melbourne, Dec 1998)


*The Science of Ageing* 1:45 (Melbourne, Nov 1999)


*The Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists (ASCEPT)* (Sydney, Dec 1999)


*Proceeding of the Australian Neuroscience Society (ANS)* (Melbourne, Jan 2000)
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CHAPTER 1

Literature Review
1.1. Diabetes Mellitus: Historical prospective

Diabetes mellitus (DM) is a disease that was recognized in antiquity. The term “diabetes mellitus” was first used in the 18th century to distinguish the sweet taste of diabetic urine from other polyuric states in which the urine was tasteless. The first major breakthrough in the history of this disease occurred in 1921 when Banting and Best discovered insulin. They showed that insulin reduced the blood glucose level in diabetics and significantly improved patient’s lives. Since then, major advances in understanding diabetes have included: the characterization of the insulin molecule (1955) and elucidating its structure (1969), the measurement of insulin concentration by radioimmunoassay (RIA) (1959), the isolation of pro-insulin (1967), the identification of the insulin receptor (1971), and the sequencing of the insulin receptor gene (1985). Milestones in the management of diabetes have included: the development of long-acting insulin (Isophane) (1936), the first therapeutic use of phenformin (1957), the introduction of test strips for measuring blood glucose, and definitive proof from Diabetes Control and Complications Trial (DCCT) data (1993) (DCCT, 1993) that strict glycaemic control could slow or prevent the development of diabetic microvascular complications (Pickup and Williams, 1997). Diabetes associations, aiming to help diabetics and promote research into it, now exist in many countries.

1.2. Characterization and diagnosis

DM is a syndrome with metabolic, vascular, and neuropathic components that are all interrelated. The metabolic syndrome is characterised by alterations in carbohydrate, fat, and protein metabolism. These are secondary to absent or markedly diminished insulin secretion and / or ineffective insulin action.

The classic symptoms of this disease are excessive thirst (polydipsia), tiredness, excess passing of urine (polyuria), presence of glucose in urine (glucosuria), large food intake (polyphagia), weight loss, and presence of ketone in urine (ketonuria). Early symptoms of some individuals may also include itchy rashes, blurred vision, and pins and needles in hands and feet. These individuals will show high glucose levels in their blood stream (hyperglycaemia) which can be easily measured.
Recently it was shown that the oral glucose tolerance test (OGTT) is not reproducible, inconvenient, costly, and not often used clinically to diagnose diabetes. The World Health Organization (WHO) and the American Diabetes Association (ADA) have therefore, adopted the fasting plasma glucose test (FPG) as a preferred method to make diagnosis and to judge therapy. Individuals with an FPG of 3.5-5.5 mmol/lit (around 60-110mg/dl) are considered as healthy but anything greater than 7mmol/lit (126mg/dl) will be considered as diabetic (Davidson, 1998).

1.3. **Aetiology**

Recent understanding of the pathogenesis and aetiology of the various categories of diabetes have led to a new classification of this disease by the ADA and WHO (Davidson, 1998). According to this classification there are 2 major types of diabetes:

- Type I (previously known as juvenile or insulin-dependent DM) (IDDM)
- Type II (previously known as adult-onset or non-insulin dependent DM) (NIDDM)
  - a) Non-obese b) Obese

Diabetes may also occur as a result of malnutrition. Gestational diabetes only appears during pregnancy in women with no history of type I or type II diabetes and disappears after childbirth.

The aetiology of other specific types of diabetes is listed below:

- Pancreatic disease
- Endocrine disease
- Genetic defects of β-cell function
- Abnormalities of insulin or its receptor
- Drug or chemical induction
- Infections and uncommon immune defects
- Genetic syndromes

1.3.1. **Type I diabetes mellitus**

This accounts for only 10-15% of all cases of diabetes. Result from a study of 27 countries showed that the incidence of type I diabetes is on the rise by around 3% per year (Onkamo et al., 1999). Type I diabetes can be subdivided into a) Autoimmune and b) Idiopathic form. The autoimmune form is by far the most common and results from a
cell-mediated autoimmune destruction of the pancreatic beta cells. The peak incidence occurs in children and adolescence but individuals up to 30 years of age can develop type I diabetes. The rate of cell destruction is variable but is more rapid in children than in adults. The autoimmune destruction of beta cells has a genetic predisposition, which can be identified by human leucocyte antigen (HLA). Epidemiological studies provide strong evidence that the emergence of type I diabetes is largely determined by the effect of adverse environmental factors (Pickup and Williams, 1997) (Table 1-1).

A small proportion of patients (e.g. African Americans) with type I diabetes will fall into the idiopathic form (Umpierrez et al., 1995). This form of diabetes is strongly inherited, caused by decreased insulin secretion and lacks immunological evidence for autoimmunity.

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Table 1-1: Epidemiologically identified detriments of type I diabetes (Adopted from Pickup, J.C. & Williams, G. (1997))

1.3.2. **Type II diabetes mellitus**

Individuals with this type of diabetes have insulin resistance in combination with a relative deficiency of insulin secretion. Its aetiology is not known and frequently these patients are undiagnosed for many years because the elevated glucose concentrations are not high enough to elicit the classic symptoms of uncontrolled diabetes. Since the gap between the onset of hyperglycaemia and the diagnosis is large (9-12 years), these patients are at risk of developing microvascular complications and neuropathy during this period.

Around 80% to 90% of patients with this form of diabetes are obese, which itself adds to insulin resistance and, therefore, weight loss is a vital part of their treatment. Age, life-
style, race, family history, and genetic susceptibility are also risk factors. The insulin secretion is defective in these patients and the presence of insulin resistance poses an additional problem. Although with weight loss the insulin resistance will reduce in these patients, the genetic susceptibility component will remain. Ketoacidosis rarely occurs in these patients since there is some effective insulin action but it can occur with stress and illness (Pickup and Williams, 1997).

1.4. **Long-term complications**

Long-term complications of DM occur in both forms of the condition in humans and have an important role in the increased morbidity and mortality suffered by these individuals. Macrovascular complications include coronary heart disease, atherosclerosis, and peripheral vascular disease. Microvascular complications include retinopathy, nephropathy, and neuropathy (Pickup and Williams, 1997). Diabetic neuropathy is the most common complication of this disease and its manifestations can be divided into two broad categories, somatic (peripheral) and visceral. Disease of the large and small vessels results in myocardial infarction, stroke, and gangrene of the lower extremities.

1.4.1. **Macrovascular disease**

Diabetes is one of the six independent risk factors for developing macrovascular disease in the general population. The presence of diabetes, in addition to any or all the other 5 risk factors, approximately doubles the chance of developing macrovascular disease and the available evidence suggests that strict diabetic control does not prevent or delay these complications. The other 5 potential factors are smoking, hypertension, hyperlipidaemia, obesity, and genetic factors (Davidson, 1998).

Macrovascular complications or disease of large vessels is due to accelerated atherosclerosis and includes ischaemic heart disease, cerebrovascular disease and peripheral vascular disease (Cooper et al., 1997; McMillan, 1997). Hence, diabetic people are at a high risk of suffering from myocardial infarction, coronary heart failure, stroke, and ischaemic gangrene (Stehouwer et al., 1997).
Mechanisms by which diabetes causes acceleration of atherosclerosis are not clear. However, a previous study showed that this acceleration does not involve glucose metabolism and will remain unaffected even if near euglycaemia is achieved (Davidson, 1998). Results from advances in molecular genetics and oxidation chemistry have shown that genetic variants of lipoprotein lipase and apolipoprotein are suitable candidates for mediating diabetic vascular risk (Sememkovich and Heinecke, 1997; Mullarkey et al., 1990). For example, variants of these proteins can produce hypertriglyceridaemia. Lipoproteins retained in the arterial wall are also subject to oxidative modifications (Mullarkey et al., 1990), which could be dependent on glycooxidation, the enzyme myeloperoxides, or the reactive nitrogen species derived from nitric oxide (NO). Accelerated vascular disease in diabetes is therefore likely to be the result of complex interactions between metabolic derangement such as hyperglycaemia, mutations in genes controlling lipid metabolism, and antioxidant defence mechanisms (Sememkovich and Heinecke, 1997). It was shown that apart from hypertriglyceridaemia, other factors such as hyperinsulinaemia, increased platelet aggregability, and increased clotting activity of thrombogenic factors can potentiate atherosclerosis (McMillan, 1997).

The increased incidence of congestive heart failure and the increased mortality and morbidity in diabetics following myocardial infarction or coronary artery bypass graft can be explained by the presence of diabetic cardiomyopathy. This is characterized by an early diastolic dysfunction and a later systolic one, with intracellular retention of calcium and sodium ions and loss of potassium (Macgoub and Abdel-Fattah, 1998). This condition is associated with structural, functional, and biochemical abnormalities of the cardiac cells (Fein and Sonnenblick, 1994; Malhotra and Sanghi, 1997). Cellular changes, including defects in calcium transport and fatty acid metabolism, may lead to myocellular hypertrophy and myocardial fibrosis (Bell, 1995). A previous study also showed mitochondrial respiratory impairment in streptozotocin (STZ)-diabetic rat’s heart (2-14 weeks), which may contribute to cardiac dysfunction (Tomita et al., 1996). It remains unclear whether changes in the diabetic heart microvasculature, such as increased basement membrane thickening and increased capillary microaneurysms, could also be
significant in the development of altered myocardial functioning (Hardin, 1996; Li, 1992).

Diabetes is associated with cerebrovascular disease featuring non-fatal or small infarction especially with multiple occurrences. A previous study showed that high blood pressure and poor blood glucose control are associated with the higher incidence of cerebral infarctions in diabetic patients (Kameyama et al., 1994). It is also a risk factor for ischaemic but not haemorrhagic stroke. Impaired autoregulation of cerebral blood flow, impaired cerebrovascular reactivity, and damage to large and small extra- and intra-cranial cerebral vessels have been found in humans and animals with diabetes (Cipolla et al., 1997; Mankovsky et al., 1996). In one study, it was shown that acute glucose exposure dilates posterior cerebral arteries with intrinsic tone and impairs cerebrovascular reactivity to transmural pressure via an endothelium-mediated mechanism that involves NO and prostaglandins (Cipolla et al., 1997).

Peripheral vascular disease (PVD) in diabetes characteristically affects the arterioles below the knee, such as the tibial and peroneal arteries and their branches. Such vessels are also commonly affected by atherosclerosis and may display capillary basement membrane thickening. Narrowing of the arteriolar lumen often leads to tissue ischaemia causing ulceration and gangrene of the lower extremities of the diabetic and ultimately amputation of the affected area (Armstrong and Lavery, 1998; Ellenberg and Rifkin, 1983). There are multiple risk factors involved in the development of diabetic PVD and no universal agreement as to which is the most important. These are blood pressure, smoking, age, duration of diabetes, blood glucose, and lipids (e.g. cholesterol, triglycerides) (Cooper et al., 1997).

1.4.2. Microvascular disease

Important functional changes associated with microvascular complications are increased organ blood flow, increased vascular permeability, abnormal blood viscosity, and abnormal platelet and endothelial function (Stehouwer et al., 1997). Increased microvascular flow and pressure results in injury to the endothelium, which can in turn cause adaptive microvascular sclerosis. This contributes to a loss of vasodilatory reserve
and autoregulatory capacity with increasing disease duration (Took, 1996). The structural hallmark of diabetic microangiopathy is thickening of the capillary basement membrane (McMillan, 1997). These changes may lead to occlusive angiopathy and tissue hypoxia and damage (Dahl-Jorgensen, 1998).

The major microvascular complications of diabetes are retinopathy, nephropathy, and neuropathy. Mechanisms by which diabetic microangiopathy develop are not clear, but may include genetic influences. Several biochemical changes are involved in causing these complications, one important change being increased protein glycation (Wautier and Guillausseau, 1998) (section 1.5.2). Reactive oxygen species (ROS) or free radicals are also involved in causing tissue damage in diabetes (Giugliano et al., 1996). ROS generation is enhanced by non-enzymatic glycation and by increased activity of the polyol pathway, while the ability to neutralize free radicals is reduced in diabetic tissues (Cameron et al., 1992; Mak et al., 1996) (section 1.5.3). A role for endothelin (ET) in diabetic microvascular complications (Deng et al., 1999) has also attracted a great interest (section 1.8.4).

All these studies and those mentioned in the following chapters have enriched the knowledge base, to different degrees, of what factors may contribute to diabetic vascular complications. Delayed skin wound repair is one of the major complications of diabetes, especially among the older population. Adequate microvascular blood flow is an essential prerequisite for tissue repair and it is known that sensory nerves play an important role in modulating microvascular blood flow (section 1.7.2). Previous studies have indicated that the function of sensory nerves is compromised at the early stage of diabetes, leading to changes in skin microvascular blood flow (Gyorfi et al., 1996; Rendell et al., 1993). Previous studies have also shown that factors such as ETs, free radicals, advanced glycated end-products (AGEs) and hyperglycaemia were involved in long-term vascular complications of diabetes mellitus (sections 1.8.4, 1.5.3, 1.5.2 & 1.8.5.3).

It is the aim of this PhD study to provide information about the mechanisms underlying changes in skin microvascular blood flow in early diabetes and its effect on tissue repair
processes. First, the involvement of ETs, free radicals, AGEs, and hyperglycaemia in modulating skin microvascular blood flow in 4 weeks streptozotocin (STZ)-induced diabetic rats was established. Then, by using specific inhibitors / antagonists, the possibility of preventing the deleterious effects of these factors and improving microvascular blood flow and tissue repair processes was examined.

1.4.2.1. **Retinopathy**

Diabetic retinopathy and blindness from diabetes became a significant problem after the discovery of insulin in 1922 due to the increase in the life expectancy of diabetics. Diabetes causes various changes in the retinal circulation, causing two forms of retinopathy: non-proliferative and the most severe proliferative retinopathy. Non-proliferative retinopathy is recognised by the formation of microaneurysms in the retinal capillaries, possibly due to slight or moderate dilatation of venules. A previous study in diabetic rats (3-4 weeks) showed an increase in the production of vasodilatory prostaglandins (E and I₂) in the rat retinal vasculature (Johnson et al., 1999), which may contribute to abnormal retinal haemodynamics in early diabetic retinopathy. However, another study provided direct evidence to show that an increase in diacylglycerol (DAG) and subsequent protein kinase C (PKC) activation are the primary biochemical sequel responsible for the development of this abnormality in diabetic (2 weeks) rats (Bursell et al., 1997).

The presence of small retinal haemorrhages from which erythrocytes and serious fluid may leak is also common; serum leakage or oedema results in formation of hard exudates (Ellenberg and Rifkin, 1983; Nathan, 1993). Such disturbances do not usually lead to loss of vision. However, if serum leaks from defective microvessels near the maculae, visual acuity may be affected (Nathan, 1993). Proliferative retinopathy follows from the development of increasingly severe non-proliferative retinopathy. Initially, what are known as 'cotton wool spots' develop, which represent the infarction and ischaemia of retinal tissue. Retinal vessel ischaemia leads to retinal neovascularization and glial proliferation; the newly formed vessels are particularly fragile and prone to haemorrhaging. If haemorrhaging occurs into the vitreous cavity, loss of vision will be the result (Ellenberg and Rifkin, 1983; Nathan, 1993). Endothelin-1 (ET-1) is a potent
vasoconstrictor of the porcine ophthalmic microcirculation and, thus, may play an important role in the physiological control of ocular perfusion and diabetic retinopathy (Ruffolo, 1995). It was shown that ETs might alter extracellular matrix protein’s gene expression in the retina leading to the development of structural changes (Deng et al., 1999).

1.4.2.2. **Nephropathy**

Diabetic nephropathy occurs in approximately one third of both types of diabetics (O'Bryan and Hostetter, 1997) and can be divided into 5 stages. Stage I occurs at the onset of the disease and is characterised by 30%-40% increase in the glomerular filtration rate (GFR) above normal. Other changes are increase in kidney size, increased glomerular diameter, and tubular size (Marshall, 1998; Nathan, 1993). An increase in GFR is mainly driven by increases in plasma flow and glomerular capillary pressure, which may cause a transient increase in albumin excretion. Renal blood flow can be reduced by ET-1 resulting in the reduction of GFR. It was suggested that the alteration in ET activity in diabetic kidney might contribute to the increase in GFR (Lam et al., 1995). Elevation in the capillary pressure could damage glomerular endothelial, epithelial, and mesangial cells. Many factors have been suggested to be involved in increasing the capillary pressure such as alterations in the endogenous level of atrial natriuretic peptide, endothelial-derived relaxing factor (EDRF), angiotensin II, prostaglandins, thromboxanes, and kinins among others (Anderson and Vora, 1995). PKC activity is altered in the kidney of 4 weeks diabetic rats (Kang et al., 1999). There is also evidence to suggest that PKC may regulate renal haemodynamics by increasing or decreasing NO production (Nishio and Watanabe, 1996) and this may be important in kidney vascular blood flow.

Ultrastructural thickening of the glomerular basement is not present at the time of onset of insulin dependence and occurs over time (Ellenberg and Rifkin, 1983). The messenger ribonucleic acid (mRNA) level of extracellular matrix components such as glomerular alpha 1 (I), (III), and (IV) collagen, laminin B1 and B2, and growth factors such as tumour necrosis factor (TNF)-alpha, platelet-derived growth factor (PDGF)-B chain, transforming growth factor (TGF)-beta, and basic fibroblast growth factor (bFGF) all
increase in glomeruli with duration of diabetes (Flyvbjerg et al., 1994). Increase in ETs may play an important role in causing structural changes in the glomeruli since this up-regulation was completely blocked by treatment with endothelin-A (ET-A) receptor antagonists (Nakamura et al., 1995). Mesangial cell abnormalities including cell expansion and hypertrophy and increased mesangial cell matrix also occurs with nephropathy about 2-3 years after the onset of diabetes (Ellenberg and Rifkin, 1983). Stage II is characterised by normal excretion of albumin regardless of the duration of the diabetes. Stage III or incipient diabetic nephropathy is characterised by microalbuminuria at rest, which can be detected by a sensitive method such as radioimmunoassay. Diabetic patients often, at this stage, have higher blood pressure than non-diabetics do. It was shown that endothelial dysfunction leads to the onset of microalbuminuria, which was observed at this stage, and the level of glycaemic control seems to be the strongest factor influencing the transition between normoalbuminuria to microalbuminuria (Marshall, 1998). Stage IV or overt diabetic nephropathy is characterized by clinical proteinuria and even higher blood pressure than stage III. The development of proteinuria and decreased GFR identify end-stage renal disease (Marshall, 1998; Nathan, 1993).

1.4.2.3. Neuropathy

Diabetic neuropathies are complex, heterogeneous disorders that encompass a wide range of abnormalities affecting both peripheral and autonomic nervous systems. They may be diffuse or focal (acute onset neuropathy), proximal or distal, and involve somatic and autonomic nerves (Vinik, 1999). Diffuse neuropathy includes peripheral and autonomic neuropathy, whereas Focal neuropathy, which is less common, may affect single or multiple peripheral nerves, cranial nerves, brachial and lumbosacral plexuses, and nerve roots (Davidson, 1998; Vinik, 1999). Focal syndromes are classified as 1) mononeuropathies or 2) entrapment syndromes. Mononeuropathies are usually due to an inflammatory, vasculitic, or autoimmune condition.

The cause of diabetic neuropathy is poorly understood but many studies have suggested that neurotrophic proteins such as nerve growth factors (NGFs) are involved (Apfel et al., 1994; Hellweg and Hartung, 1990; Schmidt et al., 1999). In 4 weeks diabetic rodents, a reduction in NGF level was observed in the serum and the sciatic nerve (Hellweg and
Hartung, 1990). Additionally, decreased NGF receptor expression in dorsal root ganglia (DRG) of STZ-treated rats (Maeda et al., 1996) supports a role for impaired NGF-mediated neurotrophism in the pathogenesis of experimental diabetic neuropathy. Another animal study suggested that neuropathy may arise as a consequence of the loss of neurotrophic insulin-like growth factor (IGF) activity due to diabetes (Zhuang et al., 1996).

Peripheral neuropathy is a generalized sensorimotor polyneuropathy of gradual onset that is usually progressive. It is the earliest and most widely recognized form of diabetic neuropathy, which affects the lower limbs. Patients initially experience sensory manifestations (e.g. burning sensation, paraesthesia, and dysesthesias). Loss of deep tendon reflexes and poor sensation (vibration, touch) can also be present. Poor sensation in the feet can lead to foot problems and may contribute to the development of diabetic ulcers. Involvement of motor fibres can cause muscle weakness and atrophy leading to feet deformities, which may also contribute to ulcers (Davidson, 1998).

Previous animal studies showed that peripheral impairments develop within weeks after induction of diabetes (Biessels et al., 1999; Ralevic et al., 1995a; Tong et al., 1996). In STZ-treated diabetic rats (6-14 weeks) this is characterised by abnormalities in thermal pain threshold, reductions in DRG levels of substance P (SP) and calcitonin gene-related peptide (CGRP), and slowing of nerve conduction velocity (Apfel et al., 1994). Early changes (after 1 week) in the sciatic nerve endoneurial blood flow and function were also shown (Cameron et al., 1991). However, no detectable structural basis has been found for these early functional abnormalities i.e. reduction in nerve conduction velocity, pain threshold, and blood flow (Walker et al., 1999). PKC may play a role in diabetic neuropathy but the exact nature of this role is not clear (see also section 1.8.5.4).

Autonomic neuropathy is thought to encompass alterations to motor, sensory, and reflex functions of the cardiovascular, gastrointestinal and genitourinary systems, impaired sudomotor and vasomotor thermoregulatory mechanisms, disturbance of autonomic control of endocrine secretion, changes in pupillary reflex function, and disturbances in respiratory control (Davidson, 1998; Ellenberg and Rifkin, 1983; Vinik, 1999) (Table 1-
2). Symptoms may be non-specific, ranging from minor disturbances to extreme disability.

<table>
<thead>
<tr>
<th>Location of autonomic neuropathy</th>
<th>Clinical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular system</td>
<td>Resting tachycardia</td>
</tr>
<tr>
<td></td>
<td>Postural hypotension:- dizziness, vertigo, nausea, vomiting, faintness, syncope</td>
</tr>
<tr>
<td>Gastrointestinal system</td>
<td>Oesophageal atony:- gastric reflex, Dysphagia.</td>
</tr>
<tr>
<td></td>
<td>Gastric atony:- nausea, vomiting, anorexia, postprandial bloating</td>
</tr>
<tr>
<td></td>
<td>Diabetic diarrhoea</td>
</tr>
<tr>
<td></td>
<td>Colonic atony:- constipation</td>
</tr>
<tr>
<td>Genitourinary system</td>
<td>Bladder atony:- loss of bladder sensation, urinary stasis, incontinence</td>
</tr>
<tr>
<td></td>
<td>Impotence and retrograde ejaculation</td>
</tr>
<tr>
<td>Thermoregulatory system</td>
<td>Sudomotor dysfunction:- anhydrosis</td>
</tr>
<tr>
<td></td>
<td>Loss of reflex vasodilatation and vasoconstriction of skin vessels</td>
</tr>
<tr>
<td>Others</td>
<td>Pupillary disturbances:- decreased resting pupillary diameter, delayed response to light</td>
</tr>
<tr>
<td></td>
<td>Defective respiratory reflexes:- cardiorespiratory arrest</td>
</tr>
</tbody>
</table>

Resting tachycardia is usually the initial clinical cardiovascular effect of autonomic neuropathy. The parasympathetic arm of the autonomic nervous system is often affected earlier and more profoundly than the sympathetic one. A reduction in the resting heart rate, and an increase in the heart rate variability were observed in diabetic rats (1-17 weeks) but the mean arterial pressure was unchanged (Schaan et al., 1997). Exercise intolerance may occur when the sympathetic nervous system is affected, because the necessary increase in cardiac output and skeletal blood flow is blunted. Standing results in venous pooling in the legs and splanchnic beds, with a decrease in venous return and cardiac output. Normally, increased sympathetic outflow to the heart and peripheral vessels will maintain blood pressure, and such reflex responses are mainly maintained via the carotid sinus and aortic baroreceptors. Autonomic neuropathy of these reflex pathways in diabetes is believed to contribute to postural hypotension (Davidson, 1998). A study in diabetic rats (2 weeks) showed a reduction in the diastolic pressure and an impairment in the cardiovascular response evoked by baroreflex and chemoreflex activation (Dall'Ago et al., 1997). It was suggested that alterations in cardiovascular responses might be secondary to the autonomic dysfunction of cardiovascular control. Autonomic neuropathy of the heart muscle is also believed to contribute to the increased incidence of painless myocardial infarction in diabetes, which can lead to sudden death (Davidson, 1998).

Disturbance in most regions of the gastrointestinal system has been demonstrated to involve damage to the parasympathetic, sympathetic, and afferent sensory fibres (Davidson, 1998). Symptoms include oesophageal atony, gastric atony, delayed gastric emptying, gall bladder atony, and colonic atony leading to constipation. However, the most commonly recognised manifestation of autonomic neuropathy involving the gut is diabetic diarrhoea. In rats, diabetic diarrhoea was observed after only 1 week pre-treatment with STZ (Musabayane et al., 1995). Although the precise cause is unknown, proposed mechanisms include pancreatic insufficiency, electrolyte imbalance, altered gastrointestinal hormonal production, altered bile acid metabolism, and gastric vagal fibre damage leading to decreased acid secretion and gut stasis, which may promote bacterial overgrowth and small intestine contamination (Beebe and Walley, 1992).
Another major system demonstrating obvious alterations is the genitourinary system. Neurogenic bladder disturbances may contribute to bladder atony, loss of bladder sensations, incontinence, and urinary stasis (Ellenberg and Rifkin, 1983). Impotence may also result in diabetic males following damage to the parasympathetic fibres of erectile tissue (Faerman et al., 1974). For example, erectile dysfunction was documented in 4 weeks diabetic rats (Ari et al., 1999). This was shown to be initially reversible by insulin treatment but with time (more than 6 months) it became irreversible. Further problems that may arise from autonomic neuropathy include sweating disorders and vasomotor instability. Loss of sweating and inability of the arterioles to respond to temperature changes may result from damage to the sympathetic nerve pathways to the skin. For example, in the case of minor tissue injury with secondary infection fluid loss, dryness, and cracking of the skin can further exacerbate the infection. In diabetics this may lead to gangrene and amputation. Complete loss of spontaneous variation in arterial blood flow (e.g. in the feet) also indicates loss of reflex vasomotor responses in these diabetic patients (Ellenberg and Rifkin, 1983). Hypoglycaemic unawareness, pupillary abnormalities, and disturbed respiratory control have also been attributed to autonomic neuropathy (Davidson, 1998). The relationship between changes in sensory nerve function and diabetes and its relevance to this thesis is discussed later in this chapter (sections 1.7.2. and 1.7.3).

1.5. Mechanisms of diabetic complications

Chronic hyperglycaemia may contribute to the pathogenesis of the long-term complications of DM, on the basis that effective glycaemic control reduces the incidence of these complications in both animal models and patients with diabetes (Chase et al., 1989; Reichard et al., 1993; Service et al., 1985). Results from a study by the DCCT in 1993 supported this hypothesis by showing that intensified insulin treatment delayed the development and progression of diabetic retinopathy, microalbuminuria, and clinical neuropathy (DCCT, 1993).

It is not clearly understood how hyperglycaemia contributes to tissue damage and functioning but progress to date suggests that glucose-induced derangement of several
biochemical pathways in diabetes may be linked to the generation of diabetic complications.

### 1.5.1. Polyol pathway

Aldose reductase is the first and rate-limiting enzyme of the polyol pathway, which converts monosaccharides (e.g. glucose), to their polyols or sugar alcohols (e.g. sorbitol). This enzyme is widely distributed throughout the body, including those tissues that are susceptible to chronic diabetic complications (e.g. retina, lens, cornea, glomeruli, schwann cells of peripheral nerves, and endothelium) (Greene et al., 1987; Harrison et al., 1989). Aldose reductase has a low affinity for glucose, which at physiological glucose concentrations is preferentially channelled into the glycolytic or pentose-phosphate pathway under the action of hexokinase, which has a much higher affinity for glucose (Fig 1-1). Under hyperglycaemic conditions in tissues where insulin does not regulate glucose entry, intracellular glucose levels rise, and glucose is then increasingly diverted through the polyol pathway to be converted into sorbitol. Sorbitol is poorly diffusible and metabolized only slowly to fructose and therefore tends to accumulate within these tissues (Pickup and Williams, 1997).

![Fig 1-1: Glucose metabolism and the polyol pathway. Glucose enters the polyol pathway only under conditions of hyperglycaemia {Adenosine diphosphate (ADP), Adenosine triphosphate (ATP), Nicotinamide adenine dinucleotide (NAD), Nicotinamide adenine dinucleotide hydrogen (NADH), Nicotinamide adenine dinucleotide phosphate (NADP), Nicotinamide adenine dinucleotide hydrogen phosphate (NADPH)} (Adopted from Pickup, J.C. & Williams, G. (1997))](image)

Increased flux through the polyol pathway is associated with various other cellular and metabolic abnormalities. These include depletion of myo-inositol, reduced activity of Na⁺/K⁺-ATPase, an abnormality of kinase C-dependent protein phosphorylation, a
reduction in intracellular reduced glutathione concentrations, and an alteration in the redox state, which may impair the activity of many cellular enzymes (Hotta, 1997). An example is a study by Obrosova et al in 1999 showing reduction in the free NAD⁺/NADH ratio and glutathione concentration in 3 weeks STZ-treated rats (Obrosova et al., 1999).

In the lens, sorbitol accumulation is thought to cause osmotic effects leading to cataract (Kinoshita and Nishimura, 1988) and in the nerve, the reduced Na⁺/K⁺-ATPase activity may be related to falls in the conduction velocity and impaired axonal transport (Mayer and Tomlinson, 1983). In the kidneys, an increase in polyol pathway activity in early diabetes can increase prostaglandin production, which may ultimately lead to changes associated with diabetic nephropathy (Keogh et al., 1997). An enhanced polyol pathway may lead to an increase in oxidative stress, which can inactivate NO (Honing et al., 1998).

Development of aldose reductase inhibitors (Sarges and Oates, 1993) has permitted investigation of the polyol pathway’s involvement in contributing to diabetes-induced defects. Many animal studies showed that aldose reductase inhibitors are mainly effective in preventing diabetic complications from developing rather than reversing the established tissue damage (Cameron et al., 1997a; Hotta et al., 1996; Tomlinson, 1993). In humans, clinical benefits of aldose reductase inhibitors are less convincing. For example, although some positive effects were reported from clinical trials on patients with diabetic neuropathy and retinopathy, overall results were inconsistent (Christensen et al., 1985; Clark and Lee, 1995; Gonen et al., 1991).
1.5.2. **Advanced Glycated End-products (AGEs)**

**Synthesis/Structure/Function**

The glycation process is a natural biochemical event occurring as a consequence of the glucose-rich milieu that constantly permeates living tissues. When proteins or lipids are exposed to aldose sugars, they undergo non-enzymatic glycation and oxidation. Initially this leads to the formation of early glycated products, Schiff bases, and amadori products (Maillard, 1912). Early glycated products are still in equilibrium with plasma glucose, but when glucose levels fall they can dissociate to the native proteins. Alternatively, if glycation continues, further molecular rearrangements (glycation, glycoxidation, and antioxidative glycosylation) occur to generate the irreversible, heterogeneous AGEs (Friedman, 1999) (Fig 1-2). Glycation is a slow process under normal ambient sugar concentrations.

![Figure 1-2: Scheme of AGEs formation in the “Maillard” reaction](image)

The Maillard reaction has been implicated in the development of pathophysiology in age-related diseases such as DM, atherosclerosis, Alzheimer’s disease, and in dialysis-related amyloidosis (Nawroth et al., 1999).

AGEs are fluorescent yellow-brown pigments, which are found on red blood cells, platelets, extracellular matrix, and vessel wall components including endothelial and smooth muscle cells (Wautier and Guillausseau, 1998). AGE formation changes the
Some of AGEs biological properties are listed below (Bierhaus et al., 1998):
- Induction of RAGE, one of the known receptors for AGE
- Impairment of vasodilatory effects by quenching NO
- Increased vasoconstriction by inducing ET-1
- Induction of intracellular oxidative stress and activation of transcription factor NF-κB
- Lipid peroxidation
- Reduction of intracellular antioxidative defence mechanisms (e.g. glutathione, vitamin C)
- Increased macrophage uptake of AGE-low density lipoprotein (LDL) and possible atheroma formation
- Induction of cytokines and growth factors (Interleukin (IL)-1α, TNF, IGF-1A, PDGF) by monocytes / macrophages folowed by vascular cell proliferation
- Induction of smooth muscle cell proliferation
- Enhancement of deoxyribonucleic acid (DNA) mutation rate

**AGE receptor**

RAGE, a cell surface receptor for AGE, is a member of the immunoglobulin superfamily and exists in two forms, 35kDa and 45kDa (Thornalley, 1998). Hyperglycaemia was identified as one of the factors that stimulates RAGE expression (Thornalley, 1998) and this could play an important role in diabetic complications. The 45kDa form of RAGE is known as soluble RAGE or sRAGE with a proteolytic property which is used, in vivo, as a pharmacological agent to prevent the vascular effects of AGEs (acts as an antagonist) in experimental diabetes (Renard et al., 1997). For example, vascular hyperpermeability in diabetic rats was inhibited in the presence of sRAGE in a dose-dependent manner (Wautier et al., 1996). Anti-RAGE IgG can also inhibit AGE-RAGE interaction and the rise of vascular complications in diabetes (Schmidt et al., 1995; Wautier et al., 1994; Yan et al., 1994).

Normally, AGE-modified proteins are either repaired or replaced and degraded in vivo. The recognition and degradation of such proteins is mediated by specific AGE receptors, which are found on monocytes (Schmidt et al., 1996), macrophages (Nawroth et al., 1999), endothelial cells (Lalla et al., 1998; Wautier et al., 1994; Yan et al., 1994), vascular smooth muscle cells, pericytes, podocytes, astrocytes, microglia, and neurons (Thornalley, 1998). Interaction of AGE with RAGE on macrophages stimulates these
cells to produce and release cytokines, growth factors, proteolytic enzymes, and increase expression of extracellular matrix proteins and vascular adhesion molecules, which are all required for normal tissue remodelling (Nawroth et al., 1999) (Table 1-3).

---

**AGEs in atherosclerosis**
- Accumulation in the vascular matrix → narrowing & occlusion
- Vascular endothelial dysfunction (e.g. inactivating NO) → procoagulant state, vasoconstriction, hypertension
- Glyoxidation of LDL → slow degradation of LDL, lipid peroxidation, oxidative stress
- Trapping of plasma proteins → initiation of complement activation, oxidation
- Monocyte chemotaxis/activation → cytokine & growth factor release → vascular tissue proliferation
- Increased endothelial cell permeability → vascular leakage

**AGEs in renal disease**
- Matrix expansion, vascular leakage & basement membrane thickening → glomerular hypertrophy & glomerular sclerosis
- Glomerular sclerosis → albuminurea
- Delayed clearance of AGE-peptide → uremic complications

**AGEs in diabetic neuropathy**
- Accumulation in vasa nervosum → wall thickening & occlusion, ischemia
- Vascular endothelial dysfunction → occlusion, ischemia
- Glycation of myelin → myelin damage
- Glycation of growth factors (NGF, fibroblast growth factor(FGF)) → loss of function
- Accumulation on macrophages → macrophage activity → myelin & vascular degeneration

**AGEs in diabetic retinopathy**
- Increased endothelial cell permeability → vascular leakage & retinal damage
- Vessel wall thickening → occlusion, ischemia
- Coagulation → occlusion, ischemia
- Induction of autocrine vascular endothelial growth factor (VEGF) synthesis → angiogenesis, neovascularization

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**Table 1-3:** Some of the consequences of AGE formation and deposition (Adopted from Bierhaus, A. et al (1998) & Vlassara, H. et al (1994))

Functional alterations or saturation of the macrophage system would allow anomalous tissue accumulation of AGEs leading to reduced structural protein turnover, increased collagen cross-links, and excessive degeneration and / or proliferation of tissue
components as observed in ageing and diabetes (Bierhaus et al., 1998; Grossi and Genco, 1998; Sensi et al., 1991) (Table 1-3).

AGE-RAGE interaction can also alter cellular phenotypes generating a pro-inflammatory environment where induction of vascular perturbation, development of vascular lesions, and impairment of normal reparative responses are initiated (Lalla et al., 1998) independent of macrophage activity (Wautier et al., 1994; Yan et al., 1994). Introduction of AGEs into the extracellular matrix can also interfere with the endothelial function at different levels. For example, AGEs quench NO, during its passage from endothelial cell to smooth muscle cells, inhibiting its vasodilatation and antiproliferative actions (Bucala et al., 1991).

Collagenous proteins are especially prone to non-enzymatic glycation because they contain several dibasic amino acid residues with free amino groups, have a very slow turnover rate, and are exposed to ambient levels of glucose. The main functional property of collagen is to provide a supporting framework to almost all tissues. AGEs inhibit a normal network formation by type IV collagen and decrease heparan sulphate proteoglycan binding by vitronectin and laminin in diabetes (Brownlee, 1994). AGEs fusion with albumin was also shown to interact with the glomerular basement membrane inducing glomerular sclerosis and albuminuria in rats (Vlassara et al., 1994).

AGEs and diabetes

Under hyperglycaemia and / or conditions where the protein and lipid turnover period is prolonged, the advanced glycation process is enhanced (Lalla et al., 1998; Nawroth et al., 1999). For example, AGEs were identified in mesenteric vessels of STZ-treated diabetic rats within 3 weeks (Rumble et al., 1997) and in their skeletal muscle arteries within 4-6 weeks (Hill and Ege, 1994).

It was postulated that AGEs contribute to the development of vascular diseases associated with diabetes (Chibber et al., 1997; Makita et al., 1995; Wautier and Guillausseau, 1998). AGEs formation on the matrix component of the vessel wall can cause structural damage by decreasing elasticity, increasing thickness, rigidity, and narrowing of the vessel lumen (Bierhaus et al., 1998; Turk et al., 1999). AGEs increase collagen cross-linking leading to
the arterial stiffness that is commonly observed in normal ageing but at an accelerated rate in diabetes (Odetti et al., 2000; Wolffenbuttel et al., 1998). In diabetic rats, some AGEs were found in the aortic collagen after 4 and 12 weeks but this was significantly increased by 20 weeks (Turk et al., 1999).

AGEs change endothelial cell properties, relevant to pathogenesis of vascular disease, by forming cross links, recognizing RAGE on cellular surfaces (Yan et al., 1994) and generating ROS by undergoing autoxidation (Chappey et al., 1997; Schmidt et al., 1994). Interaction of AGEs with RAGE on vascular matrix proteins may generate superoxide anions (Galle et al., 1998), which can inactivate NO (Bucala et al., 1991). This is supported by previous studies showing glycated haemoglobin (Hb) induced endothelial dysfunction in rat (Angulu et al., 1996) and human vessels (Vallejo et al., 2000). AGEs also induce ET-1 expression and change endothelial function towards vasoconstriction (Quehenberger et al., 1995). An in vitro study demonstrated that ET-1 could potentiate the oxidized LDL-induced superoxide anion formation (Galle et al., 2000). Hence, it is possible that ET-1 could play a role in inhibiting NO in the diabetic condition via this mechanism. A previous study supported this hypothesis and showed that reduction of cytochrome C by glycated proteins, a process involving superoxide anions, could induce lipid peroxidation (Ceriello, 1999). It was suggested that increased formation of ROS (e.g. superoxide anions and hydroxyl radicals) participate in the vascular endothelial cell dysfunction (Gryglewski et al., 1986; Suzuki et al., 1991) (section 1.5.3).

It is known that transmembrane signalling of contractile cells is generally inhibited by high glucose. In the kidney, it was hypothesized that glycated macromolecules with RAGE might inhibit transmembrane calcium signalling (Mene et al., 1999). Also, reduced function of the L-type calcium channel that occurs in retinal pericyte culture in high glucose maybe due to the glycation of a channel protein (McGinty et al., 1999). Both free glucose (Booth et al., 1997) and protein-glucose adduct (Taniguchi et al., 1996) could undergo oxidation in the presence of trace amounts of metal ions, to generate free radicals and reactive carbonyls. Amadori products undergo metal-free oxidation to form superoxide anions (Mossine et al., 1999). In one study, incubation of cultured smooth muscle cells with early glycated products (after 2 weeks) and cupric ion (Cu²⁺) was
shown to be capable of damaging these cells (Sakata et al., 1998). It was hypothesized that the generated hydrogen peroxide, under this condition, was a candidate for this damage. It is possible that hydrogen peroxide damages smooth muscle cells by collagen cross-linking (Elgawish et al., 1996).

ROS formed by glucose and protein-glucose adducts may be involved directly in the formation of AGEs and in AGE-induced pathologic alterations in gene expression (Brownlee, 2000). This was supported by previous studies, in vitro, showing antioxidants (e.g. vitamin E, alpha-lipoic acid, dimethyl sulfoxide) can inhibit the hyperglycaemia-induced AGE-formation (Bierhaus et al., 1997; Giardino et al., 1996). AGE-RAGE interaction also depletes the cellular antioxidant defence mechanism (e.g. glutathione, vitamin C, superoxide dismutase (SOD)) in diabetes (Bierhaus et al., 1997; Ceriello, 1999; Mak et al., 1996). This was supported by a previous study demonstrating inactivation of Cu-Zn-SOD by the non-enzymatic glycation (Kashiwagi et al., 1996). Also it was reported that the level of glycated Cu-Zn-SOD was increased in patients with diabetes (Taniguchi et al., 1996). It is possible therefore, that the combination of both an increase in ROS and the reduction in antioxidant efficiency could contribute to tissue damage in diabetes.

Evidence exists that transition metals may be involved in causing diabetic vascular and nerve abnormalities (Qian and Eaton, 2000). This author hypothesized that heavily glycated proteins, known to accumulate in diabetic patients, may gain an increased affinity for transition metals such as iron and copper. As a result, proteins such as elastin and collagen within the arterial wall can accumulate bound metal (i.e. copper), causing catalytic destruction of EDRF and engendering a state of chronic vasoconstriction. This in turn could impair nerve blood flow and ultimately cause nerve death. Administration of chelators could prevent or reverse slow peripheral nerve conduction, neuronal blood flow, as well as impaired endothelium-dependent arterial relaxation, supporting the above hypothesis (Qian and Eaton, 2000).

Diabetic neuropathy is associated with some early defects of axonal transport in STZ-treated animals (Di Giulio et al., 1995; Tomlinson and Mayer, 1984). Axonal transport is
dependent on intact microtubules and unsubstituted lysine residues of tubulin are essential for microtubule polymerization. Reduced polymerization of the “glycated” tubulin in diabetes (Williams et al., 1982) could in part be responsible for alterations of the axoplasmic transport observed in diabetic neuropathy. There was more than a 4-fold increase in the extent of glycation of tubulin in the sciatic nerve of 2 weeks diabetic rats (Cullum et al., 1991). Increased non-enzymatic glycation of myelin and myelin-associated proteins have also been reported in diabetes, both at the peripheral and central nervous system levels (Ryle et al., 1997; Sensi et al., 1991). For example, high levels of the early glycated product (furosine) and low levels of AGEs were found in the sciatic nerve and cytoskeletal preparations of 6 weeks STZ-treated rats (Ryle et al., 1997). Also, significantly lower levels of AGEs were found in the spinal cord and spinal nerve of these rats. It was suggested that enhanced accumulation of AGEs in cytoskeletal as well as myelin and peripheral nerve proteins might contribute to the axonal degeneration polyneuropathy in diabetes (Ryle and Donaghy, 1995). Quantitative analysis of the myelinated nerve fibres (peroneal nerve) showed no significant morphometric changes in 2 (Nukada, 1993) or 4 weeks diabetic rats but a reduction in the myelinated nerve calibre was observed after 8 weeks of diabetes (Ochodnicka et al., 1995). Over-glycation of myelin, in the diabetic condition, seems to act as a signal for recognition and degradation by macrophages, causing segmental demyelination (Ryle and Donaghy, 1995). Moreover, the presence of AGE on myelin appears to modify the properties of its proteins by trapping plasma proteins (covalent bonding). Presence of these molecules can start immunological reactions responsible for demyelination. The central nervous system is not involved in this immunological phenomenon and is protected by the blood-brain barrier.

AGE-RAGE interaction in diabetes, involving erythrocytes, increases vascular permeability (Bonnardel-Phu et al., 2000). It was proposed that this increase could be mediated by free radicals since antioxidants inhibited hyperpermeability in both in vitro and in vivo conditions (Wautier et al., 1996). VEGF may also influence the tightness of the endothelial barrier. This hypothesis was based on a previous study showing AGEs
play a role in increasing the TGF-beta expression in experimental diabetes (Rumble et al., 1997), which in turn could enhance VEGF levels (Pertovaara et al., 1994).

Non-enzymatic glycation affects proteins involved in the clotting cascade and in diabetes this modification may interfere with vascular wall homeostasis. For example, in diabetes, increased glycation of platelet membrane proteins contributes to functional platelet defects and microangiopathy (Yamagishi et al., 1998). It was suggested that the underlying mechanism might involve cyclic adenosine monophosphate (cAMP). The same study also showed that AGE-RAGE interaction could inhibit the production of prostaglandin I$_2$ and up-regulate plasminogen activator inhibitor-1 (PAI-1), by microvascular endothelial cells, through a reduction in cAMP. Erythrocyte membrane proteins undergo non-enzymatic glycation over the course of the red blood cell life, and the amount of amadori products on these proteins increases in the diabetic state. Previously, it was reported that excessive membrane glycation may play a role in altering some properties of the erythrocyte membrane proteins which can affect doublet formation, microviscosity, and increased oxygen affinity (Sensi et al., 1991). It was also shown that erythrocyte AGEs increase vascular permeability due to extensive contact with the vessel wall RAGE, leading to vascular complications (Wautier and Wautier, 1999).

LDL, a lipid transport protein that plays an important role in the development of atherosclerosis, is glycated as shown in both \textit{in vitro} and \textit{in vivo} conditions (Thorp and Baynes, 1996). The increase in LDL glycation causes a reduction in the uptake and degradation of LDL by tissue fibroblasts and macrophages, contributing to premature atherosclerosis in diabetics (Vlassara, 1996). Lipid peroxidation is known to induce cross-linking of collagen with a high rate in the presence of high glucose (Fu et al., 1994). Advanced glycated collagen was shown to be capable of covalently trapping LDL (e.g. in the arterial wall) causing it to be modified oxidatively by free radicals (Mullarkey et al., 1990). It was proposed that LDL oxidation may contribute to the formation of “foam” cells (Wautier and Guillausseau, 1998).
Aminoguanidine (AG)

AG is a small nucleophilic hydrazine compound. It blocks AGE formation by specifically binding to non-protein-bound glucose-derived intermediates of early glycated products and preventing their further rearrangements (protein-protein or protein-lipid cross-links) (Brownlee et al., 1986; Friedman, 1999) (Fig 1-3). Inhibition of AGEs by AG involves interaction between the reactive hydrazine moiety of AG and the reactive carbonyl groups of amadori or glycoxidation products (Corbett et al., 1992; Yu and Zuo, 1997).

The glycation process can decrease the susceptibility of molecules to proteolysis. The reverse is true of protein oxidation, which has previously been shown to increase the susceptibility of proteins to proteolytic digestion (Skamarauskas et al., 1996). AG is capable of enhancing the proteolytic digestion of glycated bovine serum albumin (BSA), a protein shown to be protease resistant (Skamarauskas et al., 1996). High doses of AG, in vitro, inhibits catalase, generates hydrogen peroxide, and other oxidants similar to hydroxyl radicals in activity (Ou and Wolff, 1993; Skamarauskas et al., 1996). However, in vivo and at low doses, AG is regarded as virtually a non-toxic substance. It can act as an antioxidant, quenching hydroxyl radicals and lipid peroxidation in cells and tissues as well as preventing oxidant-induced apoptosis (Giardino et al., 1998).

AG can also retard or prevent the development of nephropathy (Soulis-Liparota et al., 1991), retinopathy (Hammes et al., 1991) and neuropathy (Cameron et al., 1992) in the STZ-induced diabetic rat model (Table 1-4).
Effects on the development of atherosclerotic lesions
- prevention of collagen-to-collagen cross-linking
- decreased collagen stability
- increased elasticity and decreased fluid filtration in large arteries
- decreased trapping of lipoproteins
- inhibition of glycation and oxidation of LDL
- decrease in LDL-cholesterol, very low density lipoprotein (VLDL)-cholesterol, total cholesterol and triglycerides

Effects in kidney disease
- reduction of AGE accumulation in the renal glomerulus
- prevention of diabetes-induced thickening of basement membranes
- prevention of diabetic nephropathy in animal models
- reduction of albuminuria in diabetic and hypertensive animals
- decreased trapping of IgG by basement membranes of renal capillaries

Effects on the diabetic peripheral nerve
- preservation of nerve conduction velocity
- normalization of nerve action potential amplitude and peripheral nerve blood flow

Effects on diabetic retinopathy
- prevention of lens protein cross-linking
- prevention of AGE-formation and formation of acellular capillaries in the retina
- reduction of microaneurysms
- reduction of pericyte drop-out
- inhibition of hypertension dependent accelerated diabetic retinopathy


A previous study in vivo showed that generation of AGEs at least in rat kidneys was time-dependent and closely linked to the development of metabolic and structural changes up to 32 weeks after induction of diabetes (Soulis-Liparota et al., 1996). This may be an important factor to consider when using preventative treatment regimes involving AG in diabetics with the risk of developing complications associated with AGEs (chapter 8). Furthermore, AG was shown to have no influence on the body weight or hyperglycaemia in STZ-treated rats during the 32 weeks post-diabetes (Soulis-Liparota et al., 1996). A different study showed an increase in regional vascular albumin permeation by about 2 to 3 fold in the ocular tissue, sciatic nerve, and aorta of 5 weeks STZ-treated diabetic rats which was normalized by AG treatment (Tilton et al., 1993). While AG prevents the
ongoing AGE formation, it may not be effective in patients with a long history of disease that already resulted in extensive tissue-AGE accumulation. Also, the need to remove irreversibly bound AGEs from connective tissues and matrix compounds recently led to the design of AGE-cleaving agents such as prototypic AGE cross-link “breaker” N-phenacyl thiazolium bromide (PTB). Vasan et al in 1996 performed the first in vivo and in vitro experiments to show that PTB can reduce AGE cross-links (Vasan et al., 1996) but further studies on “breakers” are required.

AGEs and skin microvasculature

Direct in vivo evidence regarding the pathogenic influence of AGEs, independent of diabetes, was presented by Vlassara et al (1992). They showed that short-term (2-4 weeks) administration of in vitro prepared AGE-albumin to normal rats and rabbits produced vascular defects similar to those associated with experimental diabetes, such as vascular leakage and mononuclear cell extravasation, as well as unresponsiveness to vasodilator agents. These defects were significantly inhibited by AG treatment in control rats. In a study by Soulis-Liparota et al (1996), using diabetic rat kidneys (32 weeks duration), it was suggested that a relationship exists between duration of AG treatment and its protective effects but not the commencement of treatment (Soulis-Liparota et al., 1996).

Many studies have investigated the effects of AGEs and AG on diabetic vascular responses in different tissues and at different stages of the disease (Bucala et al., 1991; Quehenberger et al., 1995). However, the role of AGEs in modulating skin microvascular blood flow in early diabetes has not been previously examined. The aim of chapter 8 was to assess the short- and long-term effects of early and late products of glycation (e.g. amadori and AGEs) in modulating microvascular blood flow in 4 weeks STZ-induced diabetic rats. The effect of AGEs in microvascular changes in diabetic rats was investigated with regards to the duration of diabetes and the treatment with AG.
1.5.3. Oxidative stress and antioxidants

Free radicals are defined as atoms or molecules that contain one or more unpaired electrons. The term reactive oxygen species (ROS) refers to oxygen radicals (superoxide anions \( \cdot O_2^- \)) and hydroxyl radicals \( \cdot OH^- \)) and a number of related species, such as hydrogen peroxide \( \cdot H_2O_2 \), that do not themselves contain unpaired electrons but are often involved in the generation of free radicals (Fig 1-4).

ROS are formed continuously as normal by-products of cellular metabolism and sometimes by accident and they have the potential to produce high levels of tissue damage (oxidative stress). They damage many cellular components including DNA but primarily phospholipid A-containing cellular membranes, which are converted to malonyldialdehyde (MDA) by lipid peroxidation (Stohs, 1995).

Antioxidants are substances capable of neutralizing oxygen free radicals and other similar agents. The body’s natural protection system against free radicals involves different enzymes such as SOD, that scavenges superoxide anions, and catalase and glutathione redox enzymes (especially glutathione peroxidase) that act as a secondary defence system (Thomas, 1995). Additional antioxidant defence is provided by certain vitamins (C, E, beta-carotene), minerals (selenium), and other substances with redox capability (ubiquinone, coenzyme Q10) and is obtained through intake of food since they are not synthesized by the body (Ernster and Dallner, 1995).

**Fig 1-4:** Formation of oxygen derived radicals. [molecular oxygen \( O_2 \), superoxide anion \( \cdot O_2^- \), hydrogen peroxide \( \cdot H_2O_2 \), hydroxyl radical \( \cdot OH^- \), superoxide dismutase (SOD), xanthine oxidase (XO), nitric oxide \( \cdot NO \), peroxynitrite \( \cdot ONOO^- \), nitrogen dioxide \( \cdot NO_2 \) (Adopted from Gryglewski, R.J. et al (1986), Munzel, T. et al (1997), and Kehrer, J.P. (1993))
Physiological effects of free radicals

ROS generated by endothelial cells (e.g. superoxide anions, hydrogen peroxide, and hydroxyl radicals) (Bonnardel-Phu and Vicaut, 2000a) can directly modify vascular endothelium by interfering with the availability of NO, peroxidation of membrane lipids, and up-regulation of adhesion molecules to platelets and leucocytes. ROS are important for physiological activity of smooth muscle cells and in the pathogenesis of various diseases, where the function of smooth muscles is altered. One study, using normal smooth muscles from different body parts, showed that ROS are involved in changing the muscle tone, membrane conductance, calcium homeostasis and dependent processes, as well as in eicosanoid and NO metabolism (Bauer et al., 1999).

Superoxide anions can interact directly with NO to reduce its biological activity (Gryglewski et al., 1986) causing contraction of the vascular smooth muscle (Kaustic and Vanhoutte, 1989). Hydrogen peroxide is produced by dismutation of superoxide anions either spontaneously or catalyzed by SOD (Fig 1-4). SOD can be either cytotoxic (Rubanyi, 1988) or act as a smooth muscle relaxant (Zembowics et al., 1993), depending on its concentration, where in the latter case it can accelerate NO release (Rubanyi and Vanhoutte, 1986).

In the absence of superoxide anions, NO engages in nitrosative chemistry to yield stable N-nitrosamine derivatives. As the flux of superoxide anions is increased, nitrosative reactions are suppressed and oxidative chemistry is enhanced (Jourd'heuil et al., 1997). Thus, depending upon the flux of each radical, either nitrosation or oxidation chemistry may predominate. The chemical reaction between superoxide anions and NO generates a highly reactive peroxynitrite (ONOO\(^{-}\)) (Jourd'heuil et al., 1997) (Fig 1-4), which in turn stimulates cyclo-oxygenase catalysis, lipid peroxidation, and increased prostanoid production, resulting in endothelial dysfunction (Bouloumie et al., 1997). In physiologic pH, peroxynitrite leads to the formation of hydroxyl radicals (Prasad and Bharadwaj, 1996). Interaction between hydrogen peroxide and superoxide anions also generates hydroxyl radicals (Haber-Weiss/Fenton reaction) (Fig 1-4). It is believed that these radicals can elicit contraction of the vascular smooth muscle (Kontos and Wei, 1993), however, the exact mechanism is not clear. Evidence exist that hydroxyl radicals are
capable of both stimulating and inhibiting prostaglandin synthesis and stimulating guanylate cyclase to trigger smooth muscle relaxation (Prasad and Bharadwaj, 1996; Rubanyi, 1988)

ROS also play a role in alteration of calcium homeostasis. Under normal conditions ROS, generated by the hypoxanthine-xanthine oxidase system, can mobilize calcium from intracellular stores and induce a marked rise in the cytosolic calcium level in different cells (Dreher and Junod, 1995). They can also decrease (Ca^{2+})-stimulated ATPase activity, increase (Mg^{2+})-ATPase activity and reduce sulphydryl (SH) group content (Suzuki et al., 1991).

**ROS and diabetes**

In the diabetic condition, the level of free radicals is altered and the function of the antioxidant defence system is compromised. Evidence exists that an acute increase in plasma glucose concentration may enhance the production of free radicals by autoxidation of glucose (Pieper et al., 1997b) and the advanced glycation process that attenuates the activity of antioxidant enzymes (Mak et al., 1996). The advanced glycation process can also activate the polyol pathway, intracellularly, which produces an imbalance in the NADH / NAD^{+} ratio and favours the production of free radicals (Ido et al., 1996).

The activity of total SOD (Cu-Zn SOD & Mn-SOD) was increased in the heart, aorta, and blood of rats from the 2^{nd} week after diabetes onset and continued up to the 4^{th} week, after which it tended to decline (Kakkar et al., 1996). In the rat brain, an increase in fatty acid and MDA level and a decrease in SOD and catalase activities were observed after 2 weeks treatment with STZ (Kumar and Menon, 1993). Catalase activity, on the other hand, was increased in the heart and aorta of diabetic rats (0-6 weeks) but showed significance only after 4 weeks (Kakkar et al., 1996). Activities of catalase and SOD were significantly increased after 4 weeks of diabetes in the rat brain (Ramanathan et al., 1999). No change in the level of glutathione peroxidase activity was observed in the heart, blood or brain up to 4 weeks after STZ treatment in rats (Kakkar et al., 1996; Ramanathan et al., 1999). The gene expression of catalase and SOD (both types) was not reduced in L4, L5 of DRG and superior cervical ganglion of 3 or 12 months diabetic rats
(Kishi et al., 2000). It was suggested that changes in enzyme activity might be related to duration of diabetes or due to post-translational modifications.

Pathological effects of free radicals
An increase in the production of plasma free radicals (Mullarkey et al., 1990; Ohkuwa et al., 1995; Traverso et al., 1998) and a general reduction in antioxidant defences (Kamata and Kobayashi, 1996; Mak et al., 1996) were shown to be related to diabetic complications such as endothelial dysfunction (Stehouwer et al., 1997).

Exposure to glucose causes an increase in intracellular sorbitol and fructose levels leading to a reduction in NADPH cell stores. This could inhibit NO synthase and glutathione reductase activities. Decreased levels of NO can lead to vasoconstriction and tissue injury while reduced levels of glutathione increases the susceptibility of endothelial cells to damage by hydrogen peroxide (Paolisso and Giugliano, 1996). In early (3 weeks) diabetic rat’s synaptosomes, the reduction in glutathione and alpha-tocopherol (Vitamin E) level was suggested to be the reason for an increase in the level of hydroxyl radical and hydrogen peroxide (Aragno et al., 2000).

SOD protects NO from breakdown by decreasing the concentration of superoxide anions (Diederich et al., 1994; Gryglewski et al., 1986). One study suggested that the rapid destruction of NO by superoxide anions, in STZ-treated rats, might be due to the decrease in mRNA expression of Mn-SOD and Cu-Zn-SOD (Kamata and Kobayashi, 1996). Exogenously or endogenously-derived ROS were previously shown to be capable of enhancing ET-1 production in diabetic glomeruli (1 & 4 weeks) and this was inhibited by SOD and catalase (Chen et al., 2000). Hyperglycaemia or insulin deficiency was also suggested to be partially responsible for the increase in ET-1 production in these animals. A previous study reported that ROS increased oxidation of LDL, formation of AGE, and activation of platelets and monocytes (Stehouwer et al., 1997). Hydroxyl radicals are capable of oxidizing lipids, damaging cell membranes, and oxidizing thiol groups (Munzel et al., 1997) and have been implicated in diabetes-induced endothelial dysfunction (Pieper et al., 1997b).
The presence of MDA, a product of lipid peroxidation, can easily be detected by thiobarbituric acid (TBAR) (Stohs, 1995). It was proposed that products of lipid peroxidation might reduce NO bioactivity without affecting endothelial NOS (eNOS) mass or its catalytic activity (Hayakawa and Raij, 1999). In diabetic rats (0-6 weeks), a significant increase in TBAR levels in the aorta, blood and liver was observed (Kakkar et al., 1996). Lipid peroxide levels were also significantly increased in rat pancreas and heart as early as 2 weeks after induction of diabetes (Kakkar et al., 1998; Tatsuki et al., 1997).

In oxidative stress, changes in intracellular calcium homeostasis are thought to contribute to the cell dysfunction. Sasaki et al (1993) supported the view that hydroxyl radicals are capable of potentiating the voltage-dependent influx of calcium (Sasaki and Okabe, 1993). This author hypothesized that, in smooth muscle cells, hydroxyl radicals might damage the sarcoplasmic reticulum (SR), which could reduce calcium release. A different study showed that in STZ-diabetic (4 weeks) rat aorta, superoxide anions might be responsible, at least in part, for the impaired endothelial integrity, enhanced alpha-adrenergic receptor-mediated phosphoinositil (PI) turnover, and the augmented contractility possibly through modification of calcium channels (Chang et al., 1993). Lipid peroxidation by-products may also interfere with the calcium homeostasis by inhibiting microsomal glucose 6-phosphate, plasma membrane Ca\(^{2+}/\) Mg\(^{2+}\)-activated ATPase, and the Na\(^+\)/K\(^+\) activated ATPase pump (Dianzani, 1990; Wells et al., 1997).

**Diabetes and exogenous antioxidants**

N-acetylcysteine (NAC) has widely been used, *in vivo* and *in vitro* experiments, as an exogenous antioxidant. It was shown that NAC is a powerful scavenger of hydroxyl radicals and reacts slowly with hydrogen peroxide but no reaction with superoxide anions was detected (Aruoma et al., 1989). It is also a sulphydryl donor and a precursor for glutathione synthesis (Pieper and Siebeneich, 1998; Sagara et al., 1996).

In diabetic rats, NAC corrected the decreased GSH and increased lipid peroxide levels in plasma without any effect on the level of blood glucose and nerve glucose, sorbitol or cAMP content (Sagara et al., 1996). Long-term treatment with NAC could remove
radicals released as by-products during the glycation process and prevent the defective relaxation response to acetylcholine (ACh) in diabetes (Pieper and Siebeneich, 1998). NAC was also reported to inhibit the development of functional and structural abnormalities of the peripheral nerve in STZ-induced diabetic rats (12-15 weeks) (Sagara et al., 1996).

Tirilazad mesylate (Freedox) is a potent inhibitor of lipid peroxidation (Saniova, 1997) and was the first lazaroid compound to be used in clinical practice and disease conditions where lipid peroxidation caused further tissue damage. Tirilazad mesylate cytoprotective properties include scavenging free radical intermediates, stabilizing cell, and preserving vitamin E content in the membrane (Saniova, 1997).

A previous study showed that treatment with tirilazad mesylate could normalize the increase in plasma lipid peroxides in rats after 8 weeks of diabetes without any change in the level of serum insulin or concentration of blood glucose (Pieper et al., 1997a). In rats, oral pre-treatment with tirilazad mesylate (for 8 weeks) did not prevent the diabetes-induced impairment in endothelium-dependent relaxation caused by ACh (Pieper et al., 1997a). Tirilazad mesylate also protected cells by preserving the post-injury calcium homeostasis (Saniova, 1997).

**ROS and skin microvasculature**

It was shown that ROS are important in the physiological activity of smooth muscle cells and the active species produced by endothelial cells can directly modify vascular endothelium (Bauer et al., 1999; Bonnardel-Phu and Vicaut, 2000a). ROS also play an important role in the pathogenesis of various diseases such as diabetes (Stehouwer et al., 1997). For example, the synthesis and release of hydroxyl radicals was suggested to be responsible for the impairment of nitric oxide synthase (NOS)-dependent dilatation of the basilar artery in 3-4 weeks diabetic rats (Mayhan and Patel, 1998). The beneficial effects of antioxidants in the diabetic condition have been previously documented (Kaneto et al., 1999; Pieper et al., 1997a). For example, long-term treatment (e.g. 8-13 weeks) of diabetic rats with NAC was shown to inhibit endothelial dysfunction and development of abnormalities in the peripheral nerve (Pieper and Siebeneich, 1998; Sagara et al., 1996).
However, none of the previous studies to date have examined the role of ROS in modulating skin microvascular blood flow in early diabetes.

The aim of chapter 7 was to investigate the quantitative contribution of superoxide anions, hydroxyl radicals, and lipid peroxidation products to endothelial (inflammatory) and vascular smooth muscle responses in 4 weeks STZ-induced diabetic rats. For this purpose, the changes in diabetic skin blood flow are examined after using three free radical scavengers / antioxidants (SOD, NAC, and Tirilazad mesylate). A relationship between ROS and enhancement of ET-1 was previously reported (Chen et al., 2000). Therefore, the possible interaction between the effects of ET and free radicals was also assessed, using combined treatment of the ET-1 receptor antagonist, BQ-123, and superoxide anion scavenger, SOD.

### 1.5.4. Alterations in smooth muscle reactivity in diabetes

#### 1.5.4.1. Vasoconstrictor responses

The reactivity to vasoconstrictors such as noradrenaline (NA), angiotensin II, serotonin, and potassium chloride (KCl), has been widely investigated in numerous vascular tissues from animals with chemically induced diabetes. However, results from these studies have been inconsistent, reporting an increase, decrease, or no change in the reactivity of diabetic tissues (Kawasaki, 1997; Lieu and Ried, 1994; Ralevic et al., 1995a).

Results with respect to the vascular reactivity of aorta to NA in rats varied with the duration of diabetes. Enhanced responsiveness to NA was reported in aorta from 1 and 4 weeks (Orie and Aloamaka, 1993) as well as 8-12 weeks of diabetes (Kawasaki, 1997; Wong, 1996). Similar responses were also observed in the following tissues from STZ-treated rats: the sciatic nerve (Pa and Tomlinson, 1997), mesenteric vessels (Savage et al., 1995), and kidneys from 4-6 weeks (Martinez-Nieves and Dunbar, 1999), small cremaster muscles from 2-16 weeks (Morf, 1990), mesenteric vessels from 10-12 weeks (White and Carrier, 1990), and the eye (Su et al., 1995) and tail artery after 4 weeks (Ramanadham et al., 1984).

Diabetes-induced changes in reactivity to other vasoconstrictor agents have also been examined. For example, studies using the alpha-adrenoreceptor agonists phenylephrine and methoxamine, demonstrated significantly greater contractile responses in tissues
from 3 days and 4 weeks STZ-induced diabetic rats compared to control (Chang et al., 1993; Wald et al., 1988). Further supporting studies have shown an increase in reactivity to NA, angiotensin II, serotonin, KCl, and ET-1 in aorta (Abebe et al., 1994; Murat et al., 1999), atria (Reid et al., 1989), skeletal muscle arterioles (Ungvari et al., 1999), bladder (Waring and Wendt, 2000), renal arteries (Inazu et al., 1991), rat tail artery / vein (Ramanadham et al., 1984; Tam et al., 1997), and gastric fundus (Sakai et al., 1994) after induction of diabetes with STZ (early stages).

From studies in humans, Bodmer and co-workers (1999), demonstrated an exaggerated sensitivity to NA-induced vasoconstriction in the dorsal hand vein from diabetic patients with microalbuminuria, a predictor of micro- and macrovascular complications, as compared to non diabetic patients and normoalbuminuric diabetic patients (Boder et al., 1999). Furthermore, Christlieb et al (1976) reported an increase in vascular responsiveness to infused angiotensin II and NA in subjects with retinopathy (Christlieb et al., 1976). Together these two studies suggest that in a diabetic state a non specific increase in the vascular smooth muscle function to vasoconstrictor agonists occurs (Waring and Wendt, 2000).

A range of mechanisms has been proposed to explain vascular hyperresponsiveness to vasoconstrictor agents in diabetes. One suggested mechanism relates the contractile response to the enhanced extracellular influx of calcium through either voltage-dependent or receptor-dependent calcium channels (White and Carrier, 1990). Indeed, the uptake of radioactively labelled calcium induced by NA or serotonin, was shown to be significantly greater in diabetic rat renal arteries (Inazu et al., 1991) than in control groups. Numerous studies have demonstrated that the voltage-dependent calcium channel agonist, BAY K 8644, produces a greater force of contraction in the mesenteric, renal, and isolated skeletal muscle arterioles from STZ-treated rats than in tissues from corresponding control rats (Inazu et al., 1991; Ungvari et al., 1999; White and Carrier, 1990). These authors suggested that diabetes might have increased the activity and / or number of calcium ion channels. In another report by Waring and Wendt (2000), it was postulated that there are no major impairments in either intracellular calcium regulation or the contractile function in the bladder smooth muscle after 8 weeks of STZ-induced diabetes.
However, a non-specific enhancement in the contractile force was observed in these rats, which was associated with an increase in the intracellular calcium level. This change implied that the apparent sensitivity to intracellular calcium is enhanced in the bladder smooth muscle from diabetic rats (Waring and Wendt, 2000).

Another mechanism, which may contribute to enhanced contractile responsiveness, involves the pathway wherein phosphatidylinositol 4’, 5’-bisphosphate is hydrolyzed to generate inositol triphosphate (IP₃) and DAG. IP₃ can release intracellular calcium, whereas DAG activates PKC to initiate extracellular calcium influx and vascular contraction (Abebe and MacLeod, 1990; Legan, 1989). One study showed a decrease in PI hydrolysis after 4 weeks of diabetes and suggested that the mobilization and utilization of calcium ions during contraction occur independently of the receptor-stimulated PI hydrolysis (Legan, 1989). A change in the PKC-mediated contractile response may also contribute to the hyperresponsiveness of diabetic vascular preparations. The agent phorbol dibutyrate, which mimics DAG and its activation of PKC, produced contractions in the aorta and mesenteric artery from diabetic rats which were greater than those from control rats (Abebe and MacLeod, 1990; Kawasaki, 1997). Up-regulation of PKC was also supported by other studies (Sakai et al., 1994; Ungvari et al., 1999), where each study suggested alternative possible causes. For example, the former suggested an increase in the activity of the voltage-dependent calcium channel as a possible cause and the latter study ruled out the possibility of an alteration in calcium channel density. Ramanadham and co-workers (1984) have demonstrated supersensitivity to alpha-adrenoreceptor agonists NA and methoxamine with a simultaneous decrease in catecolamine content, in a caudal artery from STZ-treated rats of 4 weeks duration. It was suggested that autonomic neuropathy, leading to the depletion of caudal artery-NA stores, could induce a supersensitivity phenomenon as a consequence of changes in alpha-adrenoreceptor numbers and / or affinity (Ramanadham et al., 1984).

Another theory was that increased vascular smooth muscle reactivity to vasoconstrictor agents in diabetes may occur as a consequence of a diabetes-induced impairment in the opposing vasodilatation response mediated by endothelium-derived NO (Chang and
Stevens, 1992). Indeed, enhanced sensitivity of aorta from diabetic rats to phenyephrine was abolished and restored to control levels after removal of the endothelium.

In contrast to these findings, there are a number of reports demonstrating a decrease or no change in the reactivity of vascular smooth muscle to vasoconstrictor agents. Diminished contractile responsiveness to NA was reported in the cremaster muscle (Mayer and Messina, 1996), aorta (Wong and Tzeng, 1992), and caudal artery (Ramanadham et al., 1984) of rats with 4 weeks of diabetes. Similar reduction in contractile responses were obtained using vasoconstrictor agents including NA, adrenaline, serotonin, KCl, and ET-1, in the kidney (1 week) (Garcia et al., 1999), mesenteric artery (2 weeks) (Makino and Kamata, 1998), aorta (2, 8, 12 and 14 weeks) (Fulton et al., 1991; Hattori et al., 1996; Hopfner et al., 1999; Orie and Aloamaka, 1993; Utkan et al., 1998), cremaster muscle arterioles (4-8 weeks) (Mayer and Messina, 1996), atria (8-12 weeks) (Lieu and Ried, 1994), and caudal artery (12-16 weeks) (Tam et al., 1997) of STZ-induced diabetic rats.

Several possible mechanisms could contribute to the diminished contractile ability of diabetic preparations. These include impaired extracellular influx of calcium, due to alterations in calcium channel activity or alterations in intracellular calcium binding and storage, contributing to defective excitation-contraction coupling; reduced ATP levels, leading to decreased contractile ability; and defective contractile protein sensitivity or function (Hattori et al., 1996; Pfaffman et al., 1982; Zemel, 1995).

Other studies have demonstrated no change in reactivity to vasoconstrictor substances by diabetes in rat caudal artery (Vo and Reid, 1995), aorta (Fulton et al., 1991) and portal vein (MacLeod and McNeill, 1985). Numerous reports have shown that effects of diabetes on vascular smooth muscle reactivity are dependent on the duration of the disease. Orie and Aloamaka (1993) showed the responsiveness of isolated aortic rings to NA and 5-hydroxytryptamine (5-HT) was significantly enhanced after 1 and 4 weeks of diabetes but not after 12 weeks (Orie and Aloamaka, 1993). In another study in vivo, direct measures were made of the arteriolar response to NA in an intact microvascular bed using rats in acute and chronic stages of diabetes (2 to 32 weeks) (Morrif, 1990). Results showed normal responses from large arterioles at all stages of diabetes studied,
whereas smaller arterioles were hyperresponsive at the acute stage of the disease but returned to normal sensitivity as the duration of diabetes progressed to the chronic stage (32 weeks). The dependency of diabetes-induced changes to the duration of this disease has similarly been demonstrated by a number of other investigators (Legan, 1989; MacLeod and McNeill, 1985; Wong, 1996).

It is apparent that the reported effects of diabetes on smooth muscle reactivity to vasoconstrictor agents are inconsistent. Factors that may contribute to differences between studies include variation in vascular preparations, the duration of diabetes, dose of the diabetogenic agent, and the route of its administration. Nevertheless, the majority of reports indicate that contractile responsiveness to NA and to other vasoconstrictor agents is enhanced by the diabetic state. This effect in itself may particularly be dependent on the duration of the disease and the type of tissue under investigation.

1.5.4.2. *Vasodilator responses*

The importance of vascular endothelium in generating a number of vasoactive substances has been well established. A physiological role for endothelium-derived NO has been proposed in the regulation of vascular smooth muscle tone and in the modulation of vascular smooth muscle reactivity (Becker et al., 2000; Ralevic et al., 1995b; Rongen et al., 1994; Singh and Evans, 1997) (see also section 1.9.1). Therefore, any alteration to the endothelium may contribute to the development of vascular complications including those that occur as a result of diabetes mellitus. Endothelial cell damage has been previously documented in animals and patients with diabetes (Chan et al., 2000; Lorenzi and Caglieri, 1991).

Morphological changes include enhanced endothelial adhesion of leucocytes, platelets, and fibrin-like material; increased endothelial cell proliferation; increased number of multinucleated endothelial cells; thickening of basement membrane; increased endothelial cell surface area; increased fluidity of endothelial cell membrane; and increased capillary diffusion capacity (Chan et al., 2000).
Previous reports are indicative of abnormal endothelium-dependent vasorelaxation in the diabetic state (Kobayashi and Kamata, 1999a; Pieper, 1999). However, studies assessing endothelial function in both animal and human models of type I diabetes have produced conflicting results. In experimental animal models, variation in some factors between different studies including duration of diabetes, vascular bed under study, and the presence or absence of diabetic complications may account for conflicting results. There is substantial evidence that vasodilatation, mediated by endothelium-derived NO, is impaired in animal models of diabetes (Azadzoi and De Tejada, 1992; Brands and Fitzgerald, 1998; Palmer et al., 1998; Teraka et al., 1999). The importance of disease duration has been clearly demonstrated in aortic tissues. For example, endothelium-dependent relaxations produced by ACh and histamine were significantly attenuated in 8-10 weeks STZ-induced diabetic rats (Kobayashi and Kamata, 1999a; Oyama et al., 1986), whereas responses to the endothelium-independent relaxant agent, sodium nitroprusside (SNP), were unaffected (Oyama et al., 1986). However, evidence exists that this defect in diabetic aorta may occur as early as 2 weeks after induction of diabetes (Zhu et al., 1999) whereas one study showed no change at 4 weeks (Kobayashi and Kamata, 1999a). These reports, however, seem to agree with results in a recent study by Pieper (1999) who demonstrated a relationship between the triphasic response of aorta and the disease duration. The author showed an increase in endothelium-dependent relaxation to ACh after 24 hrs, normal response after 1-2 weeks, but impaired after 8 weeks of diabetes, compared to controls. Again, in this study, the vascular response to another endothelium-independent relaxing agent, nitroglycerine, was unaltered at all stages (Pieper, 1999).

Further studies demonstrated diabetes-induced attenuation of endothelium responses, in vitro, in rat hind limb vessels (Brands and Fitzgerald, 1998) and in vivo, in cerebral, hindquarter (Diederich et al., 1994; Kiff et al., 1991b; Mayhan et al., 1991), and sciatic nerve arterioles of rats (Teraka et al., 1999). There is a substantial difference in timing between impairment of the endothelial-dependent relaxation and the onset of diabetes in different vascular beds. The range is from 7-11 days in rat hind limb (Brands and Fitzgerald, 1998) and intestinal arterioles (Lash and Bohlen, 1991) to between 3 and 6 weeks in rat cremaster muscles (Alsip et al., 1996) and mesenteric arteries (Diederich et al., 1994; Kiff et al., 1991b; Palmer et al., 1998). Relaxations to SNP and glyceryl
trinitrate were not significantly altered in different diabetic tissues (Brands and Fitzgerald, 1998; Kiff et al., 1991b).

A similar disparity has been observed in studies using resistance vessels, with endothelium-dependent relaxation being found to be normal (Gebremedhin et al., 1999), impaired (Alsip et al., 1996; Taylor et al., 1995) or even enhanced (Heygate et al., 1995). Again, the disease duration and differences in the vascular bed under study could play an important role in observed discrepancies.

Abnormalities in diabetic vascular tissues were shown to occur in humans. For example, in type 1 diabetic patients, using the occlusion plethysmography technique, impairment (Elliott et al., 1993; O'Driscoll et al., 1997), no change (Huvers et al., 1999), or enhancement (Makimattila et al., 1997) was observed in endothelium-dependent relaxation. Most studies using the above technique found that the vascular response to SNP was not changed. Using the vascular Doppler ultrasound technique also showed conflicting results, with endothelium-dependent vascular responses being impaired (Zenere et al., 1995) or unchanged (Enderle et al., 1998). The vascular response to glyceryl trinitrate was found to be either impaired (Zenere et al., 1995) or unchanged (Enderle et al., 1998). The difference in these results could be due to variations in the degree of acute hyperglycaemia and the effect of glycaemic control (Huvers et al., 1999; O'Driscoll et al., 1997), sex differences (Huvers et al., 1999; Makimattila et al., 1997), and effects of diabetic complications (Elliott et al., 1993; Makimattila et al., 1997). A role for a decrease in the stimulated release of endothelium-derived NO in diabetes was not supported by these studies (both diabetic animals and humans) since no change or enhancement in endothelium-dependent vascular response was observed.

Mechanisms by which diabetes contributes to endothelial dysfunction are not completely understood, but hyperglycaemia may initiate it. The first substantive evidence linking hyperglycaemia and endothelial dysfunction was shown by Tesfamariam and co-workers (Tesafemariam et al., 1990; Tesfamariam et al., 1991). These authors showed exposure of arteries to elevated concentrations of glucose (in vitro) caused impairment in endothelium-dependent relaxation.
Hyperglycaemia-induced endothelial dysfunction may result from the decreased synthesis and / or release of endothelium-derived NO, decreased responsiveness of the smooth muscle to relaxing factor, release of endothelium-derived constricting factors, and accelerated destruction of NO. Impaired functioning of other endothelium-derived vasodilator factors such as prostacyclin and endothelium-derived hyperpolarizing factor (EDHF) could also be induced by the diabetic state. EDHF is defined as the substance which produces vascular smooth muscle hyperpolarization and this effect cannot be explained by NO or by a cyclo-oxygenase product such as prostacyclin.

Decreased production of NO may be responsible for impotence in some men with diabetes, since isolated strips of corpus cavernosum from these men were less sensitive to the relaxant effect of ACh and nerve stimulation than from non-diabetic men (Saenz De Tejada et al., 1989). Pre-treatment of isolated tissues from STZ-treated rats with L-arginine (L-Arg), the precursor of NO synthesis, reversed the impairment in relaxation to ACh in diabetic aortic rings, but had no effect on responses from the control group (Pieper and Peltier, 1995). This suggests that utilization of L-Arg by NOS to produce NO may be defective in diabetes. Aldose reductase, an enzyme present in endothelial cells, requires NADPH for converting glucose to sorbitol in glucose metabolism through the polyol pathway. NADPH is also an essential cofactor for NOS and synthesis of NO, and its depletion, as a result of hyperglycaemia, could lead to a reduction in NO synthesis. Aldose reductase inhibitors have shown early promise in reversing glucose-induced changes in sorbitol and myo-inositol metabolism and endothelial dysfunction in experimental diabetic animals (Cameron et al., 1997a; Sarges and Oates, 1993) (see also section 1.5.1).

Some studies show that the release of NO is impaired in experimentally induced DM. In one study, the vasodilator effect of insulin in skeletal muscle was shown to be mediated via an increase in NO release (Steinberg et al., 1994). Therefore, in the presence of insulin deficiency in type I diabetes there could be a reduction in NO release and this could contribute to the endothelial dysfunction. Basal NO release can be inhibited by L-Arg analogue inhibitors of NOS, N\(^G\)-nitro-L-arginine methyl ester (L-NAME). The
ability of this substance to increase the mean arterial blood pressure and to produce vasoconstriction in the rat hindquarter vasculature was found to be attenuated in diabetic animals (Kiff et al., 1991b). Also L-NAME was less effective in producing decreases in skin blood flow in diabetic rats than in control rats (Lawrence and Brain, 1992). These studies raised the possibility that beside a possible reduction in NO release, vascular sensitivity to NO may also be diminished in diabetes.

There is substantial evidence that hyperglycaemia-induced endothelial dysfunction is mediated by free radicals (sections 1.5.3) produced through increased arachidonic acid (AA) metabolism (Giugliano et al., 1996; Tesfamariam and Cohen, 1992). It has been shown that in human aortic endothelial cells, prolonged exposure to high concentrations of glucose increases eNOS gene expression and NO release, which is also associated with a pronounced concomitant increase in superoxide anion production (Cosentino et al., 1997). These anions inactivate NO (Gryglewski et al., 1986) and furthermore, they interact with NO, leading to production of peroxynitrite (Jourd'heuil et al., 1997), which may contribute to endothelial dysfunction (Bouloumie et al., 1997; Galle et al., 1992) (section 1.5.3). Superoxide dismutase has been shown to normalise NO-mediated vasorelaxation impaired by increased glucose concentration (Bassirat and Khalil, 2000; Bauer et al., 1999). Interaction between superoxide anions and hydrogen peroxide as well as peroxynitrite itself can also lead to generation of hydroxyl radicals (section 1.5.3, Fig 1-4). A previous study suggested that the synthesis and / or release of hydroxyl radicals might be responsible for the impairment of NOS-dependent vasodilation in diabetic rats (Mayhan and Patel, 1998). This was further supported by Pieper et al (1997), where inhibiting hydroxyl radical formation by DETAPAC markedly enhanced relaxation to ACh in aortic rings from STZ-induced diabetic rats (Pieper et al., 1997b). These studies show that hyperglycaemia could induce increased NO production as well as a reduced NO availability due to inactivation mediated by free radicals. The role of free radicals including hydroxyl radicals in diabetes is discussed in more detail in section 1.5.3.
Activation of PKC, by an increase in hyperglycaemia-induced DAG, has also been suggested as a mechanism for endothelial dysfunction and vascular complications in diabetes (Park et al., 1999) (section 1.8.5.4). Indeed PKC activation is responsible for several vascular alterations in diabetes (Park et al., 1999) including an increase in vascular prostanoids (Tesfamariam et al., 1991). Isolated rabbit aorta exposed to increased glucose concentrations showed impaired endothelium-dependent relaxation to ACh after 10 min treatment with 4-phorbol 12-myristate 13-acetate, a PKC activator (Tesfamariam et al., 1991). The same study demonstrated that indomethacin increased relaxation induced by ACh, suggesting a role for vascular prostanoids, and this abnormal relaxation was restored with sphingosine, a PKC inhibitor (Tesfamariam et al., 1991).

Another proposed mechanism contributing to endothelial dysfunction and diabetic vascular complications is the formation of AGEs. Accumulation of AGEs over time has been shown to reduce NO availability. Previous studies in vitro and in vivo, using a rat model of STZ-induced diabetes, showed that reactive intermediates form in the early stages of the glycation pathway, which then react with and quench NO rapidly (< 5 sec) (Bucala et al., 1991) (section 1.5.2). In addition to NO inactivation, AGEs have been shown to impair the effect of NO on mesangial cell antiproliferation, an early and characteristic lesion of diabetic vasculopathy (Hogan et al., 1992). Furthermore, in diabetic rats, AG has been shown to partially restore endothelium-dependent relaxation (Bucala et al., 1991) and retard the development of diabetic nephropathy (Soulis-Liparota et al., 1996). It should, however, be noted that AG has multiple actions including direct effects on NO generation (Corbett et al., 1992). AGEs could also cause endothelial dysfunction by other means, which are described in section 1.5.2.

Vasodilator agents other than NO, such as prostacyclin and EDHF, are produced by the vascular endothelium, and may also be involved in defective endothelium-dependent vasodilation. The decreased vascular production of prostacyclin in both diabetic animals and humans has been previously reported and alterations in prostacyclin receptor number and / or turnover were also apparent (Hodgson et al., 1992). ACh is capable of producing endothelium-dependent hyperpolarization in vascular smooth muscle, independent of NO
release (Garland and Mcpherson, 1992). While the identity of EDHF is not very clear, the potassium ion (K⁺) is gaining support as a possible candidate (Edwards et al., 1998; Makino et al., 2000) (see also section 1.8.5.1). There are controversial reports on whether diabetes can attenuate functions of EDHF. Recent evidence showed that vasodilatation induced by ACh and K⁺ are both greatly impaired in mesenteric arteries of diabetic rats (Fukao et al., 1997; Makino et al., 2000). However, Endo et al (1995) demonstrated that impaired relaxation to ACh in diabetic rat aorta could not be attributed to a defect in the function of the hyperpolarizing factor-mediated component (Endo et al., 1995).

An array of findings exist describing diabetes-induced functional changes in the vascular smooth muscle reactivity to both vasoconstrictor and vasodilator agents, in both animal models and humans. The majority of reports indicate that vasoconstrictor responsiveness is increased in diabetes and endothelium-derived NO reactivity is impaired. Such alterations may therefore contribute to the development of clinical vascular complications observed in diabetes. It should be noted that vascular responses to endothelium-independent relaxants (e.g. SNP) were mainly unchanged in different diabetic tissues at all stages of diabetes. It is well known that inflammation is an important factor for the tissue repair process. Since none of these experiments were performed on skin with inflammation, further work is required to obtain the relevant information necessary for a deeper understanding of mechanisms involved in delaying wound repair in diabetes.

1.6. Animal models of diabetes

1.6.1. History

Permanent diabetes in animals offers an excellent model for studying the interaction between hereditary and environmental factors such as drugs, toxic, and infectious agents. This is also useful in the determination of therapeutic and preventative measures (Shafrir, 1990).

Surgery, viral infections, hormone administration, and chemical agents can all produce diabetes (Mendez and Ramos, 1994). DM has been documented in monkeys, dogs, rats, cats, shrews, squirrels, seals, dolphins, hippopotamus, antelopes, and different farm animals. Some animals may develop spontaneous diabetes, which is more frequent in
rodents (rats, mice, hamsters), but it is considered as an inadequate model for studying natural development of this disease (Mendez and Ramos, 1994). It is well known that the ethics code of practice in using animals for research purposes should be respected and obeyed by any qualified person who conducts these experiments. All techniques and surgical procedures conducted in this PhD study were approved by the Royal Melbourne Hospital Research Foundation (Animal Ethics Committee) and adhered to IASP (International Association for the Study of Pain) guidelines.

1.6.2. Chemical induction of diabetes

The use of chemical agents to cause diabetes allows for precise studies to be carried out on the biochemical, hormonal, and morphological changes that occur during the induction of the diabetic state and afterwards. Alloxan and streptozotocin are the most widely used diabetogenic agents because of their selectivity for beta cells of the islets of Langerhans (Mendez and Ramos, 1994). A diabetic dosage is defined as the amount of an inducing agent which in 80% of the animals of a given species produces sustained hyperglycaemia and beta cell necrosis, but does not cause harm to other organs (Goldner and Gomori, 1944). Diabetogenic dose can vary depending on the type of animal used, the weight/age, whether they are fasted or fed before the treatment, and the route of its administration.

1.6.2.1. Alloxan

Alloxan was first synthesised in 1838 by F. Wohler and J. Leibig but its diabetogenic property was first reported in 1943, showing islet necrosis in rats (Dunn and McLetchie, 1943). Alloxan is an unstable crystal that is freely soluble in water and causes diabetes in experimental animals by destroying the insulin secreting beta cells of the pancreas, leaving alpha-cells unharmed. Morphological evidence from rat islets treated with alloxan exhibited multiple cellular necrosis, marked degranulation, and extensive vesiculation of endoplasmic reticulum and golgi complex (Abdel-Rahman et al., 1992a). Mitochondrial enlargements with disrupted cristae and mitochondrial ruptures were prominent and this was one of the targets through which alloxan destroyed beta cells.
Alloxan’s stability in aqueous solution is mainly dependent on pH and temperature. Below pH 3 it is fairly stable at room temperature, whereas at pH 7 the solution must be kept under 4 °C and its life in blood is less than 1 min (Rerup, 1970) (Fig1-5).

Fig 1-5: Structure of alloxan (Adopted from Lenzen, S. & Munday, R. (1991)).

Four factors that may be important in determining the toxicity of alloxan are stability, hydrophilicity, reactivity toward thiol groups, and generation of free radicals (Lenzen and Munday, 1991). Hydrophilicity may facilitate its access to those areas of pancreatic beta cells in which its toxic effects may be expressed. Glucose transporter-2 (GLUT-2) is expressed in rodent’s pancreatic beta cells mediating glucose uptake. It was shown that selective uptake by GLUT-2 is not a prerequisite for alloxan diabetogenicity (Munday et al., 1993). Alloxan reacts with thiols and inhibits thiol enzymes such as glucokinase and hexokinase (Lenzen and Panten, 1988).

The mechanism behind alloxan’s diabetogenic effect is not very clear. However, it has been suggested that it damages pancreatic beta cells through formation of superoxide anions and hydrogen peroxide but not hydroxyl radicals (Jorns et al., 1999; Sentman et al., 1999). This causes DNA strand-breaks (Yamamoto et al., 1981) and depletes NAD+ stores (Askar and Baquer, 1994; Le Doux et al., 1988). One study suggested that the generation of free radicals through redox cycling with glutathione was not enough to cause diabetes by alloxan but it seems that hydrophilicity of this compound is a necessary condition for its diabetogenicity (Munday et al., 1993). Administration of alloxan at higher than a diabetogenic dose can cause serious kidney damage (Rerup, 1970).
1.6.2.2. *Streptozotocin*

STZ is a broad-spectrum antibiotic isolated from *streptomyces achromogenes* (Herr et al., 1959). It is a fairly effective carcinogen (Arison and Feudale, 1967) as well as an antileukaemic agent (Evans et al., 1965). During the pre-clinical toxicology studies, it became apparent that when the drug is administered intravenously at a sufficient dose, it produced hyperglycaemia in rats and dogs within a few hours of the treatment (Rakienten et al., 1963). The structure is composed of a nitrosouria moiety with a methyl group attached at one end and a glucose molecule at the other (Fig1-6).

![Structure of Streptozotocin](image)

**Fig 1-6: Structure of Streptozotocin (adopted from Weiss, R.B. (1982)).**

STZ is a colourless crystalline water-soluble powder. When it is dissolved in saline or distilled water at room temperature and neutral pH it decomposes within a few minutes (visible bubbles). In solution, this compound is stable at pH 4 and a low temperature (Weiss, 1982). Its half-life in plasma is between 5 to 35 min and concentrates in the liver, kidney and pancreas but not in the brain. The kidney rapidly eliminates STZ but renal dysfunction is the major dose-limiting toxic effect of this drug. Vomiting is the most noxious side effect of STZ reported in treated patients. Other potential troublesome side effects of this drug are myelosuppression and hepatotoxicity, which are both uncommon (Weiss, 1982).

STZ produces permanent diabetes in some animals (rat, mouse, dog, hamster, monkey and guinea pig) by destroying pancreatic beta cells similar to alloxan. Once again, the diabetogenic dose of STZ is depend on the species, weight / age, if they are fasted or fed, and the route of its administration. In rats, STZ produces hyperglycaemia with normal levels of blood ketones, plasma-free fatty acids and heart glycogen (Weiss, 1982). This is different from alloxan-treated rats where all these parameters are markedly elevated.
(Mansford and Opie, 1968). In rats, the severity of experimental diabetes and its persistence depends on the dose of STZ used. Intraperitoneal injection of STZ at a dose of 45mg/kg is known to cause a transient state of diabetes (Simon and West, 1992). Spontaneous recovery from diabetes was previously reported in rats injected with STZ in doses up to 50mg/kg (Iwase et al., 1991).

A single high dose of STZ can cause fragmentation of DNA in beta cells, through the formation of free alkylating radicals, leading to a reduction in the cellular levels of nucleotides and related compounds, in particular NAD$, causing rapid necrosis (Thulesen et al., 1997). This is supported by Hooresns et al (1999) study which showed nicotinamide, an inhibitor of the NAD$^+$-degrading enzyme (poly (ADP-ribose) polymerase), could protect islet cells against STZ-damage through protection of the intracellular NAD$^+$ pool (Hooresns and Pipeleers, 1999).

The diabetogenic action of STZ in animals is not identical to alloxan but is very similar in many aspects. It was suggested that the nitrosouria moiety is necessary for DNA alkylation and antitumour efficacy, and the sugar moiety is necessary to facilitate islet tissue uptake and minimise bone marrow toxicity (Weiss, 1982). GLUT-2 may mediate the cellular uptake of streptozotocin (Schnedl et al., 1994).

1.6.2.2.1. Role of nicotinamide and vitamin E

Vitamins E and nicotinamide have been previously shown to play an important role in the delay or prevention of diabetes onset in a model of spontaneous experimental diabetes or STZ-treated mouse islet cells (Beales et al., 1994; Kaneto et al., 1999; Strandell et al., 1989). Vitamin E may protect islet cells by reducing cytotoxicity mediated by cytokines and their products as well as inhibiting lipid peroxidation of cell membranes preventing damage by free radicals (Beales et al., 1994; Kaneto et al., 1999). The earliest report about nicotinamide protection against diabetes onset was as early as 1947 and in alloxan-treated rats (Lazarow, 1947), an effect later reproduced with STZ (Dulin et al., 1969). Nicotinamide, the amide derivative of nicotinic acid, offers protection to pancreatic beta cells against toxic stimuli. At high doses it is a free radical scavenger, and a potent inhibitor of poly (ADP-ribose) polymerase, and protects against depletion of intracellular NAD (Gale, 1996). Nicotinamide pre-treatment in, in vivo and in vitro studies, was
capable of protecting islet cells against damage as well as showing other beneficial effects (Petley et al., 1995; Strandell et al., 1989).

1.7. **Neurogenic inflammation**

1.7.1. **History**

Upon tissue injury, inflammation occurs which is a protective response that initiates the process of tissue repair. Inflammation is characterized by a vascular response involving alterations in vessel calibre and blood flow; swelling and exudation which results from increased vascular permeability; the accumulation of inflammatory cells; and changes in ground substance. Sensory nerves play an important role in mediating “neurogenic inflammation” (Brain, 1996; Holzer, 1998) and subsequent tissue repair. The notion that certain sensory nerves are involved in cutaneous inflammation can be traced back to the classical work of Stricker and Bayliss. Stricker (1876) found that stimulation of DRG (the site of the nerve bodies of primary afferent nerves) caused them to conduct impulses centrally, but also showed them to have an efferent function that produce vasodilatation (Stricker, 1876). Later, Bayliss (1901) demonstrated that the observed cutaneous vasodilatation was infact an axon reflex, whereby impulses produced in afferent nerve fibres travel antidromically through collateral fibres (Bayliss, 1901; Maggi, 1995).

The classic signs of inflammation of skin are best described by Lewis (1927) as a “triple response” (Lewis, 1927). This response consists of vasodilatation or an increase in skin blood flow through dilated arteries (manifest as redness and warmth); plasma extravasation (PE) or an increase in the permeability of post-capillary venules to blood-borne proteins, allowing these to escape into the extracellular fluid (seen as swelling); and hyperaemia (flare) arising from axon reflexes that takes place between collaterals of single sensory nerve fibres. Injury to the skin and stimulation of small diameter afferent sensory neurons can send impulses orthodromically to the brain producing the pain component of the inflammatory process and antidromically to the spinal cord producing responses similar to inflammatory “triple response”.
1.7.2. Sensory nerves

Cell bodies of human skin sensory nerves are found in the DRG of the spinal cord. Axons from these cells travel centripetally to connections in the spinal cord and peripherally in main trunks of mixed nerves to reach the cutis, where they serve functions of pain, temperature and other tactile sensations (Wood and Bladon, 1985). In the skin many sensory neurons terminate in specialized receptors but many end in unmyelinated free-nerve endings. These latter tend to form plexus from which they branch between the cells to detect touch and pain stimulus.

Pain results from the activation of nociceptors that are divided into 2 types: high-threshold mechanoreceptors (usually arising from Aδ fibres) (Burgess and Pearl, 1967), and polymodal nociceptors that are associated with 80% of C-fibres. The former responds to strong pressure, heat, and skin damage (Lynn, 1989). The latter responds to heat, pressure, and irritant chemicals and are the most common sensory unit in the skin. An important point is that polymodal nociceptors can be especially sensitized by the presence of endogenous substances including ACh, BK, histamine, and prostaglandins.

Primary afferent sensory nerves contain several neuropeptides including SP and other tachykinins (neurokinin A (NKA), neuropeptide K, neuropeptide gamma, etc), CGRP, vasoactive intestinal peptide (VIP), cholecystokinin, angiotensin II, somatostatin, galanin and dynorphin. Upon activation, some of these sensory neuropeptides are released at the peripheral terminal to initiate inflammatory responses (Maggi, 1991).

It has been shown that unmyelinated small diameter nociceptive C-fibres are prerequisite for neurogenic inflammation induced by chemical or mechanical irritations (e.g. capsaicin, electrical nerve stimulation (ES)) (Brain, 1996; Holzer, 1991; Szolscanyi et al., 1988). It was suggested that in diabetes mellitus these fibres are altered (Gyorfi et al., 1996; Walmsley and Wiles, 1991) and neurogenic inflammatory and anti-inflammatory responses are impaired (Nemeth et al., 1999b). The aim of the study, described in chapter 3, is to examine the effect of diabetes on sensory nerve function as determined by the ability of these nerves to mount an inflammatory response.
1.7.3. Contribution of SP and CGRP to Neurogenic Inflammation

Neurogenic inflammatory responses can be induced experimentally in a number of ways. They include the exogenous application of capsaicin, introducing exogenous sensory neuropeptides themselves (e.g. SP, CGRP) or indirectly through modulators such as histamine. However, the most popular technique involves the use of antidromic nerve stimulation, which is described in the next section (1.7.4). The tachykinin SP, with 11 amino acids, has been located in the small unmyelinated nerve fibres within the sensory nerves that terminate in the superficial dorsal horn of the spinal cord; and is present in both the peripheral and central endings of these nerves (Hokfelt et al., 1981). Most of the SP synthesized in the cell bodies of the DRG is transported to peripheral nerve terminals where it is released by nerve stimulation (e.g. antidromically) (Khalil et al., 1994). SP can produce many of the physiological changes associated with inflammation including vasodilatation and PE (Khalil and Helme, 1990b), the activation of macrophages (Brunelleschi et al., 1990), and degranulation of mast cells with a local release of histamine (Johnson and Erdos, 1973; Khalil and Helme, 1989).

When SP is released, it can act upon neurokinin-1 (NK-1) receptors on the endothelium to activate phosphatidyl-inositol-bisphosphate (PI) pathway. This increases intracellular calcium and activates NOS causing the release of NO. NO can then diffuse into smooth muscle resulting in its relaxation and subsequent vasodilation (Khalil and Helme, 1989; Ralevic et al., 1992). SP-induced increase in blood flow is short acting and accompanied by tachyphylaxis (Khalil and Helme, 1996; Khalil et al., 1994). SP is also capable of activating membrane-linked G-proteins on the membrane of mast cells which causes degranulation and subsequent histamine release (Johnson and Erdos, 1973; Khalil and Helme, 1989). The latter then acts on histamine-1 (H1) receptors on sensory nerve terminals causing further release of sensory neuropeptides. SP acts more on mast cells when it is administered exogenously and at relatively high concentrations (Tausk and Undem, 1995).

SP (through NK-1 receptors) and NKA are the main messengers for increase in vascular permeability (Holzer, 1998). The early phase of the neurogenic- and SP-induced extravasation arises from a direct action of the peptide on the endothelium post-capillary
venules, whereas the later phase depends in part on the release of 5-HT, histamine, prostaglandins, leukotrienes, and other mediators from mast cells (Brain, 1996; Walsh et al., 1995). The initial phase of the exudative response takes place in the superficial dermis where peptidergic afferent nerve fibres are located with few mast cells. Although there are contact sites between afferent fibres and mast cells in the skin, most mast cells are located in a deeper layer of the dermis and hence are mainly involved in the post-acute phase of neurogenic inflammation (Kowalski et al., 1990).

CGRP, a 37 amino acid peptide, is found in C- and Aδ-fibres and commonly co-localized with SP (Mione et al., 1990). Release of CGRP can occur via ES of sensory nerves (at specific stimulus parameters) (section 1.7.3), and/or activation of receptors for inflammatory mediators (such as capsaicin, histamine, and BK). NO and prostaglandins, which are produced at inflammatory sites also have potential to modulate the release and actions of CGRP (Brain, 1996). Receptors for CGRP have been identified on vascular smooth muscle and two receptor subtypes have been verified and designated as CGRP₁ and CGRP₂, according to their sensitivity to the receptor antagonist CGRP₈-₃₇. The latter subtype is unaffected by actions of the antagonist (Dennis et al., 1990). CGRP₁ receptors are found in skin and cardiovascular tissues, whereas CGRP₂ receptors are located in the vas deferens, aorta, and urinary bladder.

CGRP is a potent vasodilator in many preparations (Holzer, 1992), but the most important physiological action of CGRP is its vasodilatation effect (long acting) on the microvascular system (Brain, 1996). The peptide-induced hyperaemia (e.g. in skin), which is mediated by CGRP₁ receptors that are coupled to the adenylate cyclase system, arises from a direct action on the vascular smooth muscle and does not involve NO or prostaglandins (Brain and Williams, 1988). CGRP can also induce vasodilation mediated by NO such as in rabbit skin (Hughes and Brain, 1994). One way to show that CGRP is involved in neurogenic vasodilatation was by pharmacological antagonism of the endogenously released peptide. It was reported that dilatation of cutaneous arterioles evoked by electrical or chemical stimulation of afferent fibres was inhibited by the CGRP₁ receptor antagonist, CGRP₈-₃₇. CGRP is unable to induce an increase in PE, however it is capable of enhancing exudative responses to sensory nerve stimulation, tachykinin and a variety of inflammatory mediators (Brain, 1996). This facilitation of PE
is thought to result from the peptide’s vasodilator activity, although inhibition of SP degradation by CGRP may also play a role (Holzer, 1992). Initially, SP was promoted as a major mediator of neurogenic inflammation; this was likely due to historical reasons in that SP was discovered much earlier than CGRP. However, with the development of specific receptor antagonists for the two sensory neuropeptides, interactive involvement of CGRP and SP in vascular responses has been determined. Although some interaction effects were shown between these two peptides, several lines of evidence indicate that SP is the major mediator of the PE responses. This is supported by the ability of NK1 receptor antagonists and SP immunoneutralization to inhibit neurogenic PE to a variety of stimuli (Brain, 1996; Walsh et al., 1995). On the other hand, CGRP has now been shown to be more prominent in local neurogenic vasodilator responses and is the principal transmitter involved in neurogenic dilatation of arterioles in all species that have been tested (Brain, 1996). This is supported by studies using CGRP8-37 and an NK-1 receptor antagonist (SR140333), both showing inhibition of PE responses following heat injury in the rat skin (Siney and Brain, 1996). SP is also known to be capable of terminating an existing vasodilator response to CGRP (Brain and Williams, 1988).

In addition to their action on the vascular system, SP and CGRP can influence the activity of granulocytes, monocytes and lymphocytes (Holzer, 1998). They stimulate not only adhesion of leucocytes to the vessel wall and their migration into the inflamed skin tissue (Brain, 1997) but can also affect the release of mediator substances from white blood cells. SP induces monocytes to release cytokines and prostanoids, stimulates basophils to release histamine, and activates neutrophils to produce free radicals (Brain, 1996; Brain, 1997; Holzer, 1992). Many of these mediators are vasoactive and are likely to exert a modulatory action on vessel diameter and permeability.

One possible reason for reduction in neurogenic inflammatory responses observed in experimental animals and patients with diabetes (Gyorfi et al., 1996; Nemeth et al., 1999b; Walmsley and Wiles, 1991) may be related to the reduction in tissue sensory neuropeptide content (Diemel et al., 1992; Karanth et al., 1990). So far reports are controversial and it seems that the type of tissue under investigation and duration of diabetes play a significant role in observed differences. A previous study on skin samples
(lip & footpad) of STZ-induced diabetic rats showed no change in the distribution or relative density of immunoreactive nerve fibres at 2, 4 or 8 weeks (Karanth et al., 1990). However, in the same study, a marked increase in CGRP-immunoreactive fibres was observed at 12 weeks in the dermis and epidermis in these rats. In a different study, the SP-like immunoreactivity (SPLI) was increased in skin at 11 weeks but no change was observed in atrial myocardium tissue of STZ-treated rats (Willars et al., 1989). Also, reductions in SPLI and preprotachykinin mRNA level were demonstrated in the gastrointestinal tract (11 weeks) (Willars et al., 1989) and anterior pituitary of STZ-treated rats (6 weeks) (Rittenhouse et al., 1996b). SPLI in arterial wall of 6 weeks diabetic rats was, however, increased by 2 fold (Markle and Walker, 1996) but strict insulin treatment partially (significantly) reversed this effect towards normal. Recently, treatment with insulin was also demonstrated to prevent loss of neurogenic inflammatory responses (vasodilatation and PE) in diabetic rats (8 weeks) and NGF pre-treatment managed to return these responses towards normal (Bennett et al., 1998). This author showed that insulin was more effective in reversing the neurogenic oedema but that both agents appeared equally effective in restoring the neurogenic vasodilator response. These results extended previous findings, where insulin showed beneficial effects on neurogenic inflammatory responses in diabetic rats and NGF was able to restore sensory nerve-mediated microvascular function in diabetes (Biessels et al., 1996; Diemel et al., 1992). The improvement in neurogenic responses by insulin may be related to inhibition of peptide loss. Insulin, by reducing high levels of glucose in the nerve, can prevent formation of glucose by-products against neuronal oedema and nerve ischaemia and hence prevent impairment in peptide expression and transport (Diemel et al., 1992). The role of NGF could be related to improvement in SP production, since SP and CGRP genes are known to be regulated by NGF (Lindsay et al., 1989). Results in Bennett’s study (1998) also suggested that neurogenic responses in diabetic rats were not markedly affected by modulation of SP or CGRP neuropeptide receptor-mediated mechanisms.

1.7.4. Electrical stimulation of the sciatic nerve

Neurogenic inflammatory responses can be induced experimentally by antidromic nerve stimulation (chapter 2, section 2.7) whereby sensory nerve fibres are electrically
stimulated to mimic the axon-reflex phenomenon, leading to the release of sensory neurotransmitters. The rat sciatic nerve is a frequently used peripheral nerve in experimental studies. This nerve originates from the spinal lumbar ganglia L3-L6 (Swett et al., 1991), and its branches innervate muscles and skin of the leg including the planter surface of the rat foot. Although electrical stimulation of the sciatic nerve relies on an invasive procedure in terms of nerve preparation, the main advantage of antidromic stimulation is that the physiological responses observed are induced via the endogenous release of peptides.

Antidromic vasodilatation and PE can occur across a range of frequencies (1-15Hz) but this must be accompanied by high intensity voltages that are capable of activating C-fibres (White and Helme, 1985). The parameters used in this study were 20V, 5Hz, 2mSec for the duration of 1 min (Merhi et al., 1998). These parameters were shown to cause sufficient sensory neurotransmitter release to cause vasodilatation in the rat hind footpad but does not cause PE due to the short stimulation period (Lembeck and Holzer, 1979; White and Helme, 1985). It is known that stimulation parameters that activate small unmyelinated sensory nerves will also activate the sympathetic efferents (Kawasaki et al., 1990). Neurotransmitters of sympathetic origin, including NA and neuropeptide Y (NPY), can modulate the action of sensory nerves at both pre- and post-junctional sites. The role of sympathetic nerves in modulating sensory nerve responses is described in section 1.8.3 (see also chapter 5).

When the sciatic nerve is stimulated antidromically, sensory neurotransmitters such as SP and CGRP, which are synthesized in the DRG and transported (axonal transport) to the nerve terminals, are released in the periphery to induce neurogenic inflammatory responses. A reduction in content and transport of SP and CGRP was reported in sciatic nerves of diabetic rats at 4 weeks (Di Giulio et al., 1995; Diemel et al., 1992) and 11 weeks (Willars et al., 1989). This coexisted with a decrease in SP and CGRP content of lumbar DRG (Terenghi et al., 1994) and the footpad skin (Karanth et al., 1990). It was shown that the metabolism and synthesis of SP and CGRP are dependent upon NGFs uptake and retrograde transport and this is impaired in the sciatic nerve of diabetic rats.
(3-4 weeks) (Diemel et al., 1992; Lindsay et al., 1989; Schmidt et al., 1999). Nemeth and co-workers (1999a) used isolated diabetic rat trachea and showed the release of SP, CGRP and somatostatin to be substantially diminished in response to electrical field stimulation after 4 weeks and suggested the cause to be of neuronal origin. Impairment in nerve axonal transport (both anterograde and retrograde) and motor nerve conduction velocity (Di Giulio et al., 1995; Lockett and Tomlinson, 1992) have also been reported in experimental diabetic rats. There is also evidence that abnormal sciatic nerve myoinositol metabolism (Finegold et al., 1983) in experimental diabetes impairs axoplasmic flow (Mayer and Tomlinson, 1983) and reduces Na⁺/K⁺-ATPase activity (Greene and Lattimer, 1983) by affecting phosphoinositide turnover (Lockett and Tomlinson, 1992). Insulin was shown to be able to reverse alterations in nerve conduction velocity (sciatic nerve, ascending and descending pathway of the spinal cord) up to 6 months after induction of diabetes in STZ-treated rats (Biessels et al., 1999). It was suggested that the reduction in glucose level might be responsible for this improvement although insulin’s direct effect on the vasculature was not overlooked. Some aldose reductase inhibitors were shown to have an effect on nerve conduction velocity and axonal transport in diabetic rats (Cameron et al., 1997a; Hotta et al., 1996). For example, acetyl-l-carnitine (ALCAR) prevented diabetes-induced SP loss in the sciatic nerve and spinal cord by improving altered metabolic pathways and hence preventing the reduction in synthesis and axonal transport of SP (Di Giulio et al., 1995) (section 1.5.1). All findings in this section and the previous section indicate that reduction in neurogenic inflammatory responses are probably due to a combination of reduction in synthesis and transport as well as the release of sensory peptides in diabetes.

1.7.5. Measuring NI

A variety of different techniques have been used to monitor and quantify changes in blood flow and have been reviewed by a number of authors (Oberg, 1990; Rowell, 1993). Examples of these techniques are plethysmography, Doppler ultrasound flowmetry, thermal and dye-dilution, and radioisotope infusion. Antidromic cutaneous vasodilatation has also been measured using these various methods.
For example, Newby and co-workers (1999) used venous occlusion plethysmography to measure changes in blood flow of the human forearm after SP infusion, whereas Arora and colleagues (1998) measured vasodilatation responses to iontophoresis of ACh and SNP in forearm skin using Doppler ultrasound flowmeter. Others have collected venous outflow from the hind limb (Lembeck and Holzer, 1979). While the above methods provide quantitative information about blood flow, some are invasive in nature and both demanding and time consuming to perform. Furthermore, they do not accurately represent dilatation changes in the cutaneous microvasculature since they measure overall blood flow in a larger area than skin innervated by the stimulated nerve.

Laser Doppler flowmetry is a non-invasive, simple-to-perform technique that produces reproducible and reliable measurements of relative blood flow. This technique was developed in early 1970’s but was first adopted for use in rat skin by Gamse & Saria in 1987, where they showed its suitability as a non-invasive technique for measuring antidromic vasodilatation. This technique has also been used for early diagnosis of diabetic microangiopathies (Balabolkin et al., 1994; Rendell et al., 1993). The helium-neon laser light (632.8 nm) was used to measure blood flow based on a Doppler principle, where the resulting photocurrent of reflected laser light proportional to the number and mean velocity of moving red blood cells is converted to a continuous electrical input signal (in volts). The effective depth of laser penetration into the skin has been previously reported to be approximately 1-2 mm (Halloway and Watkins, 1977; Rendell et al., 1993) and Rendell et al (1998) have estimated the area of measurement to be about 2 mm². The principle and mechanism underlying laser Doppler flowmetry and 2 different types of laser Doppler flowmeters (LDFs) used in this study (Perimed and Moor) are described in full detail in chapter 2, section 2.9.

PE, the other major feature of inflammation initiated by antidromic nerve stimulation, has also been extensively studied. This is commonly assessed using Evans blue dye or by the Bradford technique (Bradford, 1976). In experiments where inflammatory mediators were perfused over the blister base, PE was collected and the level of protein in the exudate was analyzed using the Bradford technique (see also chapter 2, section 2.12). In experiments where the sciatic nerve was stimulated electrically, it was not possible to
measure PE due to the short stimulation period (1 min) and only changes in blood flow was measured.

1.8. Modulators of sensory nerve function

1.8.1. Nitric oxide

NO was first described as an endothelium-derived relaxing factor by Furchgott and Zawadzki in 1980. Because of their substantially similar actions, it was deduced that NO and EDRF were the same agent (Ignarro et al., 1987). NO is synthesized as a mediator of vasodilatation from its precursor L-Arg by NOS, which is present in an inducible form and a constitutive form. Inducible NOS (iNOS) is cytosolic, requires protein for its expression and stimulated in immunological reactions by cytokines and bacterial wall proteins (Moncada and Higgs, 1993). This form of NOS is calcium-independent, and produces nanomoles of NO over long periods but is dependent on NADPH (Moncada et al., 1991). Many cell types including macrophages, vascular smooth muscle cells, Kupffer cells, and hepatocytes are capable of expressing iNOS. Constitutive NOS is both cytosolic and particulate, is NADPH-dependent, requires calcium / calmodulin, and releases picomoles of NO for short periods in response to receptor stimulation by calcium-mobilizing agonists (Moncada and Higgs, 1993; Moncada et al., 1991). This type of NOS is localized in vascular endothelial cells, central and peripheral nervous tissue, and platelets. NOS has been further divided into three isoforms: Neuronal NOS (nNOS) describes the constitutive form localized in neuronal tissues; iNOS identifies the inducible form; and eNOS represents the constitutive form found in vascular endothelial cells. Each of the NOS isoforms found in brain, endothelium, macrophages and various other cells from animals or humans, have been successfully purified and synthesized (Nankane et al., 1993; Ohshima et al., 1992; Pollock et al., 1991; Xie et al., 1992). The physiological actions of NO per se relate not only to its identity as a free radical species (or NO•), but also to its other redox forms: the nitrosonium cation (or NO⁺), and the nitroxy anion (or NO⁻) (chapter 1, section 1.5.3, Fig 1-4) (Butler et al., 1995). Hence, under physiological conditions, the term NO does not identify its redox forms but refers to a variety of NO-containing molecules which may be present.
NO plays an important role in cell communication, defence, and injury. The biological effect of NO depends upon both its concentration at the site of action as well as the specific location where it is generated. Small quantities of this agent are generated by constitutive NOS such as that present in the vascular endothelium, while large quantities of NO are synthesized by iNOS in response to cytokines or bacterial products. As a ubiquitous biological mediator, NO has been shown to have physiological roles in diverse settings as well as being implicated in the pathogenesis of some diseases (e.g. hypertension, dementia). There have been many reviews detailing various roles of NO (Moncada et al., 1991; Singh and Evans, 1997) but the following discussion will be limited to the issues relevant to this PhD study.

NO has a short half-life but is a potent endogenous vasodilator. Many of the target cell responses of NO, such as the regulation of cardiovascular tone and the mediation of neurotransmission, result from an increase in intracellular levels of cyclic guanosine monophosphate (cGMP) via the stimulation of soluble guanylate cyclase (Moncada et al., 1991). A continuous basal release of NO from vascular endothelial cells is suggested to provide a constant vasodilator tone in the vasculature. This is supported by previous studies in humans (Vallance et al., 1989) and rats (Rees et al., 1989) using NOS inhibitor. These findings, as well as other studies in the literature, led to the conclusion that there is a physiologic, NO-dependent vasodilator tone that is essential for regulating blood flow and pressure (Moncada and Higgs, 1993).

As an intracellular signalling molecule, NO has important physiological roles in nociception, neuronal degeneration, and neuroprotection (Lipton, 1999; Meller and Gebhart, 1993). There are growing numbers of reports to propose neurotransmitter roles for NO within the peripheral sensory nervous system. An immunological study has demonstrated the existence of NOS in neurones and fibres of the DRG (Ruda et al., 1994) suggesting that NO is released from sensory nerves. NOS immunoreactivity is also found in peripheral nerves in the myenteric plexus of the intestinal tract, in the retina and the adrenal gland (Bredt and Synder, 1990). Furthermore, NO was shown to be released from mast cells and endothelium (Moncada et al., 1991). Taken together these observations indicate that NO is well placed to mediate its effects in the periphery.
There is evidence showing that NO interacts with sensory neuropeptides such as CGRP. A previous study using the NOS inhibitor, L-NAME, in rabbit skin microvasculature showed that the release of CGRP from sensory nerves was NO-dependent but not its mechanism of vasodilator action (Hughes and Brain, 1994). In a different study it was suggested that NO could contribute to the activation and release of neurotransmitters from sensory nerve fibres (Holzer and Jocic, 1994).

In the rat skin, CGRP participates in neurogenic inflammatory processes but the cutaneous vasodilator action of exogenous CGRP and the CGRP-mediated vasodilation, evoked by antidromic stimulation of afferent nerve fibres, do not depend on NO formation (Holzer et al., 1995). Ralevic et al. (1992), on the other hand, showed that dilatation produced by ACh in the rat skin relies on a NO component (Ralevic et al., 1992). These studies suggest that perhaps vasodilatation caused by interaction between NO and CGRP may depend on the vascular tissue under study (Holzer et al., 1995). Increasing evidence indicates that NO may play a role in inflammation. In one study, treatment with inhibitors of NOS reduced the degree of acute inflammation in rats (Ialenti et al., 1992). This active agent (NO) has also been shown to have a role in the vasodilatation and inflammatory response to BK (Khalil and Helme, 1992), SP and VIP (Khalil et al., 1994; Ralevic et al., 1995b). NO generation is also involved in the SP-induced extravasation response in rats (Hughes et al., 1990), since actions of NO were inhibited by L-NAME.

The nature of endothelial dysfunction in diabetes is controversial. Increased NO production is thought to occur in early diabetes due to NOS activation mediated by hyperglycaemia (Pieper, 1998). It is also well established that the metabolic dysregulation of diabetes is associated with inhibition of NO release and action (Pieper, 1998). Considerable evidence implicated differences in the duration of exposure to diabetes in determining the nature and direction of these changes. Hyperglycaemia could activate EDRF in the short-term (section 1.8.4.1, Fig 1-10). However, as the duration of diabetes progresses atherosclerosis changes and other events such as generation of AGEs and superoxide radicals promote pathological changes in endothelial function leading to suppressed EDRF action (Bucala et al., 1991; Kaustic and Vanhoutte, 1989) (see also
sections 1.6.2 and 1.6.3). There is substantial evidence that vasodilatation mediated by endothelium-derived NO is impaired in humans and animals with type I diabetes (Took, 1996; Utkan et al., 1998). Accordingly, recent evidence showed that vasodilatation mediated by NO is accentuated in short-term (1-2 weeks) STZ-induced rats but becomes paradoxically impaired with increasing duration of diabetes (8 weeks) (Pieper, 1999). Moreover, Hopfner et al (1999) observed attenuated EDRF-mediated vasodilatation in 14 weeks, but not 2 weeks, STZ-treated diabetic rats (Hopfner et al., 1999). Alterations of NO in diabetes could also appreciably affect ET-1 activity, which is discussed in section 1.8.4.

Functional alteration of the endothelial L-Arg-NO pathway leads to impaired vasodilatation, reduced antithrombic properties, and structural changes (Cosentino and Luscher, 1998). For example, it was suggested that in early diabetes, metabolic defects may cause a decrease in synthesis of NO in either vascular endothelium or sympathetic ganglia leading to decreased nerve blood flow (Stevens, 1995).

The relation between diabetes and premature cardiovascular disease is well established (Bell, 1995). Atherosclerosis and microangiopathy are principle causes of morbidity and mortality in patients with diabetes mellitus (Dahl-Jorgensen, 1998; McMillan, 1997). Although the link between diabetes and cardiovascular disease is not understood, loss of the modulatory role of the endothelium may be implicated in the pathogenesis of diabetic vascular disease. This may be initiated by hyperglycaemia leading to decreased production of NO, increased free radical formations and / or alterations in the level of vasoconstrictors, which all can interfere directly and indirectly with NO activity (Chan et al., 2000; Cosentino and Luscher, 1998) (see also section 1.5.4.2).

In chapter 4, the role of NO in modulating microvascular blood flow in early diabetes is examined.

1.8.2. Capsaicin

An important tool used in the elucidation of the role of primary afferent nerves in various processes is capsaicin (trans 8-methyl-N-vanillyl-6-noneamide), the irritant principal of Hungarian red peppers. Capsaicin is a selective neurotoxin for certain types of sensory nerves, especially unmyelinated C-fibres with polymodal nociceptors, involving the
activation of the specific receptor (vanilloid) (Holzer, 1991). Activation of the vanilloid receptor leads to an influx of sodium and calcium ions, which at low doses (in the μg/kg range) excites afferent neurons with small cell bodies, and unmyelinated (C-fibre) or thinly myelinated (A-fibre) axons (Szallasi and Blumbeeg, 1996). This leads to release of neurotransmitters, which results in the development of neurogenic inflammation and nociception (Szolscany, 1984).

Acute topical application of capsaicin in small doses was shown to be capable of exciting polymodal nociceptors (C and Aδ fibres) causing flare and PE responses without leaving any permanent damage to these fibres (Fitzgerald, 1983). Long-term treatment with capsaicin, depending on the treatment regime, can degenerate sensory nerves leading to a decrease in their neurotransmitter content which in turn, attenuates neurogenic responses and / or desensitizes sensory fibres to stimuli (e.g. electrical and chemical) (Holzer, 1991; Szolscany, 1984; Szolscanyi et al., 1988). Systemic administration of high doses of capsaicin (in the mg/kg range) can produce ultrastructural (e.g. mitochondrial swelling) or even toxic effects (cell death) on primary afferents (Holzer, 1991). The extent of this damage can vary depending on the dosage of capsaicin, the route of administration, animal species, and age of the animals used. For example, pre-treatment of neonatal rats with capsaicin at 50mg/kg, i.p. (chapter 2, section 2.8), causes selective degeneration of up to 90% of unmyelinated primary afferent fibres (Holzer, 1992; Jancso et al., 1977) and destruction of small DRG cells (Fitzgerald, 1983). Presence of capsaicin-activated ion channels in DRG was shown before (Oh et al., 1996) and it is believed that their activation may underlie neurotoxic effects of capsaicin.

The importance of sensory nerves and wound healing became evident from experiments in animals pre-treated with capsaicin. Capsaicin-treated animals exhibit a number of trophic changes and abnormal responses to injury, diminished skin integrity, reduced inflammatory response, and poor wound healing (Gamse et al., 1980; Kjartansson et al., 1987). Deficiencies in repair processes in capsaicin-treated animals can be reversed by exogenous administration of sensory peptides. Reduced healing rate in capsaicin-treated or old rats, following a burn, can be normalized by intradermal (i.d.) injection of SP and CGRP into the wound (Khalil and Helme, 1996). CGRP can also increase the survival of
Loss of neurogenic inflammation in response to tissue injury may be an important complication of diabetes mellitus and can influence wound healing. A previous study found that there is loss of capsaicin-related neurogenic inflammation in the vasa nervorum of experimental diabetes (4 months) (Zochodne and Ho, 1993). The authors suggested that a similar deficit following nerve injury could impair the milieu for axonal regeneration in diabetes. In chapter 3, sensory-independent effects of diabetes on the microvasculature are examined using capsaicin pre-treated diabetic rats.

1.8.3. Sympathetic nerve

There is now a substantial body of evidence showing that NA, ATP and NPY act as neurotransmitters and are released from sympathetic nerves in variable proportions depending on the type of tissue, species used and parameters of stimulation (Burnstock, 1986). NA acts upon alpha (\(\alpha\)) and beta (\(\beta\)) adrenoreceptors to mediate its biological response. In most peripheral vascular beds in the skin the contractile response to NA is mediated via post-junctional alpha 1-adrenoreceptors. In the vascular smooth muscle, both alpha 1- and alpha 2-subtypes mediate vasoconstriction and beta-2 mediate vasodilatation (Bennett and Gardiner, 1996) (Fig 1-7).

**Fig 1-7:** Schematic representation of post-synaptic receptors for sympathetic neurotransmitter NA and their mechanism of action. Please note that in order to keep the diagram simple NPY and ATP are not included (Adopted from Bennett, T. & Gardiner, S.M. (1996)).
In the rat DRG, three alpha 2-adrenergic receptor subtypes have been identified, namely alpha 2A, 2B, and 2C (Gold et al., 1997). Existence of alpha 2-adrenoreceptors was previously reported in the membrane of unmyelinated nerve axons (Fuder and Selbach, 1993; Nicholas et al., 1993). Furthermore, neuropeptide release from pulmonary C-fibre afferents evoked by only low frequency electrical stimulation was also inhibited by stimulation of pre-junctional alpha 2-adrenoreceptors (De Luca et al., 1990). Functional studies have suggested that NA has two distinct actions on sensory nerves mediated by different adrenergic subtypes. NA can prevent pre-junctional neuropeptide release by acting on alpha 2B-adrenoreceptor and inhibiting voltage-gated calcium currents (Bean, 1989; Fuder, 1994; Kawasaki et al., 1990). NA can also directly inhibit primary afferent activity by acting on alpha 2C-adrenoreceptors (Khasar et al., 1995). It was also reported that C-fibre responses to nerve stimulation were reduced and delayed following sympathetic stimulation (Shyu et al., 1989).

In STZ diabetic rats, the spinal release of NA was markedly suppressed (4 weeks) (Bitar et al., 1999) and circulating NA was reduced (2-5 weeks) (Ohtani et al., 1997). A diminished tissue NA concentration was also reported in skin (Ahlgren and Levine, 1993), certain parts of the brain such as pons and medulla (Ohtani et al., 1997; Ramakrishnan and Nmasivayam, 1995), and in the duodenum (Patel et al., 1997) but significantly enhanced in kidney and liver of 4 weeks diabetic rats (Patel et al., 1997). Impairment in neurotransmitter release from both bladder sympathetic and parasympathetic efferent nerve endings have also been previously reported in early (2 weeks) experimental diabetes (Tong et al., 1996). The effect of diabetes on the vascular response to NA is strongly related to the type of tissue used and the duration of diabetes and this was discussed in section 1.5.4.1).

A reduction in the expression of messenger ribonucleic acid (mRNA) level encoding for alpha 2-adrenoreceptor subtypes was previously reported in the spinal cord of diabetic rats (4 weeks) (Bitar et al., 1999). The number of beta-adrenoreceptors, in the diabetic heart, was also decreased by 29% after 1 week and 50% after 3 weeks (Nishio et al., 1988; Yu et al., 1994). The decrease in beta-adrenoreceptor population size in 4 weeks diabetic rat’s ventricular tissue has been related to an increase in circulating NA level (Ramanadham and Tenner, 1987). The densities of beta 1- and beta 2-adrenoreceptors in
the brain of STZ-treated rats were increased in hypothalamus, thalamus, and amygdala (Bitar and DeSouza, 1990). It was previously suggested that diabetes may impair reactivity of large peripheral arteries and arterioles to activation of beta-adrenoreceptors (Mayhan, 1994). The relaxation of vascular smooth muscle induced by stimulation of beta-adrenoreceptors was also demonstrated to be decreased in humans (Harada et al., 1999) and diabetic rats (Kamata et al., 1992).

Angiotensin II is a potent vasoconstrictor and plays a vital role in regulating blood pressure. An in vitro study has shown that high extracellular glucose concentrations (15-20mM) could directly down regulate angiotensin II and its function in vascular smooth muscle cells (Williams et al., 1992). However, no difference in plasma angiotensin II levels was observed between 4 weeks diabetic rats and controls (Kihara et al., 1999). It has been hypothesized that activation of pre-junctional angiotensin II-receptors by angiotensin II may facilitate NA release mediated by beta 2-adrenoreceptors under physiological conditions (Schlicker et al., 1988; Ziogas and Story, 1991). On the other hand, reduced angiotensin II formation and enhanced prostaglandins formation might reduce NA release (Schwieler et al., 1992). An increase in angiotensin converting enzyme (ACE) activity was shown in type II diabetic patients (Ustundag et al., 2000). An increase in serum ACE level was also observed in rats, 12 days after STZ injection (Ustundag et al., 1999). Previous studies monitored diabetes-associated alterations in heart rate and blood pressure in STZ-treated rats over a 10 week period and showed a time-dependent reduction in heart rate (Fazan et al., 1999; Hicks et al., 1998). Although diastolic blood pressure and mean arterial pressure did not differ significantly, when compared to non-diabetics during this period, the pulse pressure was diminished. Renin-angiotensin system (RAS) may also play a role in the NO / ROS balance, as angiotensin II has been directly implicated in generation of superoxide anions in smooth muscle cells. Angiotensin II has been shown to regulate a membrane-bound flavin-containing NADH / NADPH oxidase that produces oxygen radicals (Fukui et al., 1997).

NPY and ATP are also released from sympathetic nerves upon stimulation but in variable proportions depending on tissue type, species, and parameter of stimulation (Burnstock,
NPY is a transmitter often co-localized with NA and co-released from sympathetic nerve endings (Bennett and Gardiner, 1996). Plasma levels of NPY are correlated with those of NA and its resting plasma levels are low in most species (Pernow, 1988). It is preferentially released at high frequency nerve stimulation (10 Hz or more) and has a long duration of vasoconstrictor action (Kennedy et al., 1997; Pinter et al., 1997). There are two NPY receptor subtypes, Y1 and Y2, and both are expressed in sensory ganglia fibres (Zhang et al., 1995). NPY acts as a neuromodulator controlling the release and action of NA and ATP (Bennett and Gardiner, 1996) and hence capable of regulating skin microcirculation (Pinter et al., 1997).

Pre-junctionally, this transmitter reduces the release of NA and ATP and post-junctionally it enhances their actions (Bennett and Gardiner, 1996). It was suggested that NPY initiates an intracellular calcium-sensitive mechanism, which increases alpha-adrenoreceptor sensitivity (Fallgren et al., 1993). This results in a significant increase of sarcoplasmic calcium and stronger contractile responses to NA. Although both NA and NPY have a vasoconstrictor effect, the action of NPY is not alpha-adrenoreceptor-mediated (Wahlestedt et al., 1986). It has also been shown that NPY release is enhanced after alpha-adrenoreceptor blockade or beta-adrenoreceptor stimulation, indicating that NPY release is also regulated pre-junctionally by NA (Dahlof et al., 1986).

Diabetes is associated with altered both central and peripheral nervous content of NPY. An increase in NPY level in various brain regions of STZ-treated rats (7 weeks) has been observed (Dunbar et al., 1992). This caused an increase in mean arterial pressure (MA) in diabetic rats but did not affect the heart rate. It was demonstrated that vascular conductances (e.g. regional blood flow) in response to NPY were attenuated in diabetic vessels (4 weeks) especially the iliac and renal arteries (Hu and Dunbar, 1997). Furthermore, vascular smooth muscle contractile responses (post-junctional event) to NPY were markedly reduced in human and diabetic animals (Andersson et al., 1992; Lind et al., 1995) and this was shown to be endothelium-independent.

ATP is present in both small and large dense-cored vesicles together with NA. It is synthesized in the mitochondria and incorporated into vesicles by carrier-mediated transport. ATP is released from sympathetic nerves and activates purinergic receptors (P1 and P2). The main role of ATP appears to be in the initiation of vascular contraction,
although its rapid degradation complicates the interpretation of its effect. Evidence has been presented that ATP acts as an excitatory co-transmitter (at least in some species) with NA causing vasoconstriction via excitatory P2X purinoceptors located on the vascular smooth muscle (Bennett and Gardiner, 1996). The contractile effect of ATP is dependent on an influx of extracellular calcium. ATP can also act as a vasodilator through P2Y receptors on endothelial cells or when it is broken down to adenosine by acting on P1 purinoceptors on the vascular smooth muscle (Pinter et al., 1997). ATP contribution in response to sympathetic nerve stimulation varies considerably in different vessels. In diabetes, there was no significant change in ATP concentration or its release between control and diabetic rats (2 and 8 weeks respectively) (Belai et al., 1991; Carlsson and Aronqvist, 1981).

ETs have also been reported to both stimulate and inhibit neurotransmitter release from post-ganglionic sympathetic neurons. For example, it was demonstrated that ET-1 (1-30 nmol/l) significantly inhibited but ET-3 (300 nM) potentiated ATP and NA release from rat isolated tail artery (Mutafova-Yambolieva and Westfall, 1998). Threshold and subthreshold concentrations of ET-1 can also enhance the contractile response to other vasoconstrictor agents (e.g. NA, 5-HT, vasopressin, histamine, and ATP). In certain tissues, this response is post-junctional and partially due to facilitation of calcium through L-type calcium channels (Reid and La, 1995). There was no change in positive chronotropic and inotropic effects of ET-1 to sympathetic nerve stimulation and NA in early diabetes (Lieu and Ried, 1994).

**Sympathetic nerve and skin microvasculature**

Under short-term physiological conditions there is a dynamic balance between sympathetic nerve-mediated vasoconstrictor tone and endothelial-mediated vasodilator tone. This balance may be altered by diabetes leading to neurovascular complications. One study demonstrated changes in skeletal muscle haemodynamics after only one week of STZ-induced diabetes (Hill et al., 1985). This was characterized by decreased perfusion and diminished ability to lower resistance by vasodilation. Alterations in the level of NA (circulating and tissue content), adrenoreceptors function and their densities
in diabetic condition were also previously reported. The overall information presented raised the possibility that sympathetic nerves may modulate skin microvascular blood flow in early diabetes, which is examined in chapter 5.

1.8.4. Endothelin

ET is a potent vasoconstrictor polypeptide that was first isolated from porcine aortic endothelial cells and sequenced in 1988 by Yanagisawa and his colleagues. Four isoforms of ET have been identified: ET-1, ET-2, ET-3, and vasoactive intestinal contractor ET (VIC). These isoforms are not species specific and share the total number of 21 amino acids, ET-2 > ET-1 > VIC >> ET-3 (Nayler, 1990).

ET-1 is produced by many cells other than endothelial cells, including vascular smooth muscle, kidney, tracheal cells, renal glomeruli, motor and dorsal horn neurones of the spinal cord, retinal endothelial cells, and even certain tumour cells (Nayler, 1990). In healthy individuals, plasma levels of ET-1 range from 1.5 to 3.7 pg/ml (Battistini et al., 1993). This can increase by approximately 2 to 10 fold in a number of cardiovascular disorders including myocardial infarction, atherosclerosis, chronic heart failure, cardiogenic shock, diabetes, renal failure, subarachnoid haemorrhage, and hypertension (Battistini et al., 1993).

ET synthesis and release is triggered by platelets, thrombin, other vasoconstrictors (angiotensin II, vasopressin, adrenaline), growth factors (platelet-derived growth factor (PDGF), TGF-beta, EGF), cytokines (IL-1), TNF-alpha, insulin, cellular stress (e.g. hypoxia), etc with no apparent feed back response (Ruffolo, 1995). The mechanism of agonist-induced ET-1 production and cellular release possibly involves the receptor-mediated mobilization of intracellular calcium and activation of PKC (Yanagisawa et al., 1989). A negative pathway involving the cGMP mechanism also regulates the production of ET-1. Agents such as NO and nitrosovasodilators reduce the secretion of ET-1 (Ruffolo, 1995). ET-1 has a short half-life (1-7 min) before it is eliminated from the circulation but its biological actions are maintained for a longer period (about 30 min).
ET isopeptides mediate their biological responses (in vivo and in vitro) through binding to receptors termed, ET-A and ET-B receptors (a third ET-C has also been identified) (Ruffolo, 1995). There is evidence that ET-A and ET-B receptors exist in the rat skin (Lawrence et al., 1995). ET-A receptors are characterised by selectivity for ET-1 and ET-2 and are found in the highest density in vascular smooth muscle to mediate vasoconstriction and smooth muscle cell proliferation (Ruffolo, 1995). ET-B receptors (and subtypes) are “non-selective” and have equivalent affinities for ET-1, -2, and -3. Pharmacological data indicate the existence of two anatomically separate and distinct ET-B receptors; an ET-B receptor on the endothelium that mediates the release of NO and prostacyclin (Fozard and Part, 1992) to stimulate vasodilatation and an ET-B receptor on the vascular smooth muscle that directly stimulates smooth muscle contraction. The endothelial ET-B receptor has been termed ET-B1 and the other receptor that stimulates vascular smooth muscle contraction has been termed ET-B2 (Gellai et al., 1996). The ET-C receptor, to which ET-3 shows a higher affinity than ET-1, has been identified in bovine endothelial cells (Emori et al., 1990). Species differences exist in ET receptors both at structural and functional levels (Lodge et al., 1995; Ruffolo, 1995).

Binding of ET to its receptor(s) is slow, pH sensitive, and calcium-independent (Ruffolo, 1995). Various pathological conditions, including hypertension, ischaemia, and STZ-induced diabetes can modify the density of ET receptor binding (Nayler, 1990). Various receptor agonists and antagonists have been developed over time to aid scientists with understanding ETs mechanisms of action under physiological as well as pathological conditions (Fig 1-8).
ET-1 has a wide range of biological actions in a variety of tissues, apart from its potent vasoconstrictor activity. A number of ET-1’s biological effects are shown in Table 1-5.
**Biological effects of ET-1**

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<tr>
<th><strong>Vascular effects</strong></th>
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<tr>
<td>Protracted constriction of vascular smooth muscle</td>
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<td>Mitogenic action in cell culture</td>
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<td>Proliferative actions <em>in vivo</em></td>
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<td>Synergy with other vasoconstrictors &amp; growth factors</td>
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<th><strong>Cardiac effects</strong></th>
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<td>Positive chronotropic &amp; inotropic effect</td>
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<td>Coronary vasoconstriction</td>
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<td>Arrhythmogenic</td>
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<td>Myocardial hypertrophy</td>
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<th><strong>Endothelium</strong></th>
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<td>Release of NO &amp; prostanoids</td>
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<td>Induction of adhesion molecule expression</td>
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<th><strong>Non-vascular smooth muscle</strong></th>
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<td>Constriction of intestinal, tracheal, mesangial, bladder, uterine, and prostatic smooth muscle</td>
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<th><strong>Renal function</strong></th>
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<td>Decrease in renal blood flow &amp; GFR</td>
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<td>Increase in sodium excretion</td>
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<td>Increase in plasma renin activity</td>
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<th><strong>Endocrine</strong></th>
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<td>Stimulation of neuropeptide &amp; pituitary hormone release</td>
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<td>Increase in release of atrial natriuretic peptide</td>
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<td>Stimulation of aldosterone biosynthesis</td>
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<th><strong>Nervous tissue</strong></th>
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<td>Modulation of neurotransmitter release</td>
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<td>Potent cerebrovascular spasm</td>
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<th><strong>Other effects</strong></th>
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<td>Increase in intraocular pressure</td>
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<td>Stimulate matrix production</td>
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<td>Increase in bone reabsorption</td>
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<td>Stimulate neutrophil superoxide production</td>
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*Table 1-5: A number of ET-1’s biological effects (Adopted from Ruffolo, R.R. Jr. (1995)).*
It has been shown that binding of ET-1 to its receptor activates a variety of signal transduction pathways to produce responses. These signal transduction processes include adenylate cyclase, guanylate cyclase, Phospholipase (PL) C, A₂, and D, PKC, and MAP kinase (MAPK) (Ruffolo, 1995). As a result, a number of second messengers are generated such as cAMP, cGMP, IP, intracellular calcium, DAG, and AA. These second messengers activate other enzymes to phosphorylate or dephosphorylate key cellular proteins, leading ultimately to biological responses (Fig 1-9).

Fig 1-9: Intracellular signal transduction
Pathways activated by ET-1 in vascular Smooth muscle cells (intracellular calcium store (Ca²⁺), receptor-operated calcium channels (ROCC), voltage-operated calcium channels (VGCC), free cytoplasmic calcium (Ca²⁺), phosphatidic acids (PA), prostaglandins E₂ (PG E₂), thromboxane A₂ (TXA₂)) (Adopted from Reid, J.J. & La, M. (1995)).

The physiological importance of ET-1 is not very well known. It is possible that, as a locally acting hormone, ET-1 exerts its physiological effects on the vascular tone by interacting with other vasoconstrictor agonists within the local vicinity of smooth muscle cells to produce or enhance vasoconstriction. Non-vascular smooth muscle, such as bronchial muscle, ileum, uterus, and vas deferens can also respond to ET but they are less sensitive than vascular smooth muscle and a higher concentration of ET will be required for their contraction (Ruffolo, 1995). The vasoconstrictor activity of ET is not mediated by alpha-adrenergic, histaminergic, cholinergic, or serotonergic mechanisms, and neither vasopressin nor angiotensin seems to be involved (Ruffolo, 1995).

1.8.4.1. Endothelin and diabetes
ET-1 synthesis and release
In patients with type I diabetes, plasma ET-1 has been reported to be both increased (Haak et al., 1992; Takahashi et al., 1990) and decreased (Smulders et al., 1994) but in patients with type II diabetes, this was either unchanged (Bertello et al., 1994; Nugent et al., 1996) or again increased (Donatelli et al., 1994; Morise et al., 1995). The status of
ET-1 plasma concentrations in type I diabetes in animals is also controversial. In STZ-induced diabetic rats, most studies agree that ET-1 concentration is increased in mesenteric artery and aorta after 8 weeks of diabetes (Hopfner et al., 1999; Makino and Kamata, 1998; Takahashi et al., 1991). However, this did not seem to be the case in earlier stages of the disease. Some showed reduction in ET-1 concentration in kidney, aorta, and cultured vascular smooth muscle before 5 weeks (Frank et al., 1993; Hopfner et al., 1998b; Shin et al., 1995), whereas others demonstrated no change (mesenteric artery, aorta) at 4 days, 2 or 4 weeks after induction of diabetes (Takahashi et al., 1991; Wu and Tang, 1998). One study showed an increase in ET-1 after 2 weeks of diabetes in the above tissues (Wu and Tang, 1998). It appears that the duration of diabetes can determine the direction of change in plasma ET-1 in STZ-treated animals (Hopfner et al., 1999). In addition, inter-study variability in the metabolic state, level of control, presence of established macrovascular complications, and methodology for measuring ET-1 are all likely to contribute to observed discrepancies. Importantly it was demonstrated that altered plasma ET-1 in diabetes could be returned to normal level by restoration of metabolic control (Hopfner et al., 1998b).

Key metabolic variables that are changed in DM such as plasma glucose, insulin, and lipids are well known to directly regulate the release of ET-1 from endothelial cells and modulate receptor expression and responses to the peptide (Hopfner and Gopalakrishnan, 1999) (see also Figs 1-10 & section 1.8.5.3, Fig 1-15). Prolonged and excessive exposure of both endothelial cells and vascular smooth muscle cells to the metabolic dysregulation of diabetes could also promote morphological changes that indirectly affect the release and action of ET-1 at various target sites (Hopfner and Gopalakrishnan, 1999). Hyperglycaemia, in vitro, can increase (Yamauchi et al., 1990), decrease (Hattori et al., 1991), or have no effect (Baumgartner-Parzer et al., 1996) on the release of ET-1 from endothelial cells. Species type as well as glucose concentration and incubation period used by these studies may have accounted for observed discrepancies. High glucose levels increase EDRF production through activation of NOS (Hopfner and Gopalakrishnan, 1999) and EDRF inhibition, through endothelial damage and formation
of AGES and superoxide radicals (Fig 1-10). Formation of the above substances could also regulate ET-1 production and action (Hopfner and Gopalakrishnan, 1999).

Studies examining the effect of high glucose levels on receptors and responses to ET-1 have yielded somewhat similar results and are summarized in Fig 1-10.

Hopfner et al. (1999) and a number of other investigators have gathered evidence to show that insulin can modulate ET-1 production and actions in both in vitro and in vivo settings and the summary of their findings is shown in Fig 1-14 (Hopfner and Gopalakrishnan, 1999) (section 1.8.5.3).

Alterations in NO in diabetes could also appreciably affect ET-1 activity. ET is the main vasoconstrictor / mitogenic mediator released by the vascular endothelium whereas EDRF modulates vasodilation anti-growth effects. At the gene level, EDRF inhibits ET-1. Conversely, ET-1 might inhibit EDRF in pathological conditions. It is well known that EDRF suppresses ET-1 release and action (Kourembanas et al., 1993) and endothelial damage is thought to be associated with increased release of ET-1 (Huszka et al., 1997; Takahashi et al., 1990). It was shown that changes in plasma concentration of ET-1 and vascular responses to ET-1 in STZ-treated rats are duration-dependent and occur in parallel with changes in endothelial function (Hopfner et al., 1999).

Raised plasma lipoproteins are present in both type I and type II diabetic patients, particularly in those with poor metabolic control. Considerable evidence indicates a
profound ability of plasma lipoproteins to affect both the release and action of ET-1. Oxidized LDL increases ET-1 gene expression and raised lipoproteins in vivo accentuate vascular responses to agonists (including ET-1) before the onset of atherosclerosis (Ruffolo, 1995). After the onset of atherosclerosis, ET receptors are decreased and ET-1 peptide increased in animal models of the disease (Hopfner and Gopalakrishnan, 1999). Moreover, endothelial damage resulting from the cascade of atherosclerotic events resulting from dyslipidaemia would be expected to modulate the release and action of ET-1. Thus, it is reasonable to suggest that hyperlipidaemia could regulate plasma concentrations of ET-1 as well as its receptors, and tissue reactivity to the peptide (Hopfner and Gopalakrishnan, 1999).

ET-receptors density and activity
Up-regulation of ET receptors was reported in the heart (2-6 weeks), retina (4 weeks), prostate, vas deferens (8 weeks), and urethra (16 weeks) after induction of diabetes (Deng et al., 1999; Nakamura et al., 1997; Saito et al., 1996; Vesci et al., 1995). However, in diabetic kidney (1-6 weeks), no change in ET-A receptor density was observed (Awazu et al., 1991; Vesci et al., 1995) but ET-B receptors were down-regulated (4 weeks) (Awazu et al., 1991; Masao et al., 1999). Diabetes-induced PKC activation was suggested to be responsible for up-regulation of ET-1 in the retina (Koya and King, 1998). In the kidney, an increase in ET-1 could down-regulate ET-B receptors by activating PKC and intracellular calcium mobilization (Masao et al., 1999). These suggest that in diabetes there may be a relationship between a high level of ET and PKC activity. Experiments on patients with type II diabetes showed no reduction in forearm blood flow when ET-1 was infused locally (Nugent et al., 1996). They presented several possible explanations for their results including down-regulation of ET-A and ET-B receptors on smooth muscle, up-regulation of endothelial ET-B receptors, or perhaps an increase in concentration of big ET, which is a weak agonist for ET receptors.

Vascular response to ET
In diabetic states, few studies have assessed haemodynamic actions of ET-1 in vivo, therefore no definite conclusions can be drawn from them. However, based on existing
evidence, this appears to be dependent on the pre-existing duration of diabetes, the status of endothelial function, and vascular bed under consideration (Hopfner et al., 1999) (see also section 1.6.4.2). The in vivo response to ET-1 in different vascular beds itself seems to depend on the expression and density of vasodilator ET-B and vasoconstrictor ET-A and ET-B receptors.

Most in vitro studies examining vascular responsiveness to vasoactive stimuli (including ET-1) in models of diabetes use aorta as the prototype vessel. In early diabetes (2 weeks), some suggested that significant attenuation of the aortic response to ET-1 (Makino and Kamata, 1998; Utkan et al., 1998) could be attributed to down-regulation of ET receptors by either PKC activation or increased ET-1 production (Awazu et al., 1991; Guillon et al., 1998). Others showed an exaggerated response to ET-1 in the same tissue (Hopfner et al., 1999; Hopfner et al., 1998b), and suggested that expression and action of calcium channels are altered or PKC is activated (Abebe and MacLeod, 1990; Reid and La, 1995) (see also section 1.6.4.1).

**Endothelins and skin microvasculature**

ET production is regulated at the level of mRNA transcription / translation and is made upon demand, for example in an emergency and / or in a defensive event such as inflammation and wound repair (Nakamura et al., 1990; Yanagisawa and Masaki, 1989). ET is a potent vasoconstrictor. In vascular smooth muscle, ET causes a biphasic increase in calcium concentration. A transient increase is believed to be related to the mobilization of intracellular calcium while a sustained increase is due to the influx of extracellular calcium (Highsmith et al., 1992). The influx of calcium induced by ET-1 may depend on the opening of L-type calcium channels, but the involvement of non-selective cation channels was also suggested (Highsmith et al., 1992; Reid and La, 1995). A previous study suggested that activation of ET receptors (ET-A or ET-B) mediates vasoconstriction in the rat cutaneous microvasculature but has no effect on the increased microvascular permeability (Lawrence et al., 1995). Arteries, arterioles, veins, and venules are all affected by ET, with veins being more sensitive than arteries but venules and arterioles are equisensitive.
Presence of ETs and their binding sites in both peripheral neurons and the central nervous system suggests a neuromodulatory role for these peptides (Ruffolo, 1995). For example, it was suggested that the vasoconstrictor action of endogenous ET may contribute to peripheral nerve dysfunction in experimental diabetes (5-8 weeks) (Stevens and Tomlinson, 1995; Zochodne et al., 1996). It also appears that ET does not contribute to SP-induced vasodilation in the rat skin (Khalil and Helme, 1996) and, in fact, inhibits vasodilatation induced by NO and nitroprusside (Chander et al., 1988). ET can also inhibit plasma extravasation induced in the rat skin by the i.d. injection of inflammatory mediators 5-HT, histamine, BK, and platelet activating factor (PAF) (Chander et al., 1988). The role of ET-1 in modulation of SP and CGRP release from capsaicin-sensitive neurones has been reported (Dymshitz and Vasko, 1994). However, in this study, it was not clear whether ET-1 acted directly on these nerves, or released some chemical mediator from other types of neurons, or from satellite cells present in DRG cultures.

There has been much speculation regarding the pathophysiological significance of ET-1. For example, convincing evidence exists that ET-1 might be directly linked to the development of hypertension (Yokokawa et al., 1991). Elevated ET-1 plasma levels have also been reported in many cardiovascular disorders and diabetes (Battistini et al., 1993; Haak et al., 1992; Takahashi et al., 1990). However, conclusions drawn about the direct association of increased ET-1 levels with these disease states needs to be interpreted with caution.

In chapter 6, the role of ETs in modulating skin microvascular blood flow in early diabetes is investigated. Specifically, the role of ET receptors (ET-A and ET-B) and their quantitative contribution to microvascular responses in 4 weeks diabetic rats is examined.

### 1.8.5. Other modulators

Several endogenous and local tissue factors appear to modulate release and action of neuropeptides from sensory nerves. However, only those factors that are relevant to this thesis are discussed. Factors which activate the efferent function of sensory nerves include serotonin (Khalil and Helme, 1990b), ACh, cholecystokinin (CCK) (Bennett and Gardiner, 1996; Maggi, 1991), and BK (Khalil and Helme, 1992). Other mediators inhibit
the efferent function at the pre-junctional level including galanin and somatostatin (Maggi, 1991). Furthermore, there are reports of drugs and / or transmitters that could play a dual effect via different receptors. For example, opioid peptides can inhibit plasma extravasation and vasodilatation responses (Khalil and Helme, 1990a), but they may also release SP from the central nervous system (Chahl, 1990). Others in this group include capsaicin, histamine, γ-aminobutyric acid, and adenosine (Maggi, 1991). The role of the sympathetic nervous system in modulating sensory nerve function is also important and is discussed in section 1.8.3.

1.8.5.1. Acetylcholine

ACh, a neurotransmitter, is synthesized in nerve cells from choline and acetyl coenzyme A in the presence of choline acetyl transferase. It is stored in synaptic vesicles and released at synapses of parasympathetic nerves and at neuromuscular junctions. ACh acts upon muscarinic and nicotinic receptors. The muscarinic receptors are on endothelial cells, smooth muscle cells, and glands that are coupled via a G-protein to adenylate cyclase or PLC. Nicotinic receptors are in motor end plates of skeletal muscle, sympathetic ganglia and many parts of the central and peripheral nervous systems (Bennett and Gardiner, 1996; Burnstock, 1988). Nicotinic receptors are ion channels that open up when ACh is present, leading to an influx of sodium ions and hence a depolarizing potential. This depolarization leads to the release of other transmitters and peptides in a cascade fashion. Stimulation of these receptors produces sensation of prickle, itch, burning pain, and flare which spreads via the axon reflex (Grunfeld et al., 1991).

ACh has a role in the control of local blood flow. It can produce smooth muscle relaxation and vasodilatation mediated by the release of EDRFs, mainly NO (Furchgott and Zawadzki, 1980), from the vascular endothelium in response to stimulation of muscarinic receptors. If ACh is applied to blood vessels with damaged endothelium, however, it may produce vasoconstriction by acting on the muscarinic receptors on the underlying smooth muscle (M₁ receptors) (Burnstock, 1988).

Prostacyclin or pre-junctional inhibition of adrenergic neurotransmission, in vivo, may also be involved in the vasodilatation response to ACh. At the level of skin
microvasculature, ACh may be released from cholinergic post-ganglionic sympathetic fibres that innervate sweat glands and from parasympathetic post-ganglionic nerve endings at the neuromuscular junction. In addition to ACh, the cholinergic nerve also contains VIP, which produces vasodilation by directly acting on vascular smooth muscle (Bennett and Gardiner, 1996). Presence of NOS in the neuronal tissue and the co-existence of NO and VIP in parasympathetic post-ganglionic nerve terminals suggest that NO may be synthesized and released from cholinergic vascular nerve terminals (Kummer et al., 1992). This is supported by a previous study where inhibition of NO production attenuated parasympathetic, non-cholinergic vasodilatation (Garthwaite, 1991).

It has been suggested that ACh released from post-ganglionic sympathetic nerve fibres contributes to the inflammatory response since antidromic vasodilation and neurogenic plasma extravasation were reduced by atropine, a muscarinic receptor antagonist (Furchgott and Zawadzki, 1980). A previous study investigating the mechanism underlying vasodilator action to ACh in the rat skin microvasculature, with intact endothelium, indicated that this response is comprised of two distinct components. One part of the response is mediated through NO, probably arising after direct stimulation of muscarinic receptors (M₃ receptors) on endothelial cells, and the other part of the response is mediated through nicotinic receptors located on sensory nerves, leading to the release of sensory neurotransmitters (Ralevic et al., 1992).

**Fig 1-11:** Receptor activation of vascular endothelium by ACh or BK (Intracellular calcium ([Ca²⁺]), Constitutive nitric oxide synthase (cNOS), L-arginine (L-Arg), Soluble guanylate cyclase (sGC), Guanosine triphosphate (GTP))(Adopted from Moncada, S. & Higgs, A. (1993)).
Superoxide anions depress, and hydroxyl radicals facilitate, endothelium-dependent relaxations caused by activation of muscarinic receptors (Rubanyi and Vanhoutte, 1986). NO is increased by ACh, which promotes the reaction between NO and superoxide anions (Prasad and Bharadwaj, 1996).

In rat arteries, muscarinic agonists such as ACh release EDHF (Edwards et al., 1998). One in vitro study, using hepatic artery, proposed that the stimulation of endothelial cells by agonists such as ACh or BK might activate apamin-and charbdotoxin-sensitive K⁺ channels. Any resulting K⁺ efflux might then hyperpolarize the adjacent smooth muscle, suggesting EDHF to be endothelium-derived K⁺ (Edwards et al., 1998). Relaxation responses, induced by potassium channel activators, were significantly reduced in STZ-induced diabetic rat aorta (Kamata et al., 1989), providing indirect evidence for the reduced potassium channels activity in these animals (section 1.5.4.2).

An in vitro study showed that basal release of ACh in the brain of STZ-treated rats, monitored for 7 weeks, started to decline after 1 week of diabetes (Welsh and Wecker, 1991). The concentration of ACh in the heart of 2 and 4 weeks diabetic rats was not significantly different from control rats (Akiyama et al., 1989). It was shown that diabetes causes an alteration in muscarinic receptor biosynthesis and function. Increase in the density of these receptors was observed in vas deferens (about 8 weeks) (Kanda et al., 1997) and the bladder (2 weeks) (Kanda et al., 1997; Tong et al., 1999) of diabetic rats. Impaired receptor activity was also reported in isolated gastric antral smooth muscles (Soulie et al., 1992). It was suggested that early alteration in the muscarinic ACh system in diabetes is associated with increased production of NO and cGMP (Wald et al., 1998). Another study hypothesized that muscarinic receptor up-regulation could be related to the polyol-pathway and activation of aldose reductase (Kanda et al., 1997). Alteration to the receptor property and function in experimental diabetes was suggested to be due to changes of GTP-binding proteins coupled to muscarinic receptors (Soulie et al., 1992).

Alteration in the vasodilator response to ACh has been described earlier by other studies (section 1.5.4.2) and inconsistency was found among results. Duration of diabetes and the type of vascular bed under study were nominated as possible contributing factors to these discrepancies (Chan et al., 2000; Pieper, 1999). In chapter 4, the role of parasympathetic...
nerves in modulation of skin microvascular blood flow in early diabetes is investigated. In this chapter, changes in microvascular responses to ACh and the function of its receptors (nicotinic and muscarinic) are examined in 4 weeks diabetic rats.

1.8.5.2. Bradykinin

Kinins are generated from plasma and tissue precursors at the site of tissue injury and contribute to many aspects of both acute and chronic inflammation including oedema formation, vasodilatation, and pain (Cambridge and Brain, 1995; Dray and Perkins, 1993; Khalil and Helme, 1992). BK, a naturally occurring polypeptide, has diverse actions on the vascular endothelium, smooth muscle, and cellular function and is a powerful analgesic agent, which can both sensitize and directly stimulate sensory nerve terminals (Dray and Perkins, 1993) (Fig 1-12). BK (10\(\mu\)M) stimulates vascular endothelial cells to produce NO and constricts vascular endothelial cells to produce plasma extravasation (Khalil and Helme, 1992). At concentrations higher than 2\(\mu\)M, BK caused a significant histamine release from rat peritoneal mast cells when extracellular calcium was removed (Zhao et al., 1996). There is evidence to suggest that BK can stimulate sympathetic nerves to release stored neurotransmitters (Jones et al., 1995) which was not supported in an in vivo situation (Cambridge and Brain, 1995). This is because the mechanism of a pro-inflammatory effect on the sympathetic nerve is not well understood. One study suggested that vascular permeability and plasma extravasation observed with BK may be mediated at least in part through activation of 12-lipoxygenase in the rat skin (Wang et al., 1999). BK also contracts a variety of smooth muscle tissues, stimulates prostanoid production in some tissues, exerts chemotactic and stimulatory effects on immune cells, and degranulates mast cells (Dray and Perkins, 1993) (Fig 1-12).

BK receptors, B1 and B2, modulate the cellular action of kinins and have been localised in animal neuronal tissues (Hall, 1997; Raidoo and Bhoola, 1998). Evidence suggests that B2 receptors are more involved in acute inflammatory events, such as oedema and inflammatory pain and this was supported by Khalil et al (1992) in a study of the rat skin (Khalil and Helme, 1992). These authors showed a significant part of the response to BK is mediated through an excitatory action on peripheral nociceptive C-fibres, involving B2 receptors, where sensory neurotransmitters are released to produce vasodilatation and
plasma extravasation via NO release from endothelial cells. B1 receptors, on the other hand, appear to be involved in chronic inflammatory responses (Farmer et al., 1991; Hall, 1997). Induction of B1 receptors and responses to des-Arg⁹-BK are increased by stimulation of macrophages as well as inflammatory mediators themselves such as cytokines and ILs (Farmer et al., 1991). B1 agonists can increase prostacyclin and EDRF in cultures of both endothelial and vascular smooth muscle cells (D’Orleans-Juste et al., 1989).

There is a relationship between the metabolism of kinins and the control of blood flow by the endothelium (Mombouli and Vanhoutte, 1995). For example, angiotensin-converting enzyme (ACE) catalyzes the degradation and inactivation of BK, SP, and other peptides. On the other hand, ACE inhibitors potentiate the action of kinins, normalize endothelial function (Mombouli and Vanhoutte, 1995), and are capable of enhancing prostaglandin production (Bennett and Gardiner, 1996).

A reduction in kallikrein has been found in diabetic individuals, suggesting that an impaired kallikrein-kinin system (KKS) contributes to the development of diabetic complications (e.g. nephropathy). High levels of urinary BK were detected in 12 weeks STZ-induced diabetic rats (Tschope et al., 1999a). It was suggested that this increase is related to increased filtration of components of the plasma KKS and / or renal kininogen synthesis in combination with decreased renal kinin-degrading activity. The same study also showed an impairment in the main kinin-forming enzymes, implying a reduced
capacity to generate BK in these animals (Tschope et al., 1999b), which was not compensated by elevation in the level of BK receptors.

In a previous study, in vitro, treatment of vascular smooth muscle cell for 24hrs with high glucose significantly increased the number of BK receptors (Velarde et al., 1997). The same study suggested that BK could play a role in atherosclerotic vascular disease in diabetes by increasing DNA synthesis and cell number, inducing MAP kinase activation, and nuclear translocation in vascular smooth muscle cells. In STZ-treated mouse (10 days), B1 and B2 receptors were over expressed in the stomach fundus but not in the urinary bladder (Pheng et al., 1997). Involvement of B1 kinin receptors in experimental diabetic mice have also been reported (Zuccollo et al., 1996).

The vascular response to BK in diabetic rats, once again, seems to depend on the vascular tissue under study as well as the duration of diabetes. It was previously reported that in cremaster muscles of diabetic rats (3 weeks), the vascular response to BK was unchanged (Alsip et al., 1996) but in the limb hindquarter of these animals (4 weeks), the response was attenuated (Kiff et al., 1991b). The author of the latter study suggested that this reduction was particularly dependent on NO synthesis that was selectively impaired in STZ-treated rats. It was reported that the endothelial release of BK-stimulated relaxing factor, in rat intestinal arterioles, was not compromised after 1-2 or 7-8 weeks of diabetes (Lash and Bohlen, 1991) (see also section 1.6.4.2).

In normal rat tail artery, BK elicits a concentration-dependent vasoconstriction that although is endothelium independent, is dependent on extracellular calcium entry and intracellular calcium release. This contraction was previously reported to be significantly reduced in diabetic rats treated with STZ (Wang et al., 1998). In the same study, it was demonstrated that B2 receptors located on vascular smooth muscle mediates this contraction. Altered B2 receptor-mediated vascular activity might play an important role in vascular complications associated with diabetes.

The role of BK in modulating skin microvascular blood flow in early diabetes is examined in chapter 4, an issue that was not previously addressed in other studies.
1.8.5.3. **Insulin**

The Insulin molecule consists of two polypeptide chains (A & B), linked by two disulphide bridges and is synthesized in the pancreas by beta cell of islets of Langerhans (Pickup and Williams, 1997). Its secretion is modulated by numerous metabolic hormones (e.g. glucagon, opioids, VIP, adrenaline, NA, etc) and neural factors (e.g. vagal, sympathetic) (Pickup and Williams, 1997). In mammals, the main physiological detriment of insulin release is the blood glucose concentration. Glucose must be metabolized within beta cells to produce ATP, which closes specific ATP sensitive channels in the cell membrane. This prevents potassium ions leaving the cell, causing depolarization, which in turn opens voltage-gated calcium channels in the membrane allowing calcium ions to enter the cell. The increase in cytosolic calcium then initiates granule translocation and exocytosis of insulin.

![Possible mechanisms involved in insulin secretion by glucose](Fig 1-13: Possible mechanisms involved in insulin secretion by glucose (Adapted from Pickup, J.C. & Williams, G. (1997)).)

Insulin exerts its biological effects by binding to cell surface glycoprotein receptors on the target tissues (Marino-Buslje et al., 1999). This causes a conformational change in the receptor and activation of tyrosine kinase, essential for insulin signalling. The post-receptor signalling mechanisms are still largely unknown.

Insulin-receptor structure and function are affected by many physiological (exercise, diet), hormonal (insulin), and pathological states (obesity, diabetes). Insulin resistance characterizes patients with type I and type II diabetes (Yki-Jarvinen and Koivisto, 1986). In type I, insulin resistance could be due to a post-receptor defect, diminished receptor tyrosine kinase activity, and / or altered receptor carbohydrate structure (Pickup and Williams, 1997). An *in vitro* study revealed that insulin receptors in skeletal muscle of
STZ-induced diabetic rats after 7 days diabetes were reduced and substrate kinase activity was diminished (Burant et al., 1986). They also observed alterations in the mobility of diabetic derived beta-subunits (structural change). A study, in vivo, on STZ-treated rats (2 weeks) suggested that tissue specific modulation of insulin receptors occurred at the level of mRNA (Sechi et al., 1992).

Insulin’s vascular effects are mediated both by the sympathetic nervous system (chapter 10) and the L-Arg NO pathway (Scherrer et al., 1996). Insulin is an endothelium-dependent vasodilator (Baron, 1999; Cardillo et al., 1999; Nava et al., 1997). A study, in vitro, showed that insulin can regulate the expression of the eNOS gene, in endothelial cells and microvessels, mediated by the activation of phosphoinositide 3-kinase (PI-3) (Kuboki et al., 2000). At physiological concentrations, insulin increases both cGMP and cAMP in human vascular smooth muscle, endothelial cells, and platelets (Aljada and Dandina, 2000; Trovati et al., 1997; Trovati et al., 1996). Increase in cGMP is via NO-mediated stimulation of soluble guanylate cyclase and increase in cAMP may be mediated by insulin-receptors. A previous study showed topical application of insulin (200μU/ml) to the hamster cremaster muscle caused a significant increase in arteriolar diameter (McKay and Hester, 1996), which could be inhibited by adenosine receptor antagonism and blockage of ATP-sensitive potassium channels. As shown in Fig 1-13, ATP-sensitive potassium channels are important in maintaining beta cell membrane potential and may provide a crucial link between metabolic and electrophysiological effects of glucose.

Evidence exists that insulin is involved in pro-inflammatory condition (Vianna and Garcia-Leme, 1995) and insulin-sensitive depletion of SP and CGRP was also reported in sensory nerves of STZ-treated diabetic rats (Diemel et al., 1992). When rats underwent daily insulin treatment soon after the onset of diabetes, for the duration of 4 weeks, SP and CGRP deficits in their sciatic nerve were reversed (Diemel et al., 1992), and alterations in vasoreactivity and endothelial cell function were prevented (Stevens et al., 1993). In diabetic rats, insulin treatment also restored neurogenic oedema towards normal (Bennett et al., 1998). Intensive insulin treatment prevented sciatic nerve ischaemia in rats with short-term diabetes (1-4 weeks) (Stevens et al., 1994) partially through
glycaemic control (Biessels et al., 1996). A previous study showed that insulin deficiency, in type I diabetes, often leads to sensory, motor, and autonomic nerve deficits (Vinik, 1999). It was suggested that type I diabetes (1 week) is associated with altered levels of insulin signalling components (Bonini et al., 1995).

Na+/K+-ATPase, an integral component of the sodium pump, is involved in the maintenance of cellular integrity and function such as contractility, growth, and differentiation (Greene and Lattimer, 1983). There is evidence that insulin stimulates Na+/K+-ATPase in skeletal muscle endothelium (Tack et al., 1996). The mechanism by which insulin stimulates peripheral sensory nerves may also involve the Na+/K+-ATPase system. This is supported by previous studies suggesting that the reduced Na+/K+-ATPase activity in diabetic nerve may be responsible for a reduction in conduction velocity and eventual structural abnormalities (Di Giuliio et al., 1995; Vinik, 1999). Activity of both Na+/K+-ATPase and Ca²⁺-stimulated ATPase but not Mg²⁺-ATPase was reported to be depressed in diabetic rat heart (1-4 weeks) (Golfman et al., 1998; Ver et al., 1997). These authors supported the view that sarcolemmal membrane abnormalities in Na⁺/K⁺-ATPase, Na⁺/Ca²⁺-exchange and Ca²⁺-pump activities may lead to the occurrence of intracellular Ca²⁺ overload in diabetes. Short-term insulin treatment of cultured vascular smooth muscle cells, in vitro, caused a significant increase in mRNA encoding the alpha 2-subunit of Na⁺/K⁺-ATPase (Tirupattur et al., 1993). On the vascular smooth muscle, it was suggested that insulin may act directly via stimulation of the Na⁺/K⁺-ATPase and Na⁺-H⁺ exchanger, leading to hyperpolarization of the cell membrane and consequent closure of voltage-gated calcium channels (Cleland et al., 1998). The Na⁺-H⁺ antiport of retinal epithelial cells was down-regulated when these cells were exposed to high glucose levels (in vitro, 26mM) for 2 weeks (Civan et al., 1994).

Insulin regulation of vasoconstriction and relaxation appears to be secondary to the regulation of intracellular calcium, as insulin attenuates both voltage- and receptor-mediated calcium influx and stimulates both the transcription and activity of calcium-ATPase in vascular smooth muscle cell (Zemel, 1995). Although intracellular calcium plays a poorly understood role in insulin signalling, increases beyond an optimal range results in impaired insulin sensitivity, possibly by calcium inhibition of insulin-induced
Dephosphorylation of insulin-sensitive substrates (Zemel, 1995). In liver cells, influx of calcium was required for the stimulation of PI-3 kinase activity (Benzeroual et al., 2000). Glucose control plays an important role in the pro-oxidant / antioxidant balance. It has been hypothesized that oxidative stress can impair insulin action through: 1) changes in the physical state of the plasma membrane of target cells, 2) an increase in intracellular calcium content, and 3) a reduction in NO availability (Paolisso and Giugliano, 1996). Evidence exists that insulin treatment reversed the increase in lipid peroxidation in diabetes (1 week) (Rungby et al., 1992). This treatment also retarded and reduced the intensity of advanced glycation process by regulating glycaemia (Turk et al., 1999). It appears that insulin is the modulator of ET-1 peptide, ET receptors, and ET-1 mediated responses in both in vitro and in vivo settings (Ferri et al., 1995; Frank et al., 1993; Hopfner et al., 1998b) (Fig 1-14).

Physical insulin concentrations can increase the release of ET-1 from both human endothelial cells and vascular smooth muscle cells (Ferri et al., 1995; Hopfner et al., 1998a; Hopfner et al., 1998b). At this concentration it can also up-regulate ETA receptor expression and ETA-mediated intracellular free calcium level signalling in aortic smooth muscle cells (Hopfner et al., 1998a). It was also hypothesized that insulin deficiency or hyperglycaemia may partly be responsible for the enhanced ET-1 production induced by ROS in 1 and 4 weeks diabetic kidney (Chen et al., 2000). Therefore, the relationship between insulin, ET-1, NO, ROS, and AGEs may be of importance in pathological condition such as diabetes.
In chapter 9, the role of insulin in the modulation of skin blood flow in early diabetes is examined. Based on information presented including the findings of previous studies the possibility was raised that adequate control of hyperglycaemia may prevent changes in skin microvasculature in early diabetes.

1.8.5.4. **Protein Kinase C (PKC)**

PKC, a family of kinases vital to intracellular signalling, consists of at least 11 isoforms. The conventional PKCs (alpha, beta I, beta II, gamma) are calcium-dependent, binding sites for DAG, and calcium / phospholipids. New PKCs (delta, epsilon, eta, theta, micro) are DAG-sensitive but calcium-independent. Multiple isoforms of PKC are expressed in each cell with several of them capable of mediating a similar range of functions that may be cell-specific (sections 1.5.4.1 & 1.5.4.2).

One study (*in vitro*) showed that PKC isoforms binding to DAG are activated by high glucose (20mM) in vascular smooth muscle cells (Haller et al., 1995). Total PKC activity was increased, in the above study, after six hours, causing translocation of alpha, beta, and epsilon isoforms into the nucleus. Also, PKC delta showed strong association with cytoskeletal structures. Previous studies have demonstrated that diabetes has a selective effect on the expression and subcellular distribution of isoforms of PKC (-alpha, -beta, -gamma and -zeta). For example, it was suggested that PKC-beta and delta-isoforms were activated preferentially in the vasculature of diabetic animals (Inoguchi et al., 1992; Koya and King, 1998). A recent immunohistochemical study revealed a marked increase in PKC-alpha immunoreactivity of cardiac capillaries, skeletal muscles and endothelial cells of larger arteries of 4 weeks diabetic rats (Kang et al., 1999). The author concluded that the increase in PKC-alpha in the endothelial cell supported a role for this isoform in the functional endothelial impairments observed in diabetes. There are controversial reports regarding PKC activity in the sciatic nerve of 4 weeks diabetic rats. One study showed around 47% reduction in calcium-dependent PKC in these rats (Gabbay et al., 1990), whereas others showed no significant change from control (Ido et al., 1994; Nakamura et al., 1999). Studies on the sciatic nerve, spinal cord, and DRG of 6 weeks diabetic rats
were more supportive of the notion that diabetes-induced changes in the expression of PKC beta II contributed to nerve damage (Roberts and McLean, 1997). The role of PKC in diabetic neuropathy is still unclear. For example, defective PKC has been implicated in impaired Na\(^+\)/K\(^-\)-ATPase activity in the sciatic nerve of 4 weeks STZ-treated rats (Borghini et al., 1994). On the other hand, PKC inhibition was shown to normalize the decrease in blood flow in diabetic animals (Cameron et al., 1997b). The effect of ROS on PKC isoforms is also controversial. Recent evidence suggested that PKC isoenzymes were subject to inactivation by ROS (e.g. glutathione) through unidentified oxidative structural modifications (Ward et al., 2000). This author showed that oxidative inactivation of PKC involves S-thiolation where endogenous thiols, such as glutathione, react with PKC. Other studies showed that PKC isoforms can be activated through tyrosine phosphorylation in response to hydrogen peroxide (Konishi et al., 1997; Li et al., 1999). It is possible that some of the hydrogen peroxide-induced PKC activation leads to vascular dysfunction in the early stages of diabetes (Konishi et al., 1997). Activation of PKC under diabetic conditions was suggested to have a modulatory role in oxidative stress-induced renal injury (Ha and Kim, 1999). Hyperglycaemia-induced oxidative stress may also mediate the adverse effects of PKC-beta isoform by the activation of the DAG-PKC pathway (Koya and King, 1998).

Numerous studies have shown a relationship between insulin, ET, and PKC activity. For example, insulin treatment prevented an increase in PKC activity and DAG levels in the heart (Inoguchi et al., 1994), aorta (4 weeks) (Okumura et al., 1991), and renal glomeruli (2 & 4) of diabetic rats (Babazono et al., 1998). Also, excessive PKC activity might interfere with insulin receptor signalling (Chin et al., 1994) under this disease condition. It was suggested that ET-1 could also interfere with insulin signalling in smooth muscle cells by both PKC-dependent and -independent pathways (Chin et al., 1994; Jiang et al., 1999). In the former study it was found that ET-1 increased the level of receptor (beta-subunit & IRS-2) phosphorylation as well as interfering with the PI-3 kinase pathway. Under diabetic condition or in the presence of high glucose, change in PKC activity can also regulate vascular permeability. It may increase permeability by direct phosphorylation of the barriers formed by endothelial cells and skin granulation tissues.
(Wolf et al., 1991), and/or indirectly by activating the vascular permeability factor (Koya and King, 1998).

In chapter 6, the role of PKC in modulating skin microvascular blood flow is examined in early diabetes.

1.9. Wound healing

Wound healing is defective in patients and animals with diabetes mellitus, which may be attributed to many factors. For example, hyperglycaemia leads to osmotic diuresis and subsequent decreased oxygenation and perfusion. It also limits polymorphonuclear leucocyte (PMN) functioning and produces malnutrition by increasing hormones that cause catabolism (Terranova, 1991). A recent study showed that impairment of the microcirculation is a major factor causing delayed tissue repair in diabetes (Pham et al., 1998). Although the exact mechanism underlying microvascular impairment is not known, dysfunction of the endothelium has emerged over the last decade, as the prominent abnormality related to vascular disease in diabetes (McMillan, 1997; Pickup and Williams, 1997).

1.9.1. Skin

1.9.1.1. Structure / extracellular matrix

The skin is one of the largest organs in the body, acting as a flexible barrier between the organism and the external environment. It protects the body against physical trauma, restricts water loss, plays a major role in temperature control, and being rich in nerves it forms an extensive sensory surface (Wood and Bladon, 1985).

It consists of two distinguishable layers:

The outer epidermis is cellular, non-vascular, and is the most superficial layer of the skin. It arises from the embryonic ectoderm and forms most of the cutaneous appendages including sweat and sebaceous glands, hair, and nails. In the human, it is 0.06-0.1mm thick on average and a highly organized kinetic system in which the cells divide, migrate, differentiate and eventually die. The epidermis consists of epithelial cells that grow in layers, the deepest cell layer resting on the dermis and attached to it by a basement membrane. Cells of the basal layer on the basement membrane continuously divide and
daughter cells migrate towards the surface of the skin during which they undergo differentiation. Ultimately they become packed with inert protein, keratin, and eventually die to form a layer of flattish, dry, horny cells. This layer is inert and covered with a thin layer of sebum, an oily, waxy material produced by sebaceous glands, to make it flexible and water-resistant.

The lower connective tissue layer or dermis forms the bulk of the skin. It is a tough, flexible, and highly elastic tissue that cushions underlying organs against mechanical injury. The dermis contains blood vessels, lymphatics, nerve endings, some sweat glands and hair follicles. It is much thicker than the epidermis (2-4mm), arises from the embryonic mesoderm and supports and nourishes the cutaneous appendages. In comparison with the epidermis, the dermis contains relatively few cells, mainly fibroblasts, mast cells, macrophages, lymphocytes, and melanocytes. The papillary layer of the dermis is adjacent to the basement membrane of the epidermis and includes ridges and papillae of fine connective tissue fibres, loops of capillaries, and some nerve endings. The reticular layer of dermis consists of coarse, dense, and interlacing collagen and elastic fibres.

Beneath the dermis is a layer of loose connective tissue called the hypodermis, sometimes recognised as a third layer of the skin. Over most of the body it forms a layer of adipose tissue which provides thermal insulation and mechanical protection and the fat represents an energy reserve.

**Extracellular matrix**

Most cells in a multicellular organism are in contact with an extracellular meshwork of interacting macromolecules, which constitute the extracellular matrix, and the dermis is no exception. Protein and polysaccharide molecules forming this matrix are secreted locally and assemble into an organised meshwork. Long and strong collagen fibres in the dermis strengthen and organise the matrix, the elastin fibres provide elasticity, while the aqueous phase of polysaccharide gel known as *ground substance* permits the diffusion of nutrients, metabolites, and hormones between the blood and tissue cells.

Collagen by volume is the principal constituent of the dermis. Collagen polypeptides (alpha-chains) are synthesized on membrane-bound ribosomes (pro-collagen) which pass...
into the endoplasm reticulum space where each pro-collagen polypeptide combines with two others to form a hydrogen-bound triple stranded helix. Later, they are removed by specific enzymes and remaining triple helical sections come together in the extracellular space to form larger collagen fibrils, except type IV collagen (basement membrane collagen), which seems to retain its extension peptides. After collagen fibrils have formed in this space they are considerably strengthened by the formation of covalent cross-links within and between the constituent collagen molecules.

Elastin appears to be synthesized by connective tissue cells and secreted into the extracellular space to form filaments and sheets in which molecules are extensively cross-linked to form a network. This network allows material to stretch and recoil like rubber, giving the skin its flexibility (Millington and Wilkinson, 1983; Wood and Bladon, 1985). Ground substance where collagen and elastin are embedded is made of a carbohydrate material known as glycosaminoglycan (GAG) (Millington and Wilkinson, 1983). This term encompasses a whole group of compounds with similar structures, namely: hyaluronic acid, dermatan sulphate, chondroitin sulphate, and heparan sulphate. Basically, they are long, unbranched polysaccharides composed of a repeating disaccharide unit. GAGs are highly negatively charged because of the presence of carboxyl and sulphate groups on many of the sugar residues. The high negative charge causes polymeric chains to adopt a stretched or extended configuration where the latter gives a high viscosity to the surrounding region. Their function may be related to the provision of normal suppleness and turgor as they are very hydrophilic and attract large amounts of water. GAGs also act as filters by allowing salts to move freely but due to their highly negative charge, they prevent large molecules such as proteins to diffuse into their domain. Hyaluronic acid is different from the other GAGs because it lacks a sulphate group, having very long chains, and not being joined to a protein core (Wood and Bladon, 1985). It is produced in large quantities in tissues through which cells are migrating during development or wound healing. It is possible that hyaluronic acid attracts water and swells the matrix, thus facilitating cell migration (Wood and Bladon, 1985).

Dermal ground substance was shown to be susceptible to the action of a number of hormones such as corticoids, sex hormones, thyroid hormones, anti-diuretics, and insulin
(Champion et al., 1970). A positive relationship between their degradation and the duration of diabetes was found. Other factors such as radiation, ageing, certain infections, as well as some dietary components (vitamin C and A) were also shown to have an effect either on synthesis of GAGs or on the level of hyaluronic acid (Champion et al., 1970).

It is believed that negatively charged molecules such as proteoglycans could interact with cells and under certain circumstances direct their migration, growth, and differentiation. Degradation and turnover of proteoglycans have not been clearly demonstrated in the skin, although polysaccharide-degrading enzymes have been found. These enzymes can react upon hyaluronic acid and chondroitin sulphate, but not on dermatan sulphate or proteases, which act upon the protein core of proteoglycans. The inability to degrade proteoglycans has been used to diagnose proteoglycan storage diseases (Pinnell et al., 1979).

1.9.1.2. Vascular supply / Endothelium

Blood vessels develop from the somatic mesoderm very early in life. They subsequently connect to the heart and once the flow is established, differentiation of smaller arteries and veins occurs. Larger arteries and veins are formed independently and, after foetal life, new vessels only arise by sprouting of the pre-existing blood vessel endothelium (Millington and Wilkinson, 1983). The vascular bed is therefore able to develop according to the requirements of the tissue concerned.

There are 4 classes of vessels in the skin: arteries, veins, capillaries, and lymphatics. Arteries of the skin pass through the deep fascia at definite points and thereafter in some sites branch to form the first plexus. From this plexus branches pass to the hypodermis where they give rise to the capillary network between fat cells. Other branches pass through this layer to form a second plexus in the deep layer of the dermis to form the capillary network around and through the sweat gland and papillae of the hair follicles. Further branches rise to give a third plexus in the outer zone of the dermis (sub-papillary) and to provide arteriovenous anastomoses (AVAs). These are specialised structures that permit total blood flow to the skin to increase without a change in the capillary flow (Millington and Wilkinson, 1983). They allow the cutaneous blood supply to perform its
dual task of thermoregulation and nutrition and are found in large numbers in the hands, feet, nose, and ears.

Blood supply of the skin plays a far greater role in regulating body temperature than just providing nutrients and removing waste products (Wood and Bladon, 1985). Highly developed nervous control is exercised particularly through the smallest arteries, which have the highest proportion of muscle in their walls, to control the blood flow in the skin. Arteriovenous shunts can short-circuit the capillary circulation in the dermis to prevent heat loss.

In circulation, muscular arteries and arterioles largely control the total blood flow by converting the irregular pulsation of the heart into a more continuous flow. Smaller arterioles eventually lead into non-contractile capillaries with variable width of 4 to 10 μm. Nutrition occurs only through skin capillaries, and variation in flow from zero up to 2.5 ml/100g tissue/min is required to cover these needs (Champion et al., 1970). The pre-capillary sphincter is the last portion of a smooth muscle, circularly disposed around arterioles. It controls the capillary flow and is influenced by local metabolic conditions rather than by nervous impulses. Larger arteries are more under the control of sympathetic nerves and less influenced by local conditions. The pre-capillary sphincters on the other hand, control the capillary flow and are influenced mainly by local metabolic conditions (e.g. histamine, CO₂) rather than by nervous impulses and the pressure gradient across the vessel wall. Post-capillary venules, although only weakly contractile, play an important role in the regulation of circulation. Although a slight increase in their tone will slightly reduce the flow rate, it makes an important difference in the rate of gas or material exchange between these vessels and a tissue. These vessels largely contribute to the normal skin colour and are involved in many pathological processes.

One of the major functions of the lymphatic system is to remove excess fluid and plasma proteins from extracellular space as well as antigenic materials from tissues. Lymph, which is similar to plasma but with different amounts of protein, moves in the lymph vessel by the compression of surrounding muscles and the valves that monitor the direction of flow. In the skin, each dermal papilla has a single lymphatic trunk that drains into a sub-papillary plexus and then a deeper one with valves. Then lymph pass centrally to the main lymphatic truncus and eventually into veins in the thoracic region. Flow in
the lymphatics is usually fairly slow but greatly increases with inflammation. In wound healing, lymphatics sprout from pre-existing lymphatics and not blood vessels.

**Endothelium**

The endothelium is composed of a single layer of flattened squamous cells joined together edge to edge to form a membrane of cells. It lines the innermost surface of blood vessels and is uniquely situated to sense and respond to changes in circulation. The endothelium plays a central role in the regulation of vascular muscle tone and ultimately arterial tone and blood flow by releasing and / or producing vasoactive substances (both vasorelaxants and vasoconstrictors) (Vane and Botting, 1994). Some of the vasoactive substances released by endothelium are EDRF, prostaglandins, kinins, ET, histamine, ACE, angiotensin II, cyclo-oxygenase (COX)-dependent constricting factor, and superoxide anions, etc (Bennett and Gardiner, 1996).

Activation of endothelial cells stimulates NO production and its release can lead to vascular smooth muscle relaxation by activation of guanylate cyclase and an increase in the cell cGMP. Continuous basal release of EDRF (NO) from most blood vessels, particularly arteries, contributes to resting vascular tone. Endothelial cell damage or inhibition of NO synthesis, on the other hand, leads to an increase in vessel tone (Bennett and Gardiner, 1996). Not all endothelium-dependent vasodilatations can be explained by the release / action of EDRF (NO).

Endothelial cells are also sources of endothelium-derived constrictor factors (EDCF), EDHF, and prostaglandins, which can be released following stimulation of endothelial cells by vasoactive substances or other physiological stimuli such as changes in blood flow, thrombin, NA, stretch, hypoxia, etc. At least three different classes of EDCFs have been recognised: 1) metabolites of AA, 2) the polypeptide ET, and 3) a still unidentified factor released from anoxic / hypoxic endothelial cells (Bennett and Gardiner, 1996).

1.9.1.3. **Innervation**

The skin is well supplied with nerves that are constantly sending information about the environment to the central nervous system. Sensory nerves have a variety of endings, many being naked (pain, touch), while others end in sensory cells (temperature, pressure).
This presumably enables a range of ‘sensations’ to be detected, from light touch to heavy pressure or pain, heat and cold, and vibrations (Wood and Bladon, 1985). Some areas such as the fingertips are much richer in nerve endings and consequently more sensitive than other regions. The mechanism of sensation (e.g. roughness, moistness) is probably brought about by varying stimuli acting on the ‘sensory unit’ in the skin, sending a mixture of temporal and spatial pattern of impulses to the cerebral cortex (Wood and Bladon, 1985). These may then reach the level of consciousness where, together with the individual’s past knowledge and experience, will be recognized as a particular sensation. Sympathetic fibres accompany cutaneous nerves and go to sweat glands, blood vessels, and erector muscles of hairs.

Skin vessels, both arterioles and venules, are under reflex neural control from a rich sympathetic adrenergic nerve supply. These active vasoconstrictor nerve fibres are the afferent arms from 4 types of sensory input: a) cutaneous thermoreceptors, b) baroreceptors of arteries and the cardiac-pulmonary system, c) chemoreceptors, and d) receptors activated by upright posture and exercise. Sympathetic nerves reflexly control the volume in veins but their responsiveness is modified by changes in local temperature. Veins are not responsive to baroreceptors but they do constrict during exercise. There are no parasympathetic nerves in the skin, only those from the sympathetic axis giving an adrenergic innervation to the smooth muscle of blood vessels, nipple, and dartos (Champion et al., 1970).

1.9.2. Mechanism of tissue repair

Upon tissue injury, the complex process of wound repair is initiated which consists of three phases: inflammation, proliferation, and tissue remodelling (Stadelmann et al., 1998; Waldorf and Fewkes, 1995). The inflammatory phase is marked by platelet accumulation, coagulation, and leucocyte migration. The proliferative phase is characterized by re-epithelialization, angiogenesis, fibroplasia, and wound contraction. Finally, the remodelling phase takes place over a period of months, during which the dermis responds to injury with production of the collagen and matrix proteins and then returns to its pre-injury phenotype. Wound healing is an important area of research but an in-depth discussion regarding the healing mechanism of skin wounds is beyond the scope
of this thesis. Therefore, the mechanism of wound healing in the skin is briefly discussed and only certain factors relevant to this thesis are mentioned.

**Inflammatory phase**

Following cutaneous injury, the endothelial integrity is disrupted while antidromic release of substances in an axon reflex induces a number of vascular changes. This is followed by active vasodilation that usually becomes most pronounced within 20 min after the injury and is accompanied by an increase in capillary permeability. Histamine is believed to be a key chemical mediator of these actions. Platelet aggregation then follows with concurrent stimulation of the coagulation cascade. Platelets are also a storehouse for a great number of growth factors and vasoactive substances such as PDGF, TGF-beta, FGF, platelet-derived angiogenesis factor (PDAF), epidermal growth factor (EGF), beta-thromboglobulin, serotonin, BK, prostacyclins, thromboxane, and histamine (Greenhalgh, 1996). Platelet aggregation generates a coagulation factor, thrombin, which in turn recruits additional platelets to release agents such as fibronectin and fibrin to promote platelet adhesion and to assist with the reflux of monocytes and macrophages.

Fibronectin deposition (24-48 hrs after injury) creates scaffolding on which fibroblasts can migrate into the wound. It is the fibroblast population that will become the dominant cell in the healing wound once the inflammatory phase has subsided. A variety of cell populations such as PMNs and mononuclear leucocytes will enter the trauma site as a result of increased vascular permeability and initially mature into wound macrophages and later into lymphocytes. Monocytes are considered to be the most vital component of the early phase of normal wound healing, however, PMNs are essential for protecting the wound against infection. Macrophages / PMNs destroy bacteria and remove devitalized tissue fragments and debris. Activated neutrophils release free radicals and lysozymal enzymes to help infection and clean the wound. Mediators such as thrombin and kallikrein are chemoattractant to circulating leucocytes, as well as being capable of releasing cytokines, which facilitate subsequent cellular chemotaxis, proliferation, and extracellular matrix production. The cytokine PDGF is a potent mitogen for fibroblasts and smooth muscle cells, and a chemoattractant for many other cells (Hosgood, 1993).


**Proliferation**

Fibroblasts appear in the wound within 2-3 days and dominate the wound population of cells within a week. They migrate to the wound site via scaffolding made by fibronectin and hyaluronate, the early extracellular matrix. Fibroblasts produce a variety of substances essential for tissue repair such as GAG and collagen (section 1.9.1.1). GAGs form ‘ground substance’ and collagen levels rise up to 3 weeks (increasing wound tensile strength) until a point of homeostasis is achieved. During the first 2-3 days fibroblasts mainly replicate and migrate rather than synthesize collagen, so very little in wound strength occurs during this period, often referred to as the ‘lag phase’. However, this term has been abandoned due to an enormous amount of cellular metabolism and fibroblastic activity that occurs during this period. Collagen synthesis is a seminal feature of fibroplasia. Fibroblasts are the major source of collagen and wound connective tissue. Angiogenesis accompanies this fibroblast phase where new capillary growth accompanies advancing fibroblasts into the wound to provide for their metabolic needs. If angiogenesis fails, fibroblast migration will be arrested and wound healing fails to proceed.

Wound re-epithelialization represents a sequence of steps involving mobilization, migration, mitosis, and cellular differentiation of epithelial cells. The epithelial cells immediately adjacent to the wound are stimulated to begin migration after loss of contact inhibition resulting in their growth away from adjacent intact epithelial cells (to cover the wound) (Stadelmann et al., 1998).

The phenomenon of wound contraction occurs at about 1 week after injury. At this time a portion of the wound fibroblasts undergo transformation into specialized cells, myofibroblasts. These cells are able to form secure intercellular attachments and since they are adherent to one another as well as the wound margins, the entire granulation bed contracts, drawing the wound edges closer together (Waldorf and Fewkes, 1995). Simultaneously, collagen is synthesized, deposited, and cross-linked to form a rigid scaffold holding the wound in place.
Tissue remodelling
After 3 weeks (post-injury), wound remodelling begins and continues for up to 2 years. Although there is no net increase in collagen content during this period, there is reorganization of collagen fibres into a more organized lattice structure determined by local mechanical factors. During this phase, the wound continues to increase in tensile strength. The majority of type III collagen fibres placed down early in the healing process are replaced by type I. Type I, II, and III collagen are responsible for most of the tensile strength in the body. During the tissue remodelling phase, GAGs are steadily degraded and remodelling continues until the GAG concentration and collagen ratio returns to that found in normal skin.

1.9.2.1. Role of sensory nerves
An intact nociceptor system of capsaicin-sensitive primary afferent sensory nerves is important for the initiation of the inflammatory process and successful tissue repair (Brain, 1997; Holzer, 1991). Unmyelinated afferent nerves with polymodal nociceptors mediate early components of the neurogenic inflammatory response, namely early neutrophil migration (Eglezos et al., 1991), plasma extravasation (Khalil and Helme, 1989), local vasodilatation (Khalil and Helme, 1990b), and modulation of cellular components of the immune response (Eglezos et al., 1991). These are mediated by neuropeptides (e.g. SP, CGRP) contained in peripheral terminals of capsaicin-sensitive nerves (Brain, 1997; Khalil and Helme, 1996). Healing of the injured tissue requires adequate organisational and functional reactions of the primary afferent sensory nerves and the microcirculatory system.

SP and CGRP are involved in wound healing. A recent immunohistochemical study of human burn wound healing demonstrated the presence of SP in the viable dermis in early burns and in a fibrous network of older burns, suggesting the involvement of this peptide in several steps in the repair process (Dunnick et al., 1997). A previous study also showed that CGRP increased survival of a musculocutaneous flap, in rats with skin injury, an effect independent of its vasodilator activity (Kjartansson et al., 1987). These growth promoting effects of sensory neuropeptides are thought to be involved in wound healing and contribute to the trophic functions of C-afferent fibres. A recent study has also been
supportive of the hypothesis that sympathetic efferents are important for wound healing (Kim et al., 1998).

NO is a potent vasodilator with the ability to increase vascular permeability and therefore may be important in inflammation, tissue injury and / or wound healing (Shuler et al., 1995). On the other hand, NO can also impose deleterious effects on healing process by inhibiting platelet adherence / aggregation (Radomski et al., 1990) and vascular smooth muscle proliferation (Garg and Hassid, 1989). The conflicting data produced by previous investigations on the physiological role of NO in the wound healing could be due to the major differences in the injury model, the type of animal used, and the timeframe of the investigation.

1.9.2.2. Role of growth factors

Growth factors are important signal transducers in the wound microenvironment. They are known to play a role in cell division, migration, differentiation, protein expression, and enzyme production (Greenhalgh, 1996). Almost all growth factors are peptides that bind to a cell surface receptor protein causing intracellular reactions that are not entirely understood.

There are 7 major families of growth factors: EGF, TGF-beta, IGF, PDGF, FGF, ILs and colony stimulating factor (CSF); but this is not a complete list of all growth factors known. They are a group of hormone-like polypeptides that play a central role in different phases of wound healing. Growth factors attract cells into the wound, stimulating their proliferation, and have a profound influence on extracellular matrix formation (Ashcroft et al., 1995; Greenhalgh, 1996; Martin et al., 1992; Okumura et al., 1996; Stoscheck et al., 1992). Sensory peptides are important for the chemotactic activity of monocytes, macrophages, and T-lymphocytes in skin wounds and for the subsequent availability of transforming growth factor (TGF)-alpha, which is essential for healing (Richards et al., 1997). Sensory neuropeptides and growth factors could have an overlapping range of actions during the wound healing process.

The event of early wound healing reflects a finely balanced environment in which proteolytic activity and matrix synthesis occur under tight regulation, leading to uncomplicated and rapid tissue repair. In chronic wounds, this fine balance is lost causing
delayed healing. Since describing roles of all these growth factors is beyond the scope of this thesis, only a few were discussed. Several studies provide evidence that EGF stimulates rapid epithelialization of skin wounds with an increase in EGF-receptor expression in the epidermis during the proliferation and migration of epidermal keratinocytes (Nanney, 1990; Stoscheck et al., 1992). Basic FGF (bFGF) can also improve healing largely by enhancing angiogenesis and granulation tissue formation (Okumura et al., 1996). There is evidence that IGF-1 stimulates GAG biosynthesis (Cechowska-Pasko et al., 1999) and stimulates keratinocyte proliferation (*in vitro*) (Blakytny et al., 2000). PDGF and TGF-beta are both primarily secreted from platelets but also from activated macrophages and fibroblasts. They not only possess chemotactic properties but also increase collagen content and strength in wounds in normal and compromised tissues (Hosgood, 1993).

### 1.9.3. Effect of diabetes

#### 1.9.3.1. Structural changes

Wound healing data derived from human and animal models of diabetes revealed a reduction in contraction of open wounds, decreased number of fibroblasts, and decreased number of PMNs (Franzen and Roberg, 1995; Hehenberger et al., 1999; Stadelmann et al., 1998).

A previous study demonstrated diminished wound strength in STZ-induced diabetic rats accompanied by a reduced collagen content (Verhofstad and Hendriks, 1994). In this study, the fact that the diabetic skin is thinner than normal individuals was ruled out as a reason for the decrease in the wound breaking strength (Bitar, 1998). In a different study, using 4 weeks diabetic rats with circular full-thickness skin wounds, decreased granulated tissue was suggested to be due to the increase in wound collagenase level (Ramamurthy et al., 1998). Many local and systemic factors can delay wound repair such as tissue ischaemia, intra- and extra-vascular fibrin deposition, vasodilatation of non-nutritive microcirculation, necrosis, infection, impaired migration of the epithelial cells of the ulcer edge, and inadequate cytokine pattern (Morian and Colen, 1990). For example, in diabetic mice, decreased neovascularization and less organization of the granulation
tissue during the late inflammatory phase of wound healing were suggested to be due to altered leucocyte infiltration and wound fluid IL-6 levels (Fahey et al., 1991).

A relationship between IGF-1 and GAG biosynthesis and their role in full thickness wounds in diabetic animals has also been documented (Cechowska-Pasko et al., 1999; Tsuboi et al., 1995). Also reduction in the synthesis and content of heparan sulphate, a proteoglycan, in the skin of acute diabetic animals (Cechowska-Pasko et al., 1996b; Rohrbach et al., 1983) was shown to be inversely related to the level of plasma glucose. By normalising the glucose level, using insulin treatment, the level of synthesis was returned to normal in these animals (Rohrbach et al., 1983). Heparan sulphate in the basement membrane restricts the passage of proteins; its absence could account for the increased porosity of the basement membrane in diabetes.

1.9.3.2. Physiological changes

Physiological changes related to the wound healing process observed in diabetic rats are increased oedema, increased rate of infection, decreased capillary volume, decreased neovascularization, delayed reappearance, and abnormal reformation of epithelial basement membrane proteins (Fahey et al., 1991; Morian and Colen, 1990; Nemeth et al., 1999b; Sato et al., 1999). The role of sensory neuropeptides (SP, CGRP) as trophic agents was described earlier. Delayed healing may be related to a reduced level of sensory neuropeptides.

In diabetes mellitus, there is evidence that sensory neuropeptide level is reduced in axons and cell bodies of primary afferent nerves (Diemel et al., 1992; Tomlinson et al., 1988), leading to reduced inflammatory responses in these animals (Mathison and Davison, 1993; Nemeth et al., 1999b). The role of sensory neuropeptides in acceleration of wound healing was previously documented (Baker et al., 1997; Khalil and Helme, 1996; Khalil and Merhi, 2000). Khalil and Helme (1996) have provided direct evidence for the ability of sensory neuropeptides (exogenously applied) to accelerate wound healing in aged rats and indirect evidence to suggest a possible role for sensory peptides in modulating wound contraction and re-epithelialization. This latter proposition is supported by the Watcher and Wheeland (1989) study, which provided evidence that topical application of capsaicin accelerated wound contraction and re-epithelialization of full-thickness
wounds. The authors suggested that effects of capsaicin might be mediated via the release of SP from sensory nerves. Furthermore, Khalil and Helme (1996) provided evidence that interaction between these peptides can influence their ability to modulate the healing process. In a study by Khalil and Merhi (2000), tissue repair was accelerated by increased levels of neuropeptides released from sensory nerves using transcutaneous electrical nerve stimulation. In the Baker et al (1997) study, again electrical stimulation of sensory nerves using specific parameters accelerated the healing process in diabetic patients with ulcers.

Intact fibroblast function is required for normal wound healing. An in vitro study showed suppression of fibroblast proliferation in cells exposed to high glucose (Hehenberger et al., 1999) suggested that PKC and hyaluronic acid might play a role as modulators of fibroblast proliferation during the diabetic state.

A recent study showed a significant decrease in neutrophil counts and phagocytosis in 5 weeks diabetic rats with an incisional skin wound (Canturk et al., 1999). Neutrophils play a critical role in the host defence mechanism and impairment in its function can cause healing difficulties in diabetes with or without infections (Stadelmann et al., 1998). Studies on immune cells necessary for wound healing, such as PMN and fibroblasts, suggest that there is a delayed response to injury and impaired functioning of immune cells in diabetes mellitus (Rosenberg, 1990).

Elevated ROS in short-term diabetes (Mullarkey et al., 1990; Traverso et al., 1998) reduced tissue blood flow by causing deficits in nerve conduction velocity (Cameron et al., 1991), which can, in turn, affect the release of neuropeptides from sensory nerves (Nemeth et al., 1999a). ROS also cause antioxidant-preventable vascular endothelium abnormalities, neutralizing NO-mediated vasodilation, and increased reactivity to vasoconstrictors (Stohs, 1995). In both in vivo and in vitro settings, insulin increased the activity of some antioxidants (Cu-Zn-SOD, catalase, glutathione peroxidase) and the production of hydrogen peroxide in rat macrophages (Pereira et al., 1995). Insulin improved blood flow and wound matrix formation in burn patients (Pierre et al., 1998). The former effect may be due to an improvement in antioxidant defence mechanisms as
well as production of hydrogen peroxide (Pereira et al., 1995). ROS can influence collagen remodelling by affecting the collagen structure (Arisawa et al., 1996). NO plays a significant role in wound reparative collagen deposition (Schaffer et al., 1997) as well as being involved in inflammatory responses (Bennett and Gardiner, 1996). ROS are known to destroy NO (Gryglewski et al., 1986; Kamata and Kobayashi, 1996) and in this way they may contribute to delayed healing. Furthermore, endothelium-derived NO may play an important role in mediating angiogenesis by supporting endothelial cell migration (Murohara et al., 1999), in part via an integrin-dependent mechanism.

High levels of ET-1 were previously detected in both diabetic humans and STZ-treated rats (Makino and Kamata, 1998; Nakamura et al., 1997; Takahashi et al., 1990). An increase in ET-1 was suggested to be one of the factors responsible for partial necrosis of the skin flap wound contributing to the genesis of the no-reflow phenomenon (Matzuzaki, 1993). On the other hand, it was reported that ET stimulated transmigration of endothelial cells and accelerated endothelial wound healing by acting on ET-B receptors (Goligorsky et al., 1999). The author showed that, in vivo, ET enhances new vessel formation, an effect that requires functional eNOS.

In chapter 10, the information gained from previous chapters is utilized to examine the effects of specific treatment protocols (local and systemic) in modulating the repair of full-thickness skin wounds in 4 weeks diabetic rats. It was of interest to prevent the effects of a number of factors namely ETs, free radicals, AGEs, and hyperglycaemia on microvasculature in early diabetic rats, using these treatment protocols, and examine the progression of tissue repair process.
1.10. Aims and hypotheses

Unmyelinated primary afferent sensory neurones and the microvasculature innervated by these nerves play a major role in modulating the inflammatory and tissue repair processes. Alterations in these processes caused by diabetes mellitus can lead to the impaired blood flow and delayed tissue healing. The mechanisms underlying changes in skin microvascular blood flow and their effects on tissue repair processes, especially in the early stages of diabetes, are not clear and require further investigation.

Therefore, the aims of this thesis are:

1. To establish a time frame for the occurrence of skin microvascular changes in the STZ-treated diabetic rat model. Chapter 3 presents the findings of the investigation undertaken to address this aim. It was hypothesized that there is a relationship between the duration of diabetes and the occurrence of these changes.

2. To examine alterations in sensory nerve-mediated events (pre- and post-terminal levels) and identify the mechanisms underlying these alterations in 4 weeks STZ-treated diabetic rats. Once again, chapter 3 presents the findings of the investigations undertaken to address these aims. It was hypothesized that the contribution of both pre- and post-terminal mechanisms might contribute to reduced inflammatory responses in early diabetes.

3. To examine the effect of 4 weeks diabetes on both the superficial and deep compartments of the rat skin microvasculature. Chapters 3 and 4 present the findings of the investigations undertaken to address this aim. It was hypothesized that there are alterations in the function of microvessels that contribute to the reduction in skin microvascular responses in early diabetes.
4. To examine alterations in the function of parasympathetic and sympathetic nerves and identify mechanisms underlying these changes in 4 weeks STZ-treated diabetic rats. Chapter 4 and 5 present the findings of the investigation undertaken to address these aims. It was hypothesized that these mechanisms contribute to alterations in microvascular responses in early diabetes.

5. To examine the role of local tissue factors in modulating microvascular responses in 4 weeks STZ-treated diabetic rats. Chapter 4 presents the investigation undertaken to address this aim. It was hypothesized that since local tissue factors could modulate the function of sensory nerves, any alterations in their function could influence microvascular blood flow.

6. To examine the role of ETs, free radicals, AGEs, and hyperglycaemia in modulating microvascular responses in 4 weeks STZ-treated diabetic rats. Chapters 6, 7, 8, and 9 present the results of the investigations undertaken to address this important aim. It was hypothesized that ETs, free radicals, AGEs, and hyperglycaemia may be involved in altering skin microvascular function in early diabetes, which may be inhibited by early intervention.

7. To examine the effects of specific treatment protocols (local and systemic) in modulating the repair of full-thickness skin wounds in 4 weeks diabetic rats. Chapter 10 presents the findings of the investigation undertaken to address this aim. It was hypothesized that preventing the deleterious effects of ETs, free radicals, AGEs, and hyperglycaemia, using specific inhibitors / antagonists, may improve skin microvascular blood flow and tissue repair processes.
CHAPTER 2

Materials and General methods
2.1. Materials

Rat food (GR2 and mouse breeder cubes) was provided by Barastock Stockfeeds Pty Ltd (Melbourne, Australia). Neuropeptides and chemicals were purchased from different sources:

1. Nembutal (Pentobarbitone sodium, 60mg/ml): Boehringer Ingelheim Pty Ltd (North Ryde, Australia)
2. Substance P (SP), Endothelin (ET)-A receptor antagonist (BQ-123), Calcitonin gene-related peptide (CGRP): Auspep Pty Ltd (Melbourne, Australia)
3. Streptozotocin (STZ) (mixed anomers), Alloxan monohydrate, ET-B receptor antagonist (BQ-788), Sodium nitroprusside (SNP), Superoxide dismutase (SOD) and PEG-SOD (both from bovine erythrocytes), Bradykinin (BK), N\textsuperscript{G}-Nitro-L-arginine methyl ester (L-NAME), Nicotine, Muscarine chloride, N-acetylcysteine (NAC), Guanethidine monosulfate, Capsaicin: Sigma Chemical Company (USA)
4. Aminoguanidine hydrochloride (AG): Sapphire Biosciences Pty Ltd (Alexandria, Australia)
5. Bisindolylmaleimide (BIM): Calbiochem-Novabiochem Corp (Melbourne, Australia)
6. Amadori products, Advanced glycated end-products (AGEs), anti-RAGE antibodies (IgG): Peptech (Dee Why, Australia) (See Appendix C)
7. Tirilazad mesylate (Freedox) (1.5mg/ml): Pharmacia & Upjohn (Rydamere, Australia)
8. Protophane penfill (isophane insulin, 100IU/ml): NovoNordisk Pharmaceuticals Pty Ltd (Melbourne, Australia)

2.2. Animals

Male outbred Sprague-Dawley rats were used in this study as both alloxan and streptozotocin can easily cause diabetes in these animals (Dunn and McLetchie, 1943; Rakienten et al., 1963). They were purchased from Monash University animal services (Melbourne, Australia) and were delivered to the National Ageing Research Institute (NARI), where all the experiments were performed, on a regular basis. Upon arrival, rats were placed in a special room where the temperature was kept at 22°C and the light was
on a 12hr dark / light cycle throughout the year. The original weight of rats at the start of every treatment was around 130-150 g (6-7 weeks old).

Rats were anaesthetised by a single dose of pentobarbitone sodium (65mg/kg) at the start of every experiment and added doses were administered to ensure rats remained under a constant state of anaesthesia. The loss of eyelash-reflex was used as a test to ensure the maintenance of anaesthesia throughout the experiment. Animals were kept under a deep state of anaesthesia for all experimental (invasive) procedures in order to comply with the ethic’s regulation. Animals were fully conscious during pre-treatment with alloxan, streptozotocin, insulin, etc. At the end of each experiment rats were sacrificed with anaesthetic overdose.

2.3. Types of food

2.3.1. Mouse Breeder

This type of food is rich in certain nutrients, which are required for growing young rodents (Table 2-1). Some vitamins such as A, E, and niacin that are present in this type of food, seem to play an important role in delaying or preventing diabetes onset (Chapter 1, section 1.7.2.3).

2.3.2. GR2

As shown in table 2-1, the content of GR2 is suitable for slightly older rats and contains lower levels of vitamin A, E, and niacin among others (Table 2-1). In this study, more rats develop diabetes (more than 80%), using STZ / alloxan treatments, when rats were fed GR2 instead of mouse breeder cubes.
Table 2-1: Major differences between the content of Mouse breeder and GR2.

<table>
<thead>
<tr>
<th>Content</th>
<th>Mouse breeder</th>
<th>GR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>15000 IU/kg</td>
<td>9500 IU/kg</td>
</tr>
<tr>
<td>Vitamin B1</td>
<td>64.7 mg/kg</td>
<td>10.78 mg/kg</td>
</tr>
<tr>
<td>Vitamin B2</td>
<td>60 mg/kg</td>
<td>12.8 mg/kg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>11 mg/kg</td>
<td>2 mg/kg</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>50 mg/kg</td>
<td>9.4 mg/kg</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>250 mg/kg</td>
<td>45.5 mg/kg</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>30 mg/kg</td>
<td>5 mg/kg</td>
</tr>
<tr>
<td>Niacin</td>
<td>400 mg/kg</td>
<td>80.7 mg/kg</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>260 mg/kg</td>
<td>50 mg/kg</td>
</tr>
</tbody>
</table>

2.4. Induction of diabetes

2.4.1. Choosing a diabetogenic agent

In animals, diabetes can be induced chemically using a diabetogenic agent at a specific dose (Abdel-Rahman et al., 1992b; Rakienten et al., 1963). Soon after induction of diabetes:

1. High levels of glucose should be present in animal’s urine and blood
2. Animals should show physical symptoms of diabetes (i.e. weight loss, polydipsia, polyphagia, polyuria)
3. Animals should respond to insulin treatment by showing a reduction in the level of glucose in urine and blood and reduction in the physical symptoms of diabetes

In this study, two diabetogenic agents namely STZ and Alloxan were used. Rats (130-150g) (6-7 weeks old), fed with GR2, were fasted for 24 hrs before injecting them with one dose of freshly prepared alloxan or streptozotocin. It was suggested that hypoglycaemia is severe in fasted rats treated with these chemicals and it can lead to frequent attacks of convulsions and finally death (Rerup, 1970). For this reason, injected rats were given 5% sucrose solution for 48hrs after the injection to avoid immediate death. The chosen diabetogenic doses and conditions resulted in more than 80% success rate in creating diabetic rats whether they were treated with alloxan or STZ.
The classic symptoms of diabetes (polydipsia, polyuria, polyphagia, weight loss) became generally apparent within 2-6 days after injection of the chemical, depending on which agent was used, and diabetes was confirmed by urine glucose test (Diastix, Bayer Australia Ltd). Before each experiment the blood glucose of every rat was also measured with Reflolux S (Boehringer Mannheim, Australia Pty Ltd) and those with blood glucose of 27.7 mmol/lit or more were included in the study (section 2.5).

2.4.2. Control rats
Control rats of similar age / weight (as above) were fasted over night. Then instead of a diabetogenic agent, they were injected intraperitoneally (i.p.) with a similar volume of 0.1M cold sodium citrate buffer. These rats were kept under the same condition as the experimental group and fed the same diet (GR2). Control rats were tested at the same day as the experimental rats. The data representing controls were used throughout the thesis to facilitate comparison with diabetic data.

2.4.3. Alloxan treatment
Alloxan (40mg/kg) was dissolved in cold sterile saline and was kept on ice to avoid decomposition. After 24hrs of fasting, rats were injected with one dose of freshly prepared (cold) alloxan solution intravenously (i.v.) into the tail vein (Abdel-Rahman et al., 1992b). Symptoms of diabetes became apparent 4-6 days later and diabetes was confirmed by urine glucose test. Before each experiment the blood glucose of every rat was also measured with Reflolux S and those with blood glucose of 27.7 mmol/lit or more were included in the study (section 2.5). Alloxan-treated rats coped well with the severity of their physical symptoms and did not require insulin injection during the experimental period.

2.4.4. Streptozotocin
STZ (75mg/kg) was dissolved in 0.1M cold sodium citrate buffer (pH 4) and kept on ice to avoid decomposition (section 2.4.6). Once again, after 24hrs of fasting, rats were injected (i.p.) with a single dose of freshly prepared (cold) STZ (Rakienten et al., 1963). Symptoms of diabetes became apparent in these rats earlier than alloxan-treated group (within 2-3 days) and their diabetic condition was confirmed by urine glucose test. Beside
classic symptoms of diabetes, other symptoms such as a change in the condition of their fur, development of cold and pale paws, severe diarrhoea in some cases, and reduced physical activity, were also observed in both alloxan- and STZ-treated rats. However, overall changes in physical symptoms were more severe in the latter group. As a result, STZ-treated diabetic rats were given one or two insulin injections, when the severity of symptoms became life threatening, to provide a temporary relief and prevent death (section 2.4.6). After insulin treatment, apart from obvious physical improvements in these animals, their survival rate was also enhanced. Rats were sacrificed at any point if they lost 15% or more of their body mass to comply with the ethic’s regulation.

2.4.5. **STZ vs Alloxan**

STZ was chosen as a preferred agent to alloxan in creating an animal model of diabetes mellitus for this study after careful consideration of both advantages and disadvantages of the two chemicals.

1. STZ treatment induced diabetes in rats in a shorter period of time (2-3 days) compared to alloxan treatment (4-6 days).
2. To achieve a high success rate (80% or more) in creating diabetic rats i.p., it was more economical to apply STZ (75 mg/kg) than alloxan (100mg/kg).
3. Diabetic condition was more severe in STZ than alloxan-treated animals (i.e. blood glucose above 27mmol/lit).
4. No spontaneous recovery was observed in STZ-treated rats.
5. The mortality rate was slightly higher in STZ than alloxan-treated groups due to severity of diabetes. However, this was easily prevented if diabetic rats were given 5% sucrose solution for the first 48hours after STZ injection.

2.4.6. **Modifications made to the original procedure**

The original adapted procedure for induction of diabetes in rats, using STZ (Rakienten et al., 1963; Takeuchi et al., 1994) and alloxan (Abdel-Rahman et al., 1992b), was modified in the following manner to produce the best success rate and reduce the total number of animals required for experiments in this thesis.
1. **Food**

Previously Mouse breeder cube was the only standard food used in our laboratory. In the original experiments, STZ treatment failed to cause diabetes in the rats that were on the Mouse breeder cube diet. Careful analysis of content of this food showed high levels of vitamin A, E, and niacin. Previous studies suggested that these vitamins could play a role in inhibiting the diabetogenic action of STZ by protecting pancreatic beta cells (Beales et al., 1994; Hoorens and Pipeleers, 1999) (see also chapter 1, section 1.6.2.2.1). Changing from Mouse breeder cube to GR2, which has lower levels of these vitamins, reduced their effect and STZ injection could induce diabetes in GR2 fed rats within 2-3 days.

2. **STZ and alloxan solution**

STZ is an unstable compound that dissolves in saline at room temperature but decomposes within a few minutes by forming a visible gas. Dissolving STZ in a cold freshly made 0.1 M citrate buffer solution of (pH 4) on ice proved to be successful in keeping this solution stable for a short period of time before injection (Rerup, 1970). Alloxan is freely soluble in water and its stability in aqueous form is, once again, dependent on pH and temperature. Below pH 3 alloxan is fairly stable at room temperature, whereas at pH 7 the solution has to be kept below 4°C to avoid rapid decomposition.

3. **STZ and Alloxan dose**

A diabetic dosage is defined as the amount of an inducing agent which, in 80% of animals of a given species, produces sustained hyperglycaemia and beta cell necrosis, but does not cause harm to other organs (Goldner and Gomori, 1944). In rats, a single i.v. injection of 50mg/kg STZ and 30-70mg/kg alloxan were reported to yield 100% diabetes (Rerup, 1970). Rerup (1970) reported that if non-i.v. injection was chosen as the route of administration for these agents, a different dose may be required to achieve the 80% or more success rate. Hence, 50 mg/kg was originally chosen as a minimum starting dose for STZ and 30 mg/kg for alloxan since a different route of administration was used. However, results showed that under our experimental conditions, the diabetogenic dose for STZ was 75mg/kg (i.p.) and alloxan was 40mg/kg (i.v.).
4. **Weight**

Using 75mg/kg STZ, only 20% of rats became diabetic if they weighed more than 150g and 100% of rats died within 48hrs after the injection due to severe diabetes if they weighed under 130g. However, 80-90% of rats in the range of 130g-150g became severely diabetic with the above dose and no fatality was observed. Similar results were observed when alloxan, at 40mg/kg, was used.

5. **5% sucrose solution**

Sucrose solution was used to overcome the severe hypoglycaemic condition of STZ- / alloxan-treated rats (Rerup, 1970). Our own original experiments showed that by providing treated rats with 5% sucrose solution for the first 48hr after injection, compared to 24hrs, the animals’ survival rate could be improved by 30% or more during the crucial period after diabetic onset (i.e. the first 10 days).

6. **Insulin treatment for survival**

In general, insulin treatment was not used to control the diabetic condition, except were indicated (chapters 9 and 10), but to give regular respite from the catabolic dominance of the condition while allowing regular and severe hyperglycaemia (Willars et al., 1989). One or two injections of insulin (Protophane 2IU/100g) were given subcutaneously (s.c.) to the newly diabetic rats depending on the severity of their physical condition (e.g. little eating or drinking, weight loss to around 115g, very inactive). However, if they did not show any improvement or lost more than 15% of their body weight they were sacrificed.

2.4.6.1. Establishing a time frame

The original experiments were purely performed to establish a diabetic rat model with an appropriate time frame to examine the gradual deleterious effects of diabetes on skin peripheral microvasculature over time using established techniques. These results were then presented to the animal’s ethics committee to justify the purpose of the thesis and proposed experiments. Experimental procedures were also discussed and approval was obtained for the use of these animals in this study.
Changes in microvascular blood flow in STZ-treated rats were observed during the first 10 weeks after diabetes onset using two protocols: 1) the blister model of inflammation and 2) electrical stimulation of the sciatic nerve (sections 2.6 & 2.7). For each week of diabetes, a minimum of 12 STZ-treated rats was used (n = 6).

2.5. Insulin treatment protocol

Insulin treatment was used under two conditions: 1) as an infrequent injection to relieve the severe life-threatening symptoms of diabetes in experimental rats and extending their survival rate, and 2) as regular injections for the purpose of controlling diabetes in rats. The former insulin treatment was available for all diabetic rats used in the thesis, except those that were used in chapter 10 (group 3 only) and chapter 9 experiments that received the latter insulin treatment. The dose of injection varied from 2-6IU/100g/day depending on the daily level of urine glucose, weight loss, and the observed physical condition of animals (level of activity, appearance, eating / drinking). Urine glucose was tested every morning, between 9am and midday, using specific test strips for urine glucose assay (Diastix, Bayer Australia Ltd). For measuring plasma glucose, a drop of blood was taken from the rat’s artery or vein and the glucose level was measured by Reflolux S (Boehringer Mannheim Australia Pty Ltd).

The type of insulin was protophane, an intermediate acting insulin with a peak effect of 4-12 hrs, and this was administered by a NovoPen. Insulin was injected (s.c.) on the back of the rat’s neck, where the skin is loose, every morning between 9am and midday.

2.6. Blister model of neurogenic inflammation

Neurogenic inflammatory responses can be induced experimentally in a number of ways; one is by exogenously administering sensory neuropeptides (chapter 1, section 1.7.3). For this type of experiment, rats were first anaesthetized with pentobarbitone sodium (65mg/kg, i.p.). Then a vacuum pressure of negative 40 kPa was applied for approximately 15-30 minutes to the glabrous skin of the hind footpad, using a metal suction cap heated to 40°C by an attached heating element. The smaller diabetic rats developed a blister on their footpad faster (15-20 min) than control animals (30 min). When a blister was established, the surface epidermis was removed to allow direct access to the blood vessels and nerve endings. Then the foot was fixed in a Perspex chamber.
with inlet and outlet ports to allow constant perfusion of substances over the blister base. Perfusion was maintained at 4ml/hr by a peristaltic pump (Microperfex, LKB, Sweden) and body temperature was maintained at approximately 37°C throughout the experiment (Fig 2-1).

The blood flow in the foot was monitored throughout the experiment via a laser Doppler flowmetry probe placed in a third port directly above the centre of the blister base. In every experiment, first a stable baseline blood flow was achieved after 20-30 minutes perfusion of Ringer’s solution over the blister base. In perfusion experiments, SP (1μM) was then perfused over the blister base for 30 minutes, during which time the perfusate (plasma extravasation) was collected every 10 minutes (prior, during, and post stimulation). The concentration of SP used in these experiments was previously established in our lab as the submaximal concentration that induces a significant plasma extravasation response (Andrews et al., 1989). The protein content of the perfusate was measured using Bradford colorimetric assay (Bradford, 1976). After SP perfusion, a 20 minutes washout with Ringer’s solution was allowed to establish the baseline for the second time. Changes in the smooth muscle-dependent vasodilation were then examined using SNP (100μM, 10 min), the direct smooth muscle vasodilator. After that, once again Ringer’s solution was perfused for 10 minutes or until the base line was re-established for the third time. If applicable, then CGRP (1μM, 10 min) was perfused until the maximum height of the response was achieved. CGRP perfusion was last in all experiments since its vasodilator action in the skin, unlike SP, does not undergo desensitization and can continue for a few hours (Brain, 1996). Depending on the type of laser Doppler flowmeter used, the results were calculated differently and this is described in section 2.9.

SNP, SP, and CGRP were perfused over the blister base in most experiments throughout this thesis. The independent smooth muscle vasodilator, SNP, was used to examine independent changes in smooth muscle reactivity. A sensory neuropeptide, SP, was used to examine changes in sensory mediated events at both pre- and post-terminal levels. Another sensory neuropeptide, CGRP, was used to examine changes in sensory mediated
events (at the post-terminal level) with mechanisms different to those involved in SP and SNP responses.

2.7. Electrical stimulation of sciatic nerve

Among many nerve preparations currently in use, the sciatic and saphenous nerves are the most common as they are the major nerves of the hind limbs and are easily accessible (Baker et al., 1979-80). To prepare the sciatic nerve for electrical stimulation (ES), once again rats were anaesthetised and a blister was formed in their hind footpad as described in section 2.6. Then the rat’s right leg was shaved at the mid-thigh region and under anaesthesia and aseptic condition, a cut was made to expose the sciatic nerve. Using blunt dissection through biceps femoris, the nerve was then separated from the surrounding tissue and cut as proximally as possible. The distal portion of the cut nerve was placed over bipolar platinum electrodes and immersed in liquid paraffin warmed to 37°C and contained in a “bath” that was formed by raising the skin flaps of the wound (Fig 2-1). The electrodes were fixed in such a position that electrical leakage to adjacent nerve and tissue structures was minimised.

![Fig 2-1: A schematic representation of an experimental set up for electrical stimulation of the sciatic nerve.](image)
For examining the vascular response to electrical nerve stimulation, the rat’s sciatic nerve was stimulated (Grass S48 stimulator, Grass medical instruments, Quincy, U.S.A.) every 20 minutes (20 V, 5 Hz, 2msec for the duration of 1 minute). These parameters have been previously used to stimulate efferent C-fibre responses (Helme et al., 1985; Lembeck and Holzer, 1979), to evoke an immediate increase in local blood flow. Ringer’s solution was perfused during and for at least 20 minutes after stimulation of the nerve at the speed of 4ml/hr. The time elapsed from the start of the blister induction until sciatic nerve stimulation did not exceed 60 minutes. Again, SNP (same concentration as before) was used to measure the smooth muscle-dependent vasodilatation in these rats. This was performed before stimulating the sciatic nerve to devoid our results from the sympathetic nerve contribution.

2.8. Neonatal capsaicin pre-treatment

Capsaicin was prepared in normal saline containing 10% Tween 80 and 10% ethanol. Neonatal rats of 4 days old were then injected (50mg/kg, s.c.) with a single dose of capsaicin on the back of the neck (Fitzgerald, 1983; Jancso et al., 1977). The effectiveness of the neonatal pre-treatment (up to 90% degeneration of unmyelinated primary afferents and destruction of small DRG) was assessed around 2-3 months of age. Prior to each experiment, a drop of capsaicin solution (0.1% capsaicin in ethanol) was applied to the cornea of treated animals (Fitzgerald, 1983); if the number of eye wipes were less than 25% of controls, rats were considered capsaicin denervated. Those that were not sensory denervated were euthanized (by anaesthetic overdose).

Capsaicin pre-treated rats (130-150g) were then made diabetic by STZ injection using the same protocol as described before. Two and four weeks later, they were anaesthetized and a blister was induced on the hind footpad. Once again the foot was secured in a Perspex chamber and the blood flow was monitored using the Perimed or the Moor laser Doppler flowmeters (PLDF or MLDF). Since the MLDF has dual ports, the blood flow was monitored in both feet simultaneously. SP and SNP (same concentrations as before) were perfused over their blister bases as described before (section 2.6).
2.9. Blood flow measurements using laser Doppler flowmeter (LDF)

Laser Doppler flowmetry is a non-invasive technique that is simple to perform yet reproducible and reliable. This is suitable for long-term monitoring of the circulation and examination of the effects of short-term alterations to the circulation e.g. caused by chemical sensory nerve stimulation. LDFs were developed in the early 1970’s (Halloway and Watkins, 1977; Stern, 1975) but Gamse and Saria (1987) were first to adopt this technique as a method for measuring antidromic vasodilation in rat skin.

A laser Doppler flowmeter has a solid-state laser diode as a light source, which is delivered by a fibre optic line to the probe and tissue. In the skin, light from the probe is absorbed, scattered or reflected (5-7%) because the epidermal layers act as absorption filters limiting the depth of the light’s penetration (Anderson and Parish, 1981). Since short light wavelengths are absorbed and scattered more than longer ones, red and far-red lasers with wavelengths from 600-1200 nm have been preferred in most LDF applications. This ensures the light’s maximum depth of penetration into the dermal layers of the skin (Gush and King, 1991). A portion of backscattered light is then collected by the LDF where it is analyzed.

The principle of laser Doppler flowmetry is based on the fact that moving red blood cells induce a Doppler (Frequency) shift of the wavelength. Therefore, the scattered light from a moving element will have a different frequency to the original light. The output signal from the laser Doppler is directly proportional to the perfusion of blood cell flux (Oberg, 1990), which is defined as

\[ \text{The number of moving cells} \times \text{mean velocity} = \text{blood flux} \]

Two LDFs have been utilised in this study, the Perimed periflux and Moor DRT4.

2.9.1. Perimed LDF (PLDF)

The Periflux model (PF2B, Perimed, Sweden) uses a laser beam of 633nm (Visible red light) which penetrates approximately 1mm deep into the skin where superficial capillaries (around 10μm in diameter) are located. Capillaries are arising from the papillae of the corneum and returning to subpapillary venous plexus. The blood flux (Volts) is electronically processed and is plotted directly onto a chart recorder where each
unit (2.5 cm in height) represents a magnitude of 1 volt of blood flux. Then the surface area under the response curve (cm²) is measured using a digital planimeter (section 2.9.1.1.). The area under the response curve is the most accurate measure that represents the total change in blood flux incorporating not only the height but also the duration of the response curve.

2.9.1.1. Digital Planimeter

Digital planimeter (Planix, Tamaya & company, Japan) is an instrument that is used to measure the area under a response curve (changes in blood flux) recorded by the PLDF on a chart recorder. Each area was measured three times (Area ± 0.2 cm²), and the average area was calculated.

2.9.2. Moor LDF (MLDF)

The DRT4 model (Moor Instruments Limited, U.K) uses an advanced microprocessor-controlled Doppler monitor and it offers power and flexibility to investigate microvascular perfusion. Similar to the Perimed, the Moor also contains solid-state laser diodes but it operates mainly at 780-820 nm wavelength. These long-range wavelengths can penetrate 2-3 mm deep into the skin where arterioles, venules, and arteriovenous anastomoses (AVAs) (around 100μm in diameter), are present. AVAs arise from arterioles and because of their smaller diameter, the blood flow per unit length is far greater than in capillaries. The MLDF software calculates perfusion data relating to the mean red blood cell flux, red blood cell concentration, and mean red blood cell speed and converts them into percent above basal using the following formula:

\[(\text{Value}-\text{PRL}) / (\text{PRL}-\text{PZL}) \times 100\]

PRL= Percent reference level
PZL= percent zero level

These parameters can be displayed graphically or digitally on a bright LCD display. Unlike the Perimed, data from the Moor is automatically stored for later analysis (locally or by computer) and printout (Moor DRT4 User Manual, 1995). Simultaneous use of both the PLDF and the MLDF, with probes of different wavelengths, could distinguish blood flow differences between the superficial capillaries and the deeply located vessels in the skin.
2.10. Sympathetic nerve blockade

Guanethidine monosulfate, a sympathetic neurone blocker, prevents the release of vasoconstrictors from sympathetic nerves. When a single high dose of guanethidine is injected (i.p.), it is taken up from the synaptic cleft into sympathetic terminals by means of the noradrenaline (NA) re-uptake mechanism. The normal vesicular content of the sympathetic terminals is then depleted, flooding tissues with NA. This NA release leads to the acute sympathomimetic effect of guanethidine. After uptake, guanethidine replaces NA in the sympathetic terminals and acts as a “false transmitter”, thus causing short-term functional nerve blockade. This treatment does not cause any structural damage to the sympathetic fibres or terminals or to the sensory fibres (Shir and Seltzer, 1991).

Sympathetically released neurotransmitters can modulate sensory nerve function by inhibiting peptide release when sensory nerves are stimulated. In this study, guanethidine monosulfate powder was dissolved in saline (50mg/kg) and a single dose was injected (i.p.) into either control or diabetic (4 weeks) rats one hour before sensory nerve stimulation. Administration of guanethidine (50 mg/kg) in this manner (i.p.) in adult rats is known to cause temporary (up to 2 weeks) functional sympathetic nerve blockade (Shir and Seltzer, 1991).

2.11. Wound healing

2.11.1. Induction of thermal burn

The rat fur on the interscapular region was removed with animal clippers and a cosmetic depilatory agent. Rats were anaesthetized with sodium pentobarbitone (65mg/ml, i.p.), 24-48 hrs after this treatment, and a thermal burn was induced in the treated region by using a CO₂ laser (one stimulation of 25 watts power, 2 seconds duration, and a spot diameter of 5 mm). This technique delivers standard energy levels over a given area of skin and therefore gives a similar and reproducible thermal burn. This injury results in a large circular wound area (4 cm²). The wound area increases to 4.5-5 cm² by day 2 due to progressive loss of microcirculation as thermal injury damages capillary endothelial cells. This shape and size of wound allows for healing primarily by contraction and re-epithelialization over approximately 2.5-3 weeks in diabetic rats. It is also known that
round wounds do tend to contract more uniformly, rarely deform into two or more wounds, and are associated with less measurement error (Richey et al., 1989). Thermal burn was induced in control and diabetic rats (day 28) at the same time. After induction of wounds, rats were kept in groups of 2 per cage. General health of the animals and condition of their wounds were monitored carefully, especially diabetic rats. The cages were cleaned every day for the animal’s comfort and to prevent infection.

2.11.2. Drug treatments

Among diabetic rats involved in wound healing experiments, one group was not treated but the rest were treated with:

1. Insulin (2-6IU/100g), daily, s.c. - Commenced on day 3 after diabetes onset and its confirmation (n = 5-6)
2. Aminoguanidine (AG) (25mg/kg), daily, i.p. - Commenced on day 3 after diabetes onset and its confirmation (n = 5-6)
3. Tirilazad mesylate (Freedox) (5mg/kg), every 2 days, i.p. - Commenced on day 3 after diabetes onset and its confirmation (n = 5-6)
4. Combined Superoxide Dismutase-Polyethylene Glycol (PEG-SOD) (100U/ml) + BQ-123 (100μM) (150μl, first 5 days after induction of thermal wound, i.d.) -Injection commenced on day 28 after diabetes onset and its confirmation (n = 5-6)

    PEG-SOD (50μl) + BQ-123 (100μl) = 150μl

This solution was injected at 2 sites, 1cm distance to the burn edge daily and at the same time of the day. Five consecutive days of treatment was chosen based on our own previous study on wound healing showing the most significant change in wound size occurs within the first week of its induction (Khalil and Helme, 1996).

2.11.3. Measurement of healing

The area of burn was traced daily and measured with a digital planimeter. An observer who was unaware of the treatment protocol measured the wounds. Burns were left uncovered and observed daily for any sign of infection. Although scab formation is minimal in full-thickness wounds, when it occurred, the scab was gently removed. The
purpose of this action was to keep all wounds comparable, as a previous study by Snowden et al (1982) showed that scab formation induces a transient decrease in the rate of wound contraction. It was also easier to accurately measure the open wound area without the overlying scab. The healing endpoint was determined, as the time when full wound contraction had occurred. It should be noted that although wound contraction is only one parameter of wound healing, it accounts for large portions of wound closure in full-thickness wounds.

2.12. **Protein assay technique**

Bradford protein assay technique is a simple colorimetric assay that measures the protein content of the perfusate, which is a measure of plasma extravasation (Bradford, 1976). First, a standard curve was established using a series of bovine serum albumin (BSA) concentrations (e.g. 0-400µg/ml). Then, 100µl of the perfusate was placed in a tube and 5ml of coomassie brilliant blue dye was added and left for 10 minutes. The colour of the sample was measured at 590 nm with a spectrophotometer (Beckmen DU 530, USA) and readings were recorded. Using the standard curve, colorimetric readings were converted to concentrations of protein present in the samples. The concentration of every collection (prior, during, and after stimulation) was recorded separately for individual animals in a group then the mean values were calculated.

2.13. **Statistical methods of analyses**

Results from vasodilatation responses were either calculated as areas under the response curves or percent above basal or percent of control. Plasma extravasation (PE) responses are presented as percent above basal. All data are expressed as mean ± standard error of the mean (SEM). The absolute values and 95% confidence intervals (95%CI) of those values are quoted in the text. Presentation of 95% confidence limits would allow the reader to assess both distribution and significance of results.

The statistical analyses employed were chosen according to the type of experiment and the properties of its dependent and independent variables. The statistical analyses described in this thesis were performed utilising SPSS for Windows Statistical package version 9.0.
Analysis of Variances (ANOVA) (one-way or two-way) were used to examine whether there were any group differences or interactions between control and diabetic rats (with and without treatments). If ANOVA indicated a significant main effect, then post-hoc comparisons using one or more of the following tests (t-test, Dunnet’s test, Tukey HSD test) was performed. One-way repeated measure ANOVAs were performed to identify whether there were significant group differences (control and diabetic) according to the PLDF and the MLDF, in responses to SP at different time points of perfusion (SP10, SP20, SP30). Again, where a significant main effect was detected, post hoc independent sample t-tests were conducted. A repeated measure ANOVA (one-way or two-way) with priori planned comparisons was performed to investigate whether there were significant differences in PEs between control and diabetic groups (with and without treatments) at different time points of collection. Wound healing data were analysed using a combination of one-way and repeated measure ANOVAs to identify group or treatment differences followed by either priori-planned or post hoc comparisons. In all cases a value of $p < 0.05$ was set to denote statistical significance.

2.14. Limitations of this study

1. It was not possible to examine the longer-term effect of diabetes (over 8 weeks) without insulin treatment due to ethical reasons.

2. The role of food ingredients such as vitamins in protecting pancreatic beta cells against diabetogenic agents proved interesting, based on the experience during the development of the STZ-model, and should be further investigated. In this study, the role of food was not part of the original plan and therefore time was not allocated to pursue this line of investigation.

3. Reflolum S, a blood glucose-monitoring device, used by human patients can accurately measure glucose levels up to 27.7mmol/lit. This posed a limitation in accurately measuring blood glucose above this level in diabetic rats. In turn, this limitation affected the dose of insulin used, especially in these experimental groups where the purpose of insulin treatment was to control the blood glucose level in diabetic rats (chapter 9 and chapter 10).
4. It would have been a great advantage if some of these experiments could have been performed on a type II model of diabetes. It was considered that by developing a type II model of diabetes, using STZ in Sprague-Dawley rats, a direct comparison of results would have been possible. Several attempts were made using an STZ injection in neonatal rats but this method proved to be very difficult and time consuming. Since the aim of the thesis was to examine changes in microvascular blood flow in a type I diabetic rat model, and the fact that time and resource were not originally allocated to this experiment, further attempts to develop a type II model of diabetes were not made.
CHAPTER 3

Diabetes and sensory nerve function
3.1. Neurogenic inflammation: Part 1 (Post-terminal events)

3.1.1. Introduction

Inflammation is a protective response that is essential for tissue repair. The neurogenic component of the inflammatory response is mediated via a subset of sensory nerves, namely unmyelinated primary afferent sensory neurones. Upon stimulation, sensory peptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) are released from terminal endings of primary afferent fibres causing dilatation of adjacent microvessels as well as increasing the permeability of post-capillary venules (plasma extravasation) (Holzer, 1998).

There are numerous reports showing a significant reduction in the inflammatory responses in diabetes (Bennett et al., 1998; Gamse and Jancso, 1985; Mathison and Davison, 1993; Nemeth et al., 1999b; Walmsley and Wiles, 1991). Evidence suggests that the endothelium is damaged (Huszka et al., 1997) and nitric oxide (NO) production is reduced in streptozotocin (STZ)-treated diabetic rats at early stages of the disease (Kiff et al., 1991a; Stevens, 1995). Also, a reduced amount of SP and CGRP was observed in the axons and cell bodies of the primary afferent nerves in diabetic rats (Karanth et al., 1990; Rittenhouse et al., 1996a). It was suggested that the function of unmyelinated sensory C-fibres is impaired in diabetes mellitus (Gyorfi et al., 1996), altering the production and / or release of inflammatory mediators (Nemeth et al., 1999a; Rittenhouse et al., 1996a). This in turn can lead to inadequate tissue blood supply in this disease condition (Rendell et al., 1993).

Endothelial cells can also alter the contractile magnitude of the vascular smooth muscle cells by releasing a number of constricting factors (Bennett and Gardiner, 1996) (chapter 1, sections 1.5.3 & 1.8.4). It has been suggested that in the diabetic condition some of these factors may play a role in deactivating NO (Gryglewski et al., 1986). It is also known that intracellular calcium overload can occur in early diabetes. This may be due to the reduction in the activity of associated enzymes and abnormalities in sarcolemmal membrane Na⁺/Ca²⁺-exchange and calcium pump (Golfman et al., 1998; Ver et al., 1997) (chapter 1, section 1.8.5.3). An increase in the activity of voltage-dependent calcium
channels has also been suggested to play a role in regulating intracellular calcium in diabetes (Kamata et al., 1992). The role of endothelins (ETs) and free radicals in intracellular calcium homeostasis in early diabetes could not be ignored (chapter 1, sections 1.8.4 & 1.5.3). Overall these studies suggest that damage to endothelial cells in diabetic condition may alter skin microvascular blood flow by affecting the release of vasoactive peptides (e.g. NO) and altering the contractile magnitude of vascular smooth muscles.

In this study, the blister model of neurogenic inflammation using the rat hind footpad was used. This model was developed in our laboratory (Helme and White, 1983; Helme et al., 1985) to investigate the complex interactions between mediators of neurogenic inflammation (Khalil et al., 1988; Khalil and Helme, 1989; Khalil and Helme, 1990b; Khalil and Helme, 1991). By forming a blister on the rat hind footpad, separating the epidermis from the dermis and then removing the former, easy access to the extracellular space with intact skin blood vessels can be achieved. Sensory neurotransmitters (e.g. SP, CGRP), when applied over the blister base, can cause inflammatory responses such as vasodilatation and / or plasma extravasation (post-terminal events) via mechanisms that are mainly post-terminal in nature.

Two different laser Doppler flowmeters (LDFs), with different laser light penetration capabilities, were used to examine changes in microvascular blood flow in STZ-diabetic rats at different skin levels (superficial and deep). The Perimed LDF (PLDF) and the Moor LDF (MLDF) were used respectively to record blood flow at superficial and the combined superficial and deep compartments of the skin (see also chapter 2, section 2.9). The simultaneous use of these two LDFs could distinguish differences in blood flow between the superficial capillaries and the deeply located vessels in the skin (Gush and King, 1991), thus distinguishing which type of blood vessel is more susceptible to the effects of early diabetes. Results from these experiments could provide valuable information regarding early mechanisms that might eventually contribute to the delay in repair of skin wounds in diabetic patients.
The relationship between the duration of diabetes and changes in skin microvascular blood flow in STZ-treated rats has not been previously studied. Also, the possible underlying mechanisms for the above changes in diabetic rat skin are not clear. Therefore, the aims of chapter 3, part 1 are:

1. To examine the changes in the inflammatory response in diabetic rats over time (i.e. to establish a time frame for the development of microvascular changes).
2. To examine independent changes in smooth muscle reactivity overtime using the independent smooth muscle vasodilator sodium nitroprusside (SNP).
3. To examine the function of sensory nerves in 4 weeks diabetic rats (as determined from aim 1 to be the most suitable) in relation to changes at the post-terminal level using local perfusion of SP and CGRP over the blister base. It was hypothesized that there may be an alteration in sensory mediated events at the post-terminal level.
4. To examine sensory independent effects of diabetes on the microvasculature using sensory denervated (capsaicin pre-treated) rats (with or without diabetes). It was hypothesized that there could be alterations in microvascular function that may be independent of sensory nerve related changes.
5. To examine the effect of diabetes (4 weeks) on the microvasculature at both superficial and deep compartments of the rat skin. It was hypothesized that there could be an alteration in the function of capillaries in early diabetes.

Recent studies suggested that STZ toxicity was not only responsible for pancreatic cell death and development of diabetes but might also cause neuronal cell damage (Adeghate and Parvez, 2000; Prickaerts et al., 2000). This raised the possibility that changes in microvascular blood flow observed in STZ-treated diabetic rats might not be related to hyperglycaemia but to the neurotoxic effect of STZ. Therefore, changes in microvascular blood flow in 4 weeks alloxan-treated rat was also examined. It was hypothesized that in STZ-treated rats, the changes in microvasculature might be caused by a possible independent neurotoxic effect of STZ other than its hyperglycaemic effect. The possible neurotoxic effect of STZ was also previously addressed by an insulin treatment study (Schmidt et al 1983). In chapter 9 this issue was further examined by recording changes in microvascular blood flow in insulin treated diabetic (STZ treated) rats.
### 3.1.2. Method

Twenty-two groups of rats were used, 4 control, 10 STZ-treated, 2 alloxan-treated, and 6 capsaicin pre-treated diabetic (using STZ) groups. Each group consisted of 6-9 rats (n = 6-9). Control groups were injected i.p. with sodium citrate buffer solution (0.1M, pH 4) (CB-treated) (chapter 2, section 2.4.1). Other rats were made diabetic with either STZ or alloxan treatment (chapter 2, sections 2.4.3 & 2.4.4).

For the first set of experiments, ten STZ-treated diabetic groups of rats were tested after 1 to 10 weeks of diabetes (1 group every week) together with controls. In experiment 1 (peptide perfusion), first SP (1μM, 30 min), then SNP (100μM, 10 min), a direct smooth muscle vasodilator, and finally CGRP (1μM, 10 min) were perfused over the blister base. The changes in blood flow after SP (30 min), SNP (20 min), and CGRP (20 min) perfusions were recorded by the PLDF and the areas under the response curves were measured using a digital planimeter.

Data collected from the MLDF were displayed as percent above basal (see also chapter 2, section 2.9.2). The results obtained from the PLDF indicated that in STZ diabetic rats, vascular changes (at superficial skin layer) tend to occur during the first 4 weeks of diabetes. Hence, the next set of experiments focused on the microvascular changes at 2 and 4 weeks of diabetes using the combined sensory denervated and STZ-treated diabetic rats. Also, the changes at the deeper level of the microvasculature were examined at the above time points post-diabetes. For this purpose, six separate groups of capsaicin pre-treated rats were used as shown below (see also chapter 2, section 2.8). Four groups were
injected with STZ and the other two with 0.1 M citrate buffer solution (CB-treated). Two or four weeks later, capsaicin pre-treated diabetic and CB-treated groups were anaesthetized and a blister was induced on the hind footpad. Once again, blood flow was monitored using both the PLDF and the MLDF. The vascular responses to SP and SNP (same concentrations and time mentioned before) were recorded using both the PLDF and the MLDF.

In alloxan-treated rats, after the base line was established, SP, SNP, and CGRP were perfused over the blister base (same concentrations and time mentioned above) and perfusion data were collected using the PLDF. Plasma extravasation (PE) was collected from all peptide perfusion experiments. At the end of each experiment, rats were sacrificed with pentobarbitone overdose.
3.1.2.1. **Expression of data and statistical analyses**

Vasodilatation responses were calculated as areas under the response curves, percent above basal, and percent of control. PE responses are presented as percent above basal. All data are expressed as mean ± standard error of the mean (SEM). The absolute values and 95% confidence intervals (95%CI) of these values are quoted in the text.

Separate one-way Analyses of Variances (ANOVAs) were performed to investigate whether there were significant differences in basal blood flow (BBF), weight gain, PE results and responses to SNP, SP, and CGRP, between control and diabetic rats (1-10 weeks). Where ANOVA indicated a significant main effect, post hoc comparisons were conducted using Dunnett’s test. Separate one-way repeated measure ANOVAs were performed to investigate whether there were significant differences in vascular responses to SP between control and diabetic rats, recorded by PLDF and MLDF, at different times of perfusion (SP10, SP20, SP30). Where a significant main effect was detected, post hoc independent sample t-tests were performed. A two-way ANOVA was performed to investigate whether there were significant differences in BBF and responses to SNP, SP, and ES, between control and capsaicin pre-treated animals (with or without diabetes). This test should also show interaction effects between these groups. Where ANOVA indicated a significant main effect, post hoc comparisons were conducted using independent sample t-tests. To investigate whether there were significant differences in PE responses to SP (SP2, 3 & 4) between control and diabetic rats over time, a one-way repeated measure ANOVA was performed with priori planned comparisons. Separate independent sample t-tests were performed to examine other results involving control and diabetic rats.

3.1.3. **Results**

Responses in CB-treated control rats were not expected to change with time. Hence, random CB-treated control groups for weeks 0, 1, 5, and 10 were tested. Results showed BBF and other vascular responses (SNP, SP, CGRP, and ES) from CB-treated control rats at weeks 1, 5, and 10 were similar to those at week zero (Appendix A, Table A-1). Therefore, all control (CB-treated) values presented in this chapter and throughout the
thesis are those of week zero. The data representing controls were used throughout the thesis to allow comparison with diabetic data.

The BBF (measured for 20 min) in diabetic rats was generally lower than control animals. However, this reached a significant level after 4 weeks of diabetes \([(16.7 \pm 1.3 \text{ cm}^2) \ (95\%\text{CI }14.1-19.2)]\) when compared to control \([(25.1 \pm 0.9 \text{ cm}^2) \ (95\%\text{CI }23.3-26.9)]\), and remained approximately stable at this level for a further 6 weeks (Fig 3-1).

\[\text{Area under the response curve (cm}^2)\]

Fig 3-1: BBF in rats before and after induction of diabetes (changes over time), presented as mean $\pm$ SEM \((n = 6-9)\). * Denotes significance from control rats \((P<0.05)\)

Responses to SNP, SP, and CGRP were initially calculated as percentage increase above own basal blood flows as shown in Fig 3-2.
**Fig 3-2:** Vascular responses to SNP (100 μM), SP, and CGRP (both at 1μM), presented as percent above basal ± SEM (n = 6-9). * Denotes significance from control rats (P<0.05)

Vasodilatation responses to SNP, SP and CGRP in 4 weeks diabetic rats (filled bars) reached 99 ± 17.6 %, 93 ± 15.9 % and 129 ± 17.5 % above basal blood flow respectively. These were significantly different to those of control animals (unfilled bars) where the responses to SNP, SP, and CGRP reached 256 ± 18.9%, 157 ± 10.2 % and 214 ± 37.4% above basal blood flow respectively.

Vasodilatation responses were also measured as areas above basal (areas under the response curve) as shown in Fig 3-3. In 4 weeks diabetic rats, the response to SNP was significantly reduced [(7.4 ± 0.6 cm²) (95%CI 6.2-8.6)], reaching only 28% of the control value [(26.1 ± 1.8 cm²) (95%CI 22.6-29.6)]. Similarly, vascular responses to SP [(21.6 ± 3.4 cm²) (95%CI 14.9-28.3)] and CGRP [(10.4 ± 1.3 cm²) (95%CI 7.8-12.9)] were also significantly reduced in these rats, reaching 42% of their own control groups [(51.4 ± 2.9 cm²) (95%CI 45.7-57.1) and (24.8 ± 4.9 cm²) (95%CI 15.2-34.4) respectively].
Since both methods of measurement (percentage increase above basal blood flow and area above basal) showed similar significant differences between diabetic and control rats, all subsequent results for vasodilatation responses are presented as areas above baseline.

As mentioned above, a general reduction was observed in SNP, SP and CGRP responses in diabetic rats over time (Figs 3-4, 3-5, 3-6). This decline continued until week 4 where SNP, SP and CGRP responses reached a minimum followed by a slight increase before reaching a plateau. Hence, 4 weeks post-diabetes was considered as a suitable time frame for this and subsequent chapters in this study.
**Fig 3-4:** Changes in the vascular response to SNP (100 μM) in diabetic rats over time, presented as mean ± SEM (n = 6-9). * Denotes significance from control rats (P<0.05)

**Fig 3-5:** Changes in the vascular response to SP (1 μM) in diabetic rats over time, presented as mean ± SEM (n = 6-9). * Denotes significance from control rats (P<0.05)
The results also showed a significant difference in average weight gain between control and diabetic rats over the 10 weeks period. Non-diabetic animals gained an average of 30.1g/week compared to only 8.8 g/week in diabetic rats. Table 3-1 and Figs 3-7 and 3-8 show changes in weight in control and diabetic rats during this period.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Average weight at week 0</th>
<th>Average weight at week 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>147 ± 2g</td>
<td>448 ± 10.9g</td>
</tr>
<tr>
<td>Diabetic</td>
<td>147 ± 2g</td>
<td>235 ± 11.3g</td>
</tr>
</tbody>
</table>

*Table 3-1: Average weight of control and diabetic rats at week zero and week ten.*
Fig 3-7: Weight in non-diabetic rats over time, presented as mean ± SEM (n = 6-9).
* Denotes significance from week zero (P<0.05)

Fig 3-8: Weight in diabetic rats over time, presented as mean ± SEM (n = 6-9).
* Denotes significance from week zero (P<0.05)

PE was collected for control and 2, 4, 6, and 9 weeks diabetic rats. The PE response to SP (SP2, 3 & 4) was calculated as percent above basal ± SEM. SEMs were omitted from Fig 3-9 for clarity but quoted in the text. Basal PE level (R1) was significantly increased in 4, 6, and 9 weeks diabetic rats when compared to control group (Appendix B, Table B-1). The PE response to SP, in control rats, was 36 ± 19%, 72 ± 18%, and 89 ± 14% above basal respectively during the three 10 minutes perfusion time. This increase above basal was significantly reduced in diabetic rats, reaching 25 ± 15%, 60 ± 23%, and 49 ± 18% by week 2 and 9 ± 23%, 16 ± 32% and 0.1 ± 23% by week 4 (Fig 3-9).
**Fig 3-9:** PE in diabetic rats over time, presented as percent above basal (n = 6-9).

**Effect of diabetes on microvasculature at both superficial and deep microvascular compartments**

Vasodilatation responses to SNP and SP, using the MLDF, showed a significant reduction in both 2 and 4 weeks diabetic rats. The areas under the response curves from the MLDF were automatically calculated as percent above basal for each 10 minutes of the response, using Moor software. It was simpler, therefore, to convert the PLDF results into percent above basal blood flow for comparison purposes. For this reason, the total area under the response curve for SP, measured by the PLDF (for 30 min), was divided into three 10 minute periods. Then, each period was measured by digital planimeter and results were calculated as percent above basal blood flow (Figs 3-10 & 3-11).
Using the MLDF and the PLDF together allowed comparison between microvascular responses measured from all skin compartments (superficial and deep) and those measured from superficial compartments. The total vascular response to SP in control rats, calculated as a percent above basal, for the PLDF and the MLDF, was $413 \pm 20\%$ and $179 \pm 8\%$ respectively. In diabetic rats, the total response to SP measured by the
PLDF and the MLDF was reduced to 223 ± 21% and 89.5 ± 2.9% after 2 weeks and 194 ± 10% and 49 ± 3.2% after 4 weeks. The ratio of blood flow at the superficial compartment, measured by the PLDF, to that of both superficial and deep compartments, measured by the MLDF, was 2:1 in control animals (i.e. 413 / 179). After 2 weeks of diabetes, this ratio increased to approximately 3:1 (i.e. 223 / 89.5) and by 4 weeks of diabetes it reached 4:1 (i.e. 194 / 49).

**Effect of diabetes on microvasculature in sensory denervated (capsaicin-treated) rats measured by PLDF**

BBFs were significantly and equivalently reduced in rats treated with either capsaicin or STZ [(16.6 ± 1.6 cm²) (95%CI 13.5-19.7) and (16.7 ± 1.3 cm²) (95%CI 14.1-19.2) respectively] reaching 66 ± 6.5% and 67 ± 5.4% of control value [(25.1 ± 0.9 cm²) (95%CI 23.3-26.9)] respectively (Fig 3-12). When capsaicin rats were treated with STZ, BBFs were further reduced [(9.6 ± 0.9cm²) (95%CI 7.8-11.4)] reaching only 38.2 ± 3.5% of control value.

![Figure 3-12](image-url)

**Fig 3-12:** BBFs in control, diabetic (4 weeks), capsaicin pre-treated, and capsaicin pre-treated diabetic rats, presented as percent of control ± SEM (n = 6).
* Denotes significance from control rats (P<0.05)
X Denotes significance from capsaicin pre-treated rats (P<0.05)
+ Denotes significance from diabetic rats (P<0.05)
The vascular responses to SNP were slightly but insignificantly reduced in rats treated with capsaicin [(19.7 ± 1.9 cm²) (95%CI 16-23.4)] but were significantly reduced in STZ-treated rats [(7.4 ± 0.6 cm²) (95%CI 6.2-8.6)]. These responses reached 75.2 ± 7.5% and 29.3 ± 3% of control value [(26.1 ± 1.8 cm²) (95%CI 22.6-29.6)] respectively (Fig 3-13).

![Vascular responses to SNP](image)

**Fig 3-13:** Vascular responses to SNP (100 μM) in control, diabetic (4 weeks), capsaicin pre-treated, and capsaicin pre-treated diabetic rats, presented as percent of control ± SEM (n = 6).

* Denotes significance from control rats (P<0.05)

X Denotes significance from capsaicin pre-treated rats (P<0.05)

When capsaicin rats were treated with STZ, SNP responses were further reduced [(5.2 ± 0.6 cm²) (95%CI 4-6.4)] reaching 20 ± 2.5% of control. These SNP responses were significantly lower than that observed in rats treated with capsaicin alone. There was no significant difference between SNP responses in diabetic rats and capsaicin pre-treated diabetic rats.

The vascular response to SP in capsaicin pre-treated rats was significantly reduced [(27.4 ± 3.5 cm²) (95%CI 20.5-32.3)], reaching 53.8 ± 7.1% of control [(51.4 ± 2.9 cm²) (95%CI 45.7-57.1)]. On the other hand, the response to SP in STZ-treated rats [(21.6 ± 3.4 cm²) (95%CI 14.9-28.3)] (42 ± 6.8% of control), was not different to that of capsaicin rats. Treatment of capsaicin rats with STZ, however, showed a further significant
reduction in SP response [(9.1 ± 1 cm²) (95%CI 7.1-11.1)] reaching 17.6 ± 1.5 % of control and this was also significantly different from all the other responses (Fig 3-14).

**Fig 3-14:** Vascular responses to SP (1μM) in control, diabetic (4 weeks), capsaicin pre-treated, and capsaicin pre-treated diabetic rats, presented as percent of control ± SEM (n = 6).
* Denotes significance from control rats (P<0.05)
X Denotes significance from capsaicin pre-treated rats (P<0.05)
+ Denotes significance from diabetic rats (P<0.05)

**Effect of diabetes on microvasculature in alloxan-treated rats**
Rats injected with alloxan were similar in age to STZ-treated rats. Approximately 60% of alloxan-treated rats showed a blood glucose level of 27.7mmol/lit compared to the blood glucose of STZ-treated rats, where around 10% were equal to, and the rest were above 27.7 mmol/lit.

Although alloxan-treated rats (4 weeks) showed physical symptoms of diabetes, they were heavier in weight and generally in better physical condition than STZ-treated rats after 4 weeks (Table 3-2).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average weight at week 0</th>
<th>Average weight at week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>147 ± 2g</td>
<td>297 ± 4.8 g</td>
</tr>
<tr>
<td>STZ</td>
<td>147 ± 2g</td>
<td>160 ± 10.6g</td>
</tr>
<tr>
<td>Alloxan</td>
<td>147 ± 2g</td>
<td>211 ± 10.4g</td>
</tr>
</tbody>
</table>

*Table 3-2:* Average weight of STZ- and alloxan-treated rats at week zero and week four
Similar to STZ-treated rats, after 4 weeks, there was a significant reduction in BBF in alloxan-treated rats $[(18 \pm 1.4 \text{ cm}^2) (95\% \text{CI } 15.2-20.7)]$ (Fig 3-15).

**Fig 3-15:** BBFs in control, STZ-treated, and alloxan-treated diabetic rats, presented as mean ± SEM $(n = 6)$. * Denotes significance from control rats $(P<0.05)$

The reduction in vascular responses to SNP and SP in alloxan-treated rats was also similar to those in STZ-treated animals. The SNP response was reduced to $(9.4 \pm 1.3 \text{ cm}^2) (95\% \text{CI } 6.9-11.9)$ and SP to $(24.7 \pm 3.4 \text{ cm}^2) (95\% \text{CI } 18-31.4)$, reaching 36% and 48% of own control response (Figs 3-16 & 3-17).

**Fig 3-16:** Vascular responses to SNP $(100 \text{ μM})$ in control, STZ-treated, and alloxan-treated diabetic rats, presented as mean ± SEM $(n = 6)$. * Denotes significance from control rats $(P<0.05)$
The vascular response to CGRP was also diminished in alloxan-treated rats [(9.75 ± 1.8 cm²) (95%CI 6.2-13.3)], reaching 39% of control response. This reduction in CGRP response was similar to the reduction in CGRP response in STZ-treated rats [(10.4 ± 1.3 cm²) (95%CI 7.8-12.9)] (42% of control) (Fig 3-18).
3.1.4. Discussion

Diabetes mellitus is a complex metabolic disorder affecting small nerve fibres and blood vessels over time leading to severe complications (McMillan, 1997; Vranes et al., 1999; Westerman et al., 1989). It was suggested that impaired sensory C-fibres in diabetes could play an important role in inflammation and control of microvascular blood flow (Gyorfi et al., 1996).

The results demonstrated a significant reduction in diabetic rats’ inflammatory vasodilatation responses over time. These responses at the superficial skin layer reached a minimum 4 weeks after induction of diabetes (Figs 3-4, 3-5 & 3-6). Since the changes in microvascular responses in diabetic rats occurred within the first 4 weeks of the disease development, this time frame was considered suitable for the following experiments in this thesis.

Results showed a reduction in BBF and body weight in diabetic rats (Figs 3-1, 3-7 & 3-8). These data suggest that there is a significant reduction in the skin nutritional blood flow in the planter surface of diabetic rat paw. Blood flow activity is influenced by many factors such as local temperature, nervous impulses (e.g. axon reflexes involving sensory nerves and sympathetic vasoconstrictor tone), circulating hormones (e.g. catecholamines), and physical stimuli. Previously it was reported that blood flow at the planter surface of the rat paw did not change throughout the first 3 months of diabetes (Rendell et al., 1993). Studies in diabetic patients, however, showed severe reduction in the skin capillary blood flow, thus supporting the current results (Journeskog et al., 1995; Yosipovitch et al., 1996). Journeskog et al. (1995), showed that in diabetic patients the disturbances were mainly seen in the capillaries and the capillary blood flow (in the great toe) was severely reduced during reactive hyperaemia. In contrast, total skin microcirculation was normal, indicating that sufficient blood reaches the area but not enough reaches the capillaries.

Superficial capillary blood flow, in this chapter, was examined using the PLDF with short-wavelength emitting beams (633 nm) that can only penetrate to a depth of 1 mm. The current vasodilatation results, using the PLDF, showed that microvascular function at the superficial compartment of the skin is altered in the skin of diabetic rats, which is in agreement with a human study by Yosipovich et al (1996) (Fig 3-10). In that study, an
abnormal blood flow was observed in the feet of young diabetic patients with and without microangiopathy and regardless of the presence of peripheral neuropathy. It should be noted, however, that the reduction in inflammatory vascular responses in this PhD study was not simply due to the reduction in basal blood flow. This is based on the data showing that measurement of responses as a percent above basal or areas above baseline were similarly reduced in diabetic rats and capsaicin pre-treated rats, with and without diabetes, when compared to control (Figs 3-2, 3-3 & 3-12).

Evidence is provided that the reduction in inflammatory responses after 4 weeks of diabetes coincides with a 72% reduction in smooth muscle reactivity (Figs 3-4 & 3-5) (Kamata et al., 1992). One possible reason for this reduction might be related to an alteration in the intracellular calcium level in the smooth muscle. Na⁺/K⁺-ATPase and Ca²⁺-stimulated ATPase dysfunction in early diabetes and alteration in Na⁺/Ca²⁺-exchange and Ca²⁺-pump in the sarcoplasmic membrane (Golfman et al., 1998) may have caused calcium overload and smooth muscle contraction. Experiments in chapter 6, involving bisindolylmaleimide (BIM), examined possible alterations in the intracellular calcium level in smooth muscle of early diabetic rats.

The reduction in vascular responses to SNP, a NO donor, suggests that NO has a lower efficiency to induce vasodilatation. This could be due to factors like deactivation of NO (Gryglewski et al., 1986) or reduction in the efficiency of the second messenger system (cyclic guanosine monophosphate (cGMP)) (Sasaki and Okabe, 1993; Weisbrod et al., 1993), rather than factors that affect the release or production of NO. Other possibilities for reduced responses to SNP could include the presence of ET and free radicals (Bassirat and Khalil, 2000). The roles of these substances are discussed in chapters 6 and 7 respectively.

In rat skin, CGRP activates the cyclic adenosine monophosphate (cAMP) pathway to induce relaxation of the smooth muscle. The results showed a significant reduction in CGRP response in 4 weeks diabetic rats, reaching 42% of control response (Fig 3-6), suggesting that the cAMP pathway might also be inefficient. However, since the reduction in SNP response (28% of control) was greater than CGRP, it could be argued that reduction in CGRP could be totally related to the changes in smooth muscle reactivity. Furthermore, it is also possible that the cAMP system in diabetic rats functions
more efficiently to compensate for poor performance by the cGMP system (Chiappe de Cingolani, 1986).

Vascular responses to SP were examined in diabetic (2 & 4 weeks) and control rats using both LDFs. Blood flow changes in superficial capillaries in the skin (1 mm in depth) can be measured by the PLDF. However, the MLDF with longer wavelength emitting beams (780-820 nm) can penetrate deeper (2-3 mm) into the skin and measure blood flow changes in both superficial and deeper vessels (arterioles, venules, and arteriovenous anastomoses (AVAs)). AVAs are specialized structures that are controlled by thermoregulatory reflexes. The reaction of AVAs to stimuli is different to capillaries; they can permit total blood flow to the skin to increase without a change in the capillary flow (Millington and Wilkinson, 1983).

Before analysis, the Perimed results were made comparable to those obtained from Moor as described in the result section (Table 3-2). When the MLDF results were compared to those obtained from the PLDF, a greater reduction in SP responses in diabetic rats was observed, especially after 4 weeks (27% compared to 47% of own total control) (see also Figs 3-10 & 3-11). The ratio of blood flow in superficial vessels to combined superficial and deeper vessels was also increased with the duration of diabetes. This ratio increased from 2:1 in control to 3:1 after 2 weeks and 4:1 after 4 weeks of diabetes. These results suggest that, in early diabetes, both superficial and deeper vessels in the skin are altered but deeper vessels might contribute more to the reduction in SP response. The current results also showed that this reduction can be detected after only 1 week of diabetes, which is in agreement with previous studies (Brands and Fitzgerald, 1998; Lash and Bohlen, 1991; Zhu et al., 1999). The response to SP was subject to further reduction with time (4 weeks).

PE results suggest alterations in the permeability of post-capillary venules in diabetic rats (Fig 3-9). The protein content of R1 collected from diabetic rats showed an overall increase with the duration of diabetes (up to 9 weeks) (Appendix B, Table B-1). This value was increased by 63% after 2 weeks and by 135% after 4 weeks of diabetes. As diabetic post-capillary venules are already leaky they respond poorly to SP. Consequently, the ability of SP to induce sufficient PE responses is reduced (Mathison and Davison, 1993). Post-capillary venules are an important part of the circulation and
are affected by inflammation. Elevated $R_1$ in diabetic rats, especially after 4 weeks, suggest that there are possible structural disturbances in endothelial cells. This is in accord with a previous study demonstrating that elevated endothelial extravasation is one of the early vascular changes in diabetes (Chakir and Plante, 1996).

In this chapter, vasodilatation responses were also examined in sensory denervated (capsaicin pre-treated) rats, with and without diabetes. Capsaicin treatment significantly reduced SP response to 54% of control but no significant change was observed in smooth muscle reactivity (Figs 3-13 & 3-14). On the other hand, induction of diabetes caused a significant reduction in both SP (42% of control) and SNP responses (28% of control). When capsaicin rats were treated with STZ, both SP and SNP responses were further reduced reaching 18% and 20% of their own controls respectively (Figs 3-13 & 3-14).

When SP is applied exogenously, it exerts its vasodilatory effect on rat skin in two ways. The first is directly on blood vessels, causing NO release and vasodilatation (post-terminal event). The second is indirectly by releasing histamine from mast cells that subsequently excites sensory nerves, leading to release of more neurotransmitters from these nerves (pre-terminal event) (Khalil and Helme, 1989). In the former situation, SP acts upon neurokinin-1 (NK-1) receptors on the endothelium, initiating the phosphatidyl-inositol-bisphosphate pathway and activating NO synthase to produce NO, which then diffuses into smooth muscle causing its relaxation and subsequent vasodilation. In the latter situation, SP is involved in degranulation of mast cells and release of histamine, which causes more SP to be released by sensory nerves.

The reduction in SP, in capsaicin rats (54% of control), is mainly related to the direct effect of capsaicin on sensory C-fibres and dorsal root ganglia (DRG). Capsaicin treatment of neonatal rats causes reduction in SP synthesis in DRG, impairment of SP axonal transport, and depletion of 50-60% of SP in the skin (Fitzgerald, 1983; Gamse et al., 1982). Although neonatal capsaicin treatment can destroy most of the C-fibres, the remaining fibres are likely to remain normal and function efficiently. In capsaicin pre-treated diabetic rats, the further reduction in smooth muscle reactivity (by 9%) alone cannot explain the further reduction in SP response (by 24%) in these same rats. Therefore, the possibility was raised that diabetes may have also affected the remaining
intact sensory nerves (after capsaicin treatment) and altered their function. This possibility is further examined in part 2 of this chapter. Hence, the overall reduction in SP response, in capsaicin pre-treated diabetic rats, could be related to the combined loss of sensory nerves due to capsaicin treatment and the effect of diabetes on the remaining intact nerves.

The effect of diabetes on microvasculature in STZ- and alloxan-treated rats

Early pre-clinical studies revealed that STZ is toxic to pancreatic cells, causing hyperglycaemia and at high concentrations (above the diabetogenic dose), it can also damage the kidneys (Weiss, 1982). The less common myelosuppression and hepatotoxicity caused by STZ have also been previously reported (Weiss, 1982). Evidence exists that the toxic effect of STZ on pancreatic beta cells involves the NO pathway (Adeghate and Parvez, 2000). It was also hypothesized that STZ may cause neuronal damage by liberating NO from its nitroso moiety (Prickaerts et al., 2000). However, Prickaerts et al showed that administration of NO (using SNP), in vivo, did not lead to any behavioural and / or neuronal deficits.

In this chapter, alloxan-treated rats showed a significant reduction in the SP response (48% of control), which could be mainly due to the reduction in smooth muscle reactivity (36% of control) (Figs 3-16 & 3-17). The CGRP response was also reduced reaching 39% of control response (Fig 3-18). Similar responses were observed in STZ-treated rats showing significant reductions in SP, SNP, and CGRP responses (42%, 28% & 42% of own control values respectively). Collectively, the data from both STZ- and alloxan-treated rats support the hypothesis that the observed changes in diabetic microvascular responses in STZ-treated rats after 4 weeks is most likely related to the effect of hyperglycaemia. However, future investigation may be required to further clarify this issue. It should be noted that since STZ and alloxan were administered via different routes, a separate set of control rats (CB-treated (i.v.)) were used for alloxan-treated animals.
In conclusion, the changes observed in vasodilation and PE results in diabetic rats support the notion that there are functional and structural disturbances in endothelial cells. It was demonstrated that the observed reduction in neurogenic inflammatory responses (SP & CGRP) is mainly due to a reduction in smooth muscle reactivity. It was suggested that a common mechanism could be involved in reducing SNP, SP, and CGRP responses in early diabetes. Inactivation of NO and / or alterations in the efficiency of cGMP and cAMP pathways were raised as possible causes for this reduction. Evidence was provided that reduction in sensory nerve function in capsaicin pre-treated diabetic rats is largely independent of smooth muscle reactivity. The results also supported the hypothesis that the observed changes in the microvasculature of STZ-induced rats are most likely related to the hyperglycaemic effect of STZ rather than a possible independent neurotoxic effect of this drug.

In chapter 3, part 1 (above), the effect of diabetes on sensory-mediated events at the post-terminal level was investigated. In chapter 3, part 2, the effect of diabetes on sensory-mediated events at the pre- and post-terminal levels was studied. By utilizing both sets of results (parts 1 & 2), changes in microvascular responses at the pre-terminal level can also be examined.
3.2. Neurogenic inflammation: Part 2 (Pre-terminal and Post-terminal events)

3.2.1. Introduction

In part 1, a neurogenic inflammatory response was induced in diabetic rats by perfusing sensory peptides exogenously over a blister base. In this section (part 2), neurogenic inflammatory responses were induced in diabetic rats using electrical nerve stimulation (ES), whereby the sciatic nerve is stimulated antidromically (at parameters known to activate C-fibres) to mimic the axon reflex phenomenon, leading to the release of sensory neurotransmitters (chapter 2, section 2.7). Previously, the release of SP and CGRP from isolated tracheas of 4 weeks diabetic rats was shown to be substantially diminished in response to electrical field stimulation (Nemeth et al., 1999a). Also, a reduction in content and transport of SP and CGRP was observed in sciatic nerves of 4 weeks diabetic rats (Diemel et al., 1992; Donadoni et al., 1995; Karanth et al., 1990). These changes in sensory neuropeptides may be responsible for the weak neurogenic inflammatory response observed in diabetic animals (Nemeth et al., 1999b).

The first aim of (chapter 3) part 2 is to examine changes in the vascular response to ES in control and diabetic rats over time. The second aim is to examine the function of sensory nerves in 4 weeks diabetic rats in relation to changes at the pre-terminal level. It was hypothesized that there is an alteration in sensory mediated events at the pre-terminal level.

Electrical stimulation of the unmyelinated C-fibres of the sciatic nerve can provide information related to sensory mediated events at both pre- and post-terminal levels. Although the ES technique relies on an invasive procedure in terms of nerve preparation, the main advantage of antidromic stimulation is that the observed vascular responses are induced by the endogenous release of peptides (e.g. SP, CGRP, etc).

Sensory denervated (capsaicin pre-treated) animals and alloxan-treated rats are also used (different groups to part 1) to determine sensory independent and hyperglycaemic independent mediated effects of diabetes on the microvasculature respectively.
3.2.2. Method

Eleven groups of rats were used in this study: four control, five diabetic, and two capsaicin pre-treated groups. Four control groups and one of the capsaicin pre-treated groups were injected with citrate buffer solution (CB-treated) (chapter 2, section 2.4.1). One group of rats was made diabetic by alloxan injection (chapter 2, section 2.4.3). The other groups, including one capsaicin pre-treated group, were made diabetic by STZ injection (see the below diagram) (see also chapter 2, sections 2.8 & 2.4.4).

Rats were anaesthetized and a blister was induced on the hind footpad. The rat sciatic nerve was prepared for ES, under aseptic conditions, as described in chapter 2 (section 2.7). Once the basal blood flow was established, first SNP (100μM, 10 min) was perfused to measure smooth muscle reactivity. Then the basal blood flow was re-established using Ringer’s solution (20 min) followed by activation of sensory nerve fibres (20V, 5Hz, 2msec for the duration of 1 minute) using Grass stimulator (chapter 2, section 2.7). In control and diabetic rats, changes in the skin microvascular blood flow over time were recorded by the PLDF.

Then, in capsaicin pre-treated rats (control and 4 weeks diabetic) and alloxan-treated animals (4 weeks), the sciatic nerve was stimulated, using the above parameters, and again the skin microvascular blood flows were recorded by the PLDF.
3.2.2.1. Expression of data and statistical analyses

Results were calculated as areas under the response curves and percent of control. All data are expressed as mean ± standard error of the mean (SEM). The absolute values and 95% confidence intervals (95%CI) of these values are quoted in the text.

Separate one-way Analyses of Variances (ANOVAs) were performed to investigate whether there were significant differences in responses to SNP and ES between control and diabetic rats over time. Where ANOVA indicated a significant main effect, post hoc comparisons were conducted using either Dunnett’s or independent sample t-tests. A two-way ANOVA was also performed to investigate whether there were significant differences in responses to ES between control and capsaicin pre-treated rats (with and without diabetes) and interaction between these groups. Where ANOVA indicated a significant main effect, post hoc comparisons were conducted using independent sample t-tests. Separate independent sample t-tests were performed to examine other results involving control and diabetic rats.

3.2.3. Results

Originally random control groups for weeks 0, 1, 5, and 10 were tested. Once again, results showed the vascular responses to SNP and ES from control rats at weeks 1, 5, and 10 were similar to those at week zero (Appendix A, Table A-1). Hence, the results of week zero were reported as controls in this section of the study.
Responses of diabetic rats to SNP and ES were all significantly reduced compared to their own control groups (Figs 3-19 & 3-20).

**Fig 3-19:** Changes in the vascular response to SNP (100 μM) in diabetic rats over time, presented as mean ± SEM (n = 6-9).

* Denotes significance from control rats (P<0.05)

X Denotes significance from 2 weeks diabetic rats (P<0.05)

**Fig 3-20:** Changes in the vascular response to ES (20V, 5Hz, 2msec for 1 minute) in diabetic rats over time, presented as mean ± SEM (n = 6-9). * Denotes significance from control rats (P<0.05)
At week 4, both SNP and ES responses reached 28% of their own controls respectively.

**Diabetes and changes in sensory nerve function in capsaicin pre-treated rats**

In capsaicin pre-treated rats, the vasodilator response to ES \([(9.7 \pm 1.5 \text{ cm}^2) (95\%CI 6.8-12.6)]\) was significantly reduced, reaching 55 ± 8.4% of control \([(17.6 \pm 1.3 \text{ cm}^2) (95\%CI 15-20.1)]\) (Fig 3-21).

![Bar chart](chart.png)

**Fig 3-21:** Vascular responses to ES (20V, 5Hz, 2msec for 1 minute) in control, diabetic, capsaicin pre-treated, and capsaicin pre-treated diabetic rats, presented as percent of control ± SEM (n = 6).

* Denotes significance from control rats (P<0.05)
X Denotes significance from capsaicin pre-treated rats (P<0.05)

This reduction in this response was similar to the reduction in SP response in these animals \((53.8 \pm 7.1\%)\) (Fig 3-14). The smooth muscle reactivity in capsaicin rats \([(19.7 \pm 1.9 \text{ cm}^2) (95\%CI 16-23.4)]\) was not significantly different from control (Fig 3-13).

In capsaicin pre-treated diabetic rats, however, the vascular response to ES was further reduced \([(5.9 \pm 1 \text{ cm}^2) (95\%CI 3.9-7.9)]\), reaching 33.5 ± 5.7% of control which was similar to the ES response in diabetic rats \((28.9 \pm 4.8\% \text{ of control})\) (Fig 3-21). The response to SNP in capsaicin pre-treated diabetic rats \([(5.2 \pm 0.6\text{cm}^2) (95\%CI 4-6.4)]\) reached 20 ± 2.5% of control which was similar to those in diabetic rats \([(7.4 \pm 0.6 \text{ cm}^2) (95\%CI 6.2-8.6)]\) (29.3 ± 3% of control) (Fig 3-13).
Diabetes and changes in sensory nerve function in alloxan-treated rats

The vascular response to ES in alloxan-treated rats was reduced \((8.2 \pm 1.8 \text{ cm}^2)\) (95%CI 4.7-11.7) reaching 46% of control ES. This was greater than the ES response in STZ-treated rats \([(5.1 \pm 2 \text{ cm}^2)\) (95%CI 1.2-9)] (Fig 3-22).

\[\text{Area under the response curve (cm}^2)\]

**Control ES**

**STZ-treated ES**

**Alloxan-treated ES**

**Fig 3-22:** Comparing the response to ES (20V, 5Hz, 2msec for 1 minute) in control, STZ-treated, and alloxan-treated diabetic rats, presented as mean ± SEM \((n = 6)\).

* Denotes significance from control rats \((P<0.05)\)

3.2.4. Discussion

The results showed a significant reduction in ES responses of diabetic rats over time (Fig 3-20). However, these reductions were similar to the reductions in the smooth muscle reactivity of the corresponding diabetic rats (Fig 3-19). For example, in 4 weeks diabetes, the responses to ES and SNP both reached 28% of their own controls. This suggests that at 4 weeks of diabetes the observed reduction in ES responses could be due to reduction in smooth muscle reactivity rather than damage to the C-fibres by diabetes, which further supports the results in chapter 3, part 1.

The role of sympathetic nerves in reducing ES responses in diabetic rats cannot be ruled out and is investigated in chapter 5. Capsaicin pre-treatment of neonatal rats causes reduction in SP synthesis in DRG, its transportation across the nerve, as well as its release from nerve terminals (Fitzgerald, 1983; Gamse et al., 1982). This may explain the reduction in ES response (55% of control) with no significant change in the smooth
muscle reactivity in capsaicin pre-treated rats. The ES and SNP responses, in capsaicin pre-treated diabetic rats, were further reduced (33.5% & 20% of own controls respectively) when compared to these responses in capsaicin pre-treated rats. This suggests that diabetes reduced SNP response in these rats by an action independent of sensory nerves. Therefore, the reduction in microvascular blood flow in 4 weeks diabetic rats may be due to changes in smooth muscle reactivity rather than an action on sensory nerves.

The vascular response to ES in alloxan-treated rats (46% of control) was less reduced when compared to those in STZ-treated animals (29% of control). In part 1, it was reported that the blood glucose level in alloxan-treated rats was approximately 27.7mmol/lit (while 90% of the STZ-treated rats were above this figure) and alloxan-treated animals were in better physical health than STZ-treated animals. These observations suggest that although both groups of rats showed severe diabetic symptoms, the alloxan-treated rats may be less diabetic. The blood glucose-monitoring device that was employed in this study, was originally designed to be used by diabetic patients and was capable of accurately measuring the blood glucose levels up to 27mmol/lit (chapter 2, section 2.4). Therefore, it was not possible to report the exact blood glucose level in diabetic rats since they were equal to or above 27.7mmol/lit. The severity of diabetes can be related to the concentration of alloxan used in this study. The minimum concentration of alloxan required for producing diabetes in 80% of rats was 40mg/kg. On the other hand, 75mg/kg of STZ proved to be the minimum concentration required for producing the same number of diabetic rats. Due to ethical issues, higher concentrations of alloxan were not used. Therefore, direct comparison of results between these two groups of rats was not possible (see also chapter 2, section 2.4.5).

In conclusion, the results further confirmed that post-terminal changes in microvascular function are more apparent than the pre-terminal changes in early stages of diabetes. The data from part 1 and part 2 of this chapter, collectively, suggest that other factors associated with diabetes may be involved in reducing the inflammatory responses in 4 weeks diabetic rats, acting mostly at the post-terminal level (see also chapters 6 & 7). It was also demonstrated that in experimental diabetes, the type and / or the dose of the
diabetogenic agent are important factors to consider and that interpretation of comparative results should be made with caution.
CHAPTER 4

Diabetes and modulation of microvascular blood flow:
Role of local tissue factors and parasympathetic neurotransmitters
4.1. Introduction

*Changes in the vascular response to acetylcholine (ACh) with diabetes*

The interaction between blood vessels and perivascular nerves in the periphery is important for many physiological responses including neurogenic inflammation and blood flow regulation. A number of neurotransmitters have been identified in perivascular nerves and some are co-localized in: sensory nerves (substance P (SP), calcitonin gene-related peptide (CGRP) and neurokinin A (NKA)), parasympathetic nerves (ACh and vasoactive intestinal peptide (VIP)), and sympathetic nerves (noradrenaline (NA) and neuropeptide Y (NPY)), (Bennett and Gardiner, 1996). The close apposition between these nerve varicosities and endothelial cells in capillaries raises the possibility of direct interactions between nerves and endothelial cells in the microvasculature. Vasoactive substances released from these nerves and endothelial cells play a role in the control of microvascular tone, either independently or collectively. Smooth muscle is innervated by noradrenergic, and in some instances by cholinergic, nerve fibres and hence their reactivity is also important in the modulation of microvascular tone.

In diabetes, previous studies showed that the basal release of ACh was not altered at 1 week but was significantly reduced after 5 weeks (Welsh and Wecker, 1991). Diabetes may also be associated with an early alteration (2 weeks) to muscarinic receptor density and function (Soulie et al., 1992; Tong et al., 1999; Wald et al., 1998). The relaxation response to ACh in diabetic animals was different depending on the duration of diabetes and type of vascular bed under study (Chan et al., 2000; Pieper, 1999) (chapter 1, section 1.5.4.2). It was proposed that ACh-induced dilatation is mediated by multiple mechanisms that may include the endothelial-dependent production of nitric oxide (NO) and endothelial-derived hyperpolarizing factor (EDHF) (Edwards et al., 1998; Quilley et al., 1997).

Reduced production of NO (Pieper and Dondlinger, 1997) and decreased activity of superoxide dismutase in diabetes (Kobayashi and Kamata, 1999b) were suggested to be two possible causes for impaired ACh response. Evidence exists that in normal rats, free radicals can depress endothelium-dependent relaxations mediated by activation of
muscarinic receptors (Prasad and Bharadwaj, 1996; Rubanyi and Vanhoutte, 1986). In diabetes, free radicals have also been implicated in altering the function of vascular smooth muscle (Fleischhacker et al., 1999; Sakata et al., 1998), leading to development of circulatory dysfunction (chapter 1, section 1.5.3).

Previous studies in early diabetic animals did not assess the role of the parasympathetic neurotransmitters in modulation of blood flow in the skin tissue. Therefore, in chapter 4, changes in skin microvascular blood flow in diabetic rats are examined using exogenous perfusion of ACh and two related receptor agonists, namely nicotine and muscarine chloride. The role of NO in ACh-induced vascular responses in early diabetes is also examined. Furthermore, the relative contribution of the superficial and deeper vessels to vascular activity of ACh in diabetic rats (4 weeks) is determined using two laser Doppler flowmeters (LDFs), Perimed and Moor (PLDF & MLDF).

Changes in the vascular response to bradykinin (BK) with diabetes

Several endogenous local tissue factors appear to be involved in the modulation of the peripheral release of neuropeptides from sensory nerves. An important local tissue factor is BK, which is produced during inflammation and tissue injury and can both sensitize and directly stimulate sensory nerve terminals (Dray and Perkins, 1993). In rats, BK was shown to induce neurogenic inflammatory responses (Cambridge and Brain, 1995; Khalil and Helme, 1992; Miao et al., 1996), and play a role in activation of mast cells (Zhao et al., 1996). This local tissue factor also has diverse actions on vascular endothelium, smooth muscle, and cellular function (chapter 1, Figs 1-11 & 1-12).

BK actions are mediated directly via B1 and B2 receptors (Hall, 1997) on target tissues and indirectly by release or amplification of NO or other inflammatory mediators including neuropeptides and prostaglandins (chapter 1, section 1.8.5.2). Based on previous studies, including one study on rat skin, the general conclusion has been made that acute activation by BK of sensory neurones is mediated through B2 receptors (Dray and Perkins, 1993; Jones et al., 1995). However, where there is a significant inflammatory process, B1 receptors are expressed and contribute to the action of BK on sensory nerves (Farmer et al., 1991; Hall, 1997; Perkins et al., 1993).
The vascular response to BK in diabetic rats, once again, seems to depend on the vascular tissue under study and the duration of diabetes (chapter 1, section 1.8.5.2). In early diabetes (1-10 days) an increase in BK density was demonstrated in both in vivo and in vitro situations (Pheng et al., 1997; Velarde et al., 1997; Zuccollo et al., 1996). Also, alteration in B2 receptor activity was reported on the smooth muscle cells in STZ-diabetic rats (4 weeks) (Wang et al., 1998).

The role of BK in modulating microvascular blood flow was previously studied in different diabetic vascular beds (hindquarter, mesenteric artery, tail artery, cremaster muscle arterioles) (3-4 weeks) but not skin (Kiff et al., 1991b). Therefore, in this chapter, changes in skin microvascular responses to BK are examined in 4 weeks STZ-diabetic rats. The role of NO in BK-induced vascular response in early diabetic rats is also assessed.

Changes in NO involvement in microvascular responses in diabetes

The vascular endothelium synthesises a number of vasoactive substances that modulate vascular tone and smooth muscle proliferation. NO was shown to mediate endothelium-dependent relaxation (Furchgott and Zawadzki, 1980). Endothelial and neuronal nitric oxide synthase (eNOS and nNOS) are constitutively expressed and, upon activation (e.g. by ACh, BK), can generate NO within a short period of time (Khalil and Helme, 1992; Ralevic et al., 1992). Inducible NOS (iNOS), however, is not normally expressed but it can be activated by pro-inflammatory agents, leading to the generation of large quantities of NO (Moncada and Higgs, 1993).

NO plays an important role in cell communication, defence, and injury. Increasing evidence indicates that NO may play a role in inflammation (Ialenti et al., 1992). In rat skin, NO has a role in the vasodilatation and inflammatory response to BK (Khalil and Helme, 1992), SP and ACh (Khalil et al., 1994; Ralevic et al., 1995b). N\(^G\)-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase, attenuated vasodilatation and plasma extravasation (PE) responses induced by SP (Ralevic et al., 1995b), BK (Khalil and Helme, 1992) and the vasodilatation response to ACh (Ralevic et al., 1992).
Experimental evidence suggested that any one or combination of the following factors might occur in diabetes, which could contribute to vascular complications: a reduction in NO release (Steinberg et al., 1994) and its bioactivity (Bucala et al., 1991; Pieper et al., 1997b), possible defect in secondary messenger system (cyclic guanosine monophosphate (cGMP)) (Trovati et al., 1996), and defective vascular smooth muscle response to NO (Kamata et al., 1992).

In this chapter, the role of NO in modulating microvascular blood flow in 4 weeks diabetic rats is examined using a NO synthase inhibitor, L-NAME. The aim is to examine the contribution of NO to SP response in 4 weeks diabetic rats.

4.2. Method

A total of 16 groups of rats (n = 6-9) were used in this study, of which 14 groups were equally divided into control (treated with 0.1M sodium citrate buffer) and STZ-treated animals (chapter 2, sections 2.4.1 & 2.4.4). The following substances were perfused over the footpad blister base of both control and diabetic rats.

**Group 1:** ACh (100μM, 30min) → sodium nitroprusside (SNP, 100μM, 10min)

**Group 2:** Nicotine (100μM, 30min) → SNP (100μM, 10min)

**Group 3:** Muscarine Chloride (100μM, 30min) → SNP (100μM, 10min)

**Group 4:** BK (10μM, 30min) → SNP (100μM, 10min)

**Group 5:** L-NAME (100μM, 10min) → [L-NAME (100μM) + BK (10μM)] (30 min) → SNP (100μM, 10min)

**Group 6:** SP (1μM, 30 min) → SNP (100μM, 10min)

**Group 7:** L-NAME (100μM, 10min) → [L-NAME (100μM) + SP (1μM)] (30 min) → SNP (100μM, 10min)

The remaining two groups (one diabetic and one control) were used in an experiment using exogenous perfusion of ACh and blood flow measurement using the MLDF. Induction of diabetes and peptide perfusion has been described in detail in chapter 2, sections 2.4 and 2.6. PE was collected from all experiments and at the end of each experiment, rats were sacrificed with pentobarbitone overdose.
4.2.1. Expression of data and statistical analyses

Results from vasodilatation responses were calculated as areas under the response curves. In some instances responses were calculated as percent above basal or percent of control to facilitate comparisons between groups. PE responses are presented as percent above basal. All data are expressed as mean ± standard error of the mean (SEM). The absolute values and 95% confidence intervals (95% CI) of these values are quoted in the text. Separate one-way Analyses of Variances (ANOVAs) were performed to investigate whether there were significant differences in responses to BK and BK + L-NAME or SP and SP + L-NAME, between control and diabetic rats. Where ANOVA indicated a significant main effect, post hoc comparisons were conducted using the Tukey HSD test. Separate one-way repeated measure ANOVAs were performed to investigate group differences (control and diabetic) according to the PLDF and the MLDF in response to ACh at different times of perfusion (ACh10, ACh20, ACh30). Where a significant main effect was detected, post hoc independent sample t-tests were performed. To investigate whether there were significant differences in PE responses to BK, BK + L-NAME, SP, SP + L-NAME, nicotine, and muscarine chloride between control and diabetic rats, a one-way repeated measure ANOVA with priori planned comparisons was performed. Separate independent sample t-tests were performed to examine other results involving control and diabetic rats.

4.3. Results

Changes in the vascular response to ACh with diabetes

The average basal blood flow (BBF) was (24.7 ± 1.1 cm²) (95%CI 22.5-26.8) in control rats and (14.8 ± 1.3 cm²) (95%CI 12.2-17.3) in diabetic rats.
The vascular response to SNP showed a significant reduction in diabetic rats [(6.1 ± 0.8 cm²) (95%CI 4.5-7.7)], reaching 25% of control [(24.5 ± 1.9 cm²) (95%CI 20.8-28.2)] (Fig 4-1).

![Graph showing vascular responses to SNP](image)

**Fig 4-1:** Vascular responses to SNP (100μM) in control and diabetic rats, presented as mean ± SEM (n = 6-7). * Denotes significance from control rat (P<0.05)

There was no difference between vascular responses to ACh [(41.1 ± 3.3 cm²) (95%CI 34.6-47.6)], nicotine [(42.4 ± 5.2 cm²) (95%CI 32.2-52.6)], and muscarine chloride [(40.6 ± 4.3 cm²) (95%CI 32.2-49)] in control animals (Fig 4-2).

![Graph showing vascular responses to ACh, nicotine, and muscarine chloride](image)

**Fig 4-2:** Vascular responses to ACh, Nicotine, and Muscarine chloride (all 100μM) in control rats, presented as mean ± SEM (n = 6-7).
However, in diabetic rats, all these responses were significantly reduced, reaching 27-36% of their own control responses. Responses to ACh reached $(11 \pm 1.8 \text{ cm}^2)$ (95%CI 7.5-14.5), to nicotine reached $(15.3 \pm 1.9 \text{ cm}^2)$ (95%CI 11.6-19), and to muscarine chloride reached $(13.7 \pm 2.1 \text{ cm}^2)$ (95%CI 9.6-17.8) in these animals (Fig 4-3).

**Fig 4-3:** Vascular responses to ACh, Nicotine, and Muscarine chloride (all 100μM) in diabetic (Diab) rats, presented as mean ± SEM (n = 6-7).

The profile of the vasodilator response to ACh was characterized by a tachyphylaxis where there was a gradual reduction in response despite continuous perfusion. Similar profiles were observed using both the PLDF and the MLDF instruments (Figs 4-4 & 4-5).

**Fig 4-4:** Response profile to ACh (100μM, 30 min) in control rat skin microvasculature, as recorded by the PLDF.
Fig 4-5: Response profiles to SNP (100μM, 10 min) and ACh (100μM, 30 min) in control rat skin microvasculature, as recorded by the MLDF.

Using the MLDF and the PLDF together enables a comparison of microvascular responses to ACh measured from all skin compartments (superficial and deep) to those measured from superficial compartments. The total vascular response to ACh calculated as a percent above basal was similar between Perimed and Moor in control animals (165 ± 10% & 128.5 ± 14%) but was different in diabetic rats (127.5 ± 9% & 80.5 ± 6.2%) (Table 4-1). The ratio of blood flow at the superficial compartment measured by the PLDF to that of both superficial and deep compartments measured by the MLDF, was 1:1 in control animals but increased to about 2:1 after 4 weeks of diabetes.

The results from the PLDF showed the most reduction in response to ACh in diabetic rats (by 30%) was during the first 10 minutes of perfusion (Table 4-1, Fig 4-6). This was followed by 2.5% and 5% reductions during the next 20 minutes of perfusion (ACh20 and ACh30 respectively).

<table>
<thead>
<tr>
<th>PLDF (%)</th>
<th>MLDF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAT</td>
<td>ACh10</td>
</tr>
<tr>
<td>Control</td>
<td>92 ± 12</td>
</tr>
<tr>
<td>Diabetic</td>
<td>62 ± 12</td>
</tr>
</tbody>
</table>

Table 4-1: Vascular responses to ACh (100μM) in control and diabetic rats (4 weeks) measured by the PLDF and the MLDF, presented as percent above basal ± SEM (n = 6).
Fig 4-6: Vascular responses to ACh (100μM) in control and diabetic (Diab) rats (4 weeks) at 10 minutes intervals measured by the PLDF, presented as percent above basal ± SEM (n = 6).

Results measured by the MLDF, on the other hand, showed similar reduction during the first 10 minutes (by 23%) but more reduction in the following 20 minutes of ACh perfusion (15.5% & 9.5%) (Fig 4-7).

Fig 4-7: Vascular responses to ACh (100μM) in control and diabetic (Diab) rats (4 weeks) at 10 minutes intervals measured by the MLDF, presented as percent above basal ± SEM (n = 6).
Changes in the vascular response to BK and NO involvement with diabetes

The average BBF was (24.1 ± 2.1 cm²) (95%CI 20-28.2) in control animals and (15.8 ± 0.9 cm²) (95%CI 14-17.6) in diabetic rats.

The vascular response to SNP showed a significant reduction in diabetic rats [(8.2 ± 0.7 cm²) (95%CI 6.8-9.6)], reaching only 32% of control [(25.6 ± 1.3 cm²) (95%CI 23-28.1)] (Fig 4-8).

![Graph showing vascular responses to SNP (100μM) in control and diabetic rats, presented as mean ± SEM (n = 6-9). * Denotes significance from control rats (P<0.05)]

In control rats, perfusion of L-NAME significantly decreased the vascular response to BK [(38 ± 2.9 cm²) (95%CI 32.3-43.7)], reaching 64.4 ± 5% of control response [(58.3 ± 1.3 cm²) (95%CI 55.7-60.8)]. The vascular response to BK was also diminished in diabetic rats [(27.9 ± 2.5 cm²) (95%CI 23-32.8)], reaching 48 ± 4.2% of control response. Compared to control animals, L-NAME was more potent in reducing the BK response in diabetic rats [(11.8 ± 1.2 cm²) (95%CI 9.4-14.1)], reaching only 20 ± 2% of control response (Fig 4-9).
L-NAME perfusion reduced SP response in control rats [(51.4 ± 2.9 cm²) (95%CI 45.7-57.1)] to 42.5 ± 5.5% of control value [(20.9 ± 3 cm²) (95%CI 15-26.8)] (Fig 4-10).
The vascular response to SP in diabetic rats \[21.6 \pm 3.4 \text{ cm}^2 \text{ (95\%CI 14.9-28.8)}\] was further more reduced by this NO inhibitor \[(7.6 \pm 1.4 \text{ cm}^2 \text{ (95\%CI 4.8-10.3)}\], reaching \(15 \pm 3\%\) of control response.

PE was collected from control and diabetic rats. The PE response to SP (SP\(_2\), 3 & 4) was calculated as percent above basal \(\pm\) SEM. SEMs were omitted from Fig 4-11 for clarity but quoted in the text. The basal PE (R\(_1\)) did not change in either control or diabetic rats after L-NAME perfusion (Appendix B, Table B-2). However, in control rats, L-NAME caused a 20 \(\pm\) 22% reduction in PE response during the first 10 minutes of SP perfusion. This reduction became significant during the following 20 minutes reaching 45 \(\pm\) 31% at SP\(_3\) and 53 \(\pm\) 19% at SP\(_4\). In diabetic rats, there was no significant reduction in PE responses to SP at any time after L-NAME was perfused (Appendix B, Table B-2).

In control rats, PE responses to nicotine (20 \(\pm\) 9%, 52 \(\pm\) 17%, 64 \(\pm\) 22%) were weaker than SP, whereas in diabetic rats (4 \(\pm\) 9%, 9 \(\pm\) 8%, -9 \(\pm\) 10%) these responses were similar (Fig 4-11).

![Graph](image_url)

**Fig 4-11:** PE in control (SP (1\(\mu\)M), BK (10\(\mu\)M)), diabetic (Diab) (SP (1\(\mu\)M), BK (10\(\mu\)M)), control after Nicotine (100\(\mu\)M) or Muscarine chloride (100\(\mu\)M), and diabetic rats after Nicotine (100\(\mu\)M) or Muscarine chloride (100\(\mu\)M), presented as percent above basal (n = 6-9).
PE responses to BK were similar to SP in both control [(35 ± 8%, 86 ± 13%, 114 ± 30%) & (36 ± 19%, 72 ± 18%, 89 ± 14%) respectively) and diabetic rats [(3 ± 5%, 12 ± 6%, 7 ± 5%) & (9 ± 23%, 16 ± 32%, 0.1 ± 23%) respectively).

4.4. Discussion

It is known that blood vessels from humans and animals with experimental diabetes exhibit changes to vasoactive agents (Vane and Botting, 1994). This may lead to vascular dysfunction and diabetes-associated complications (Cooper et al., 1997). In this chapter, the microvascular response to ACh was examined in early diabetes. In addition, the changes in the contribution of NO to BK and SP responses were assessed in 4 weeks diabetic rats with a focus on the relative contribution of the superficial and deeper vessels to vascular reactivity to ACh in the utilized model.

In general, BBFs in control and diabetic rats (4 weeks) showed similarity to those observed in chapter 3. There was a reduction in BBF in diabetic rats when compared to controls. The similarity between SP response in control rats after L-NAME and SP response in diabetic rats suggests, in absolute terms, that the contribution of NO to this response is reduced (Fig 4-10). It is possible that NO synthesis is not reduced but the bioactivity of NO is diminished possibly due to oxidative stress. The above results also suggest that since the relative contribution of NO to SP response is not altered after 4 weeks of diabetes, any change in BBF may be related to increase in sensitivity of blood vessels to vasoconstrictors (see chapter 6). In chapter 5, the role of sympathetic nerves in reducing basal blood flow in diabetic animals is examined.

Changes in the vascular response to ACh with diabetes

Vasodilator responses to nicotine and muscarine chloride were similar to that of ACh in both control and diabetic rats with the latter showing significant reductions in all responses (Figs 4-2 & 4-3). A previous study in rat skin microvasculature showed that the vasodilator response to ACh is partially mediated by NO release when muscarinic (M3) receptors on the endothelium are directly stimulated (Ralevic et al., 1992). The other part of the response is mediated by sensory neurotransmitters (e.g. SP, CGRP) released by activation of nicotinic receptors on sensory nerves (Ralevic et al., 1992). The vasodilator
response to nicotine and muscarine chloride is also mediated by the indirect and direct effect of these substances on nicotinic and muscarinic receptors respectively, releasing NO, which may explain the similarity between these three responses.

In diabetic rats, the vascular response to ACh, nicotine, and muscarine chloride were similarly reduced, reaching 27%, 36% and 34% of their own controls respectively (Fig 4-3). Since all these vasoactive agents are known to cause the release of NO, it is possible that in diabetes, the released NO is deactivated by free radicals such as superoxide anions (Gryglewski et al., 1986), causing reduced vasodilation. Previous studies supported this notion by showing an increase in free radical concentration and a decrease in the level of antioxidants such as SOD (Ha and Kim, 1999; Kobayashi and Kamata, 1999b; Traverso et al., 1998). SOD is known to be capable of reducing the concentration of superoxide anions (Diederich et al., 1994). The vascular response to SNP (a NO donor) in diabetic rats was reduced to 25% of its own control, providing support for this proposition (see also section 1.5.4.2). The effect of free radicals on microvascular responses in 4 weeks diabetic rats is examined in chapter 7. The other possibility could be alterations in muscarinic receptor function. In diabetes, damage to the endothelium could alter muscarinic (M3) receptor function that mediates vasodilation (Huszka et al., 1997). This may explain the reduction in response to muscarine chloride in 4 weeks diabetic rats (Fig 4-3). The similarity between reduction in response after ACh and muscarine chloride in diabetic rats provides further support for this proposition.

A previous study by Kobayashi showed no alteration in ACh response in aorta of 4 weeks diabetic rats in vitro (Kobayashi and Kamata, 1999b). The discrepancy between the experimental results in this study and that of Kobayashi could be related to the difference in the vascular bed and the fact that the results in this study were from an in vivo preparation.

The relative contribution of different skin compartments to the vasodilator response to ACh was examined in this study. In diabetic rats, vascular responses to ACh, recorded by the PLDF and the MLDF, during the first 10 minutes of perfusion, were equally reduced (by 30% & 23% respectively) (Figs 4-6 & 4-7). During the following 20 minutes of the peptide perfusion, however, the MLDF showed a further 25% reduction in response
compared to only 7.5% reduction recorded by the PLDF. These results suggest that around 17.5% of the reduction in ACh response is mostly related to changes in microvascular function of deeper skin compartments (e.g. AVAs).

In normal and diabetic rats respectively, total ACh response measured by the PLDF and calculated as a percent above basal was greater (165 ± 31% & 128.5 ± 45.5%) than the MLDF (127.5 ± 25% & 80.5 ± 19%) (Table 4-1). Also, the ratio of superficial to combined superficial and deeper vessel’s blood flow was doubled in the diabetic condition. Overall, these results suggest that both superficial and deeper vessels are affected by diabetes. Also, the greater percent reduction in ACh response using the MLDF compared to the PLDF might suggest an additional involvement of deeper vessels in diabetes, further supporting the results in chapter 3.

**Changes in the vascular response to BK and NO involvement with diabetes**

In control rats, although perfusion of L-NAME significantly reduced the vascular response to BK (65% of control), it showed more potency in reducing SP response in these animals (40% of control) (Figs 4-9 & 4-10). The reduction in BK and SP responses in control rats after L-NAME perfusion is mainly attributed to inhibition of NO synthase activity and unavailability of NO. The greater effect of L-NAME on SP response can be related to a greater contribution of NO to SP-mediated vasodilation in rat skin.

A previous study showed that a significant part of the vasodilator response to BK is mediated through an excitatory action on peripheral nociceptive C-fibres where sensory neurotransmitters (e.g. SP, CGRP) are released (Khalil and Helme, 1992). SP, exogenously applied, exerts its vasodilatory effect on rat skin in two ways. The first is directly on blood vessels, causing NO release, and vasodilatation. The second is indirectly by degranulating mast cells with subsequent release of mediators that excite sensory nerves, leading to release of more neurotransmitters (Khalil and Helme, 1989). Therefore, when SP is used as a vasodilator agent, more NO is released and hence L-NAME is capable of exerting a greater inhibitory effect.

In diabetic rats, vascular responses to SP and BK were significantly diminished reaching 42% and 48% of own controls respectively (Figs 4-9 & 4-10). These reductions could be partially related to a decrease in smooth muscle reactivity (28% of control) (Fig 4-8).
Perfusion of L-NAME, in diabetic rats, caused a further (similar) reduction in SP and BK responses (15% & 20% of own controls respectively). Results from both BK and SP responses after L-NAME perfusion suggests that endothelial cells, in 4 weeks diabetic rats, are still capable of synthesizing NO. The similarity between SP response in control rats after L-NAME (40% of control) and SP response in diabetic rats (42% of control) might suggest that the relative contribution of NO to the response is similar. The overall reduction in vascular responses to SP, BK, and SNP, therefore, could be related to rapid deactivation of NO. The possibility was raised that the presence of certain substances in diabetic condition may contribute to this deactivation (Bucala et al., 1991; Pieper et al., 1997b).

*Plasma extravasation and diabetes*

The results of this study showed similarity in PE responses to BK and SP in control rats (Appendix B, Table B-2). This could be due to a similarity between mechanisms involved in inducing PE response by these agents (Khalil and Helme, 1989; Khalil and Helme, 1992). In rat skin, BK is known to stimulate sensory nerves to release SP (Khalil and Helme, 1992). Therefore, the first 10 minutes of an inflammatory response to SP or BK is possibly mediated by an action of SP at the post-capillary venules through its C-terminal, an action which is independent of histamine or prostaglandins (Khalil and Helme, 1989). Both SP and BK are capable of stimulating mast cells to release histamine (Khalil and Helme, 1992; Lee and Pearce, 1990; Zhao et al., 1996). This could contribute to the PE response after the first 10 minutes interval (Khalil and Helme, 1989).

Once again, in diabetic rats, neither BK nor SP induced a significant change in the general PE (Fig 4-11). These data confirm the results in chapter 3 and raise the notion that the ability of SP or BK to induce PE is masked by diabetes-induced endothelial changes that results in high basal PE.

In conclusion, evidence was provided that NO plays an important role in acute inflammation in early diabetes and that the reduced skin inflammatory responses in diabetic rats could be related to inactivation of NO. Evidence was provided to show that the bioactivity of NO is diminished after 4 weeks of diabetes. It was hypothesized that the
release of certain agents in early diabetes could affect NO activity, contributing to the reduced microvascular responses. This hypothesis will be further investigated in chapters 6 & 7. It was also demonstrated that both superficial and deeper vessels of the rat skin are affected by diabetes after 4 weeks. However, deeper vessels may have an additional involvement in reducing microvascular blood flow in these animals. The possibility was raised that the overall reduction in endothelial-mediated responses in early diabetes might be related to the fate of NO once it is released.
CHAPTER 5

Diabetes and modulation of microvascular blood flow:
Role of sympathetic nerves
5.1. Introduction

Blood vessels in the skin are innervated not only by sensory but also sympathetic nerve fibres and both sets of nerves release vasoactive mediators. It has been proposed that sympathetic nerve fibres strongly influence blood flow in skin microvasculature (Kurvers et al., 1997; Pinter et al., 1997), particularly small arteries, arterioles and arteriovenous anastomoses (AVAs), which are richly supplied with these fibres (Norberg and Hamberger, 1964).

It is known that stimulation parameters that activate the small unmyelinated sensory nerves can also activate the sympathetic efferents, with the release of neurotransmitters that will act on the microvasculature in an actively opposing manner (chapter 1, section 1.7.4). Most of the sympathetic nerves, which innervate blood vessels, release the potent vasoconstrictor noradrenaline (NA), which acts on alpha (α-) and beta (β-) adrenoreceptors to mediate its biological response (chapter 1, section 1.8.3). These receptors are found on smooth muscle cells and may also exist on unmyelinated nerve axons (Bennett and Gardiner, 1996; Fuder and Selbach, 1993; Nicholas et al., 1993). Neuropeptide Y (NPY) and adenosine triphosphate (ATP) could also be released from sympathetic nerves by high frequency nerve stimulation (Bennett and Gardiner, 1996; Fuder and Selbach, 1993; Kennedy et al., 1997; Nicholas et al., 1993). NPY has been shown to regulate the cutaneous microcirculation in rat skin and acts as a neuromodulator controlling the release and action of NA and ATP (Pinter et al., 1997).

Previous studies have shown that NA concentration is diminished in the skin of rats with early diabetes (4 weeks) (Ahlgren and Levine, 1993). The effect of diabetes on the vascular response to NA is strongly related to the type of tissue under investigation and the duration of diabetes (chapter 1, sections 1.8.3 & 1.5.4.1). A previous study in diabetic heart showed a reduction in the number of alpha-adrenoreceptors and a receptor supersensitivity to alpha-agonists (Wald et al., 1988). The authors hypothesized that the observed supersensitivity could be due to high activity of phospholipase C (PLC) (with an increase in diacylglycerol (DAG) production). A reduction in the number of beta-adrenoreceptors in this tissue was also observed in early diabetes (1-3 weeks) (Bitar and DeSouza, 1990; Ramanadham and Tenner, 1987).
Both sensory and sympathetic nerves control microvascular blood flow in the skin. This chapter is designed to investigate the role of sympathetic nerves in the modulation of skin microvascular blood flow in early diabetic rats. It was hypothesized that the function of sympathetic nerves may be altered in early diabetes, contributing to the reduced microvascular responses observed in these animals. Changes in microvascular responses to sodium nitroprusside (SNP), substance P (SP), and electrical stimulation (ES) of the sciatic nerve are examined in diabetic rats (4 weeks) treated with a short-term sympathetic neurone blocker guanethidine monosulfate (chapter 2, section 2.10).

5.2. Method

Eight groups of rats (n = 4-10) were used, four control and four streptozotocin (STZ)-treated diabetic groups. Control rats were treated with 0.1M sodium citrate buffer solution (CB-treated) (chapter 2, section 2.4.1). On day 28, rats (diabetic or control) were anaesthetized and a blister was induced on the hind footpad. Then rats were further prepared depending on the type of experimental protocol (i.e. ES or peptide perfusion) (chapter 2, sections 2.6 & 2.7). One hour before nerve stimulation, four groups of rats (2 control and 2 diabetic) were given a single injection of guanethidine monosulfate (50mg/kg, i.p) (chapter 2, section 2.10) and others were non-treated (NT).
In experiment 1 (peptide perfusion), SP (1μM, 30 min) and SNP (100μM, 10 min) were perfused over the blister base and plasma extravasation (PE) was collected. In experiment 2 (ES), first SNP (100μM, 10 min) was perfused over the blister base and later sensory nerve fibres were activated by a Grass stimulator (20V, 5Hz, 2 msec, for 1 minute) (chapter 2, sections 2.6 & 2.7). At the end of each experiment rats were sacrificed with pentobarbitone overdose.

5.2.1. Expression of data and statistical analyses

Results from vasodilatation responses were calculated as areas under the response curves. PE responses are presented as percent above basal. All data are expressed as mean ± standard error of the mean (SEM). The absolute values and 95% confidence intervals (95% CI) of these values are quoted in the text. A two-way Analysis of Variance (ANOVAs) was performed to investigate whether there were significant differences in basal blood flow (BBF) and responses to SNP, SP, and ES, between control and diabetic rats, based on whether or not these rats received guanethidine treatment. Where ANOVA indicated a significant main effect, post hoc independent sample t-tests were performed. A two-way repeated measure ANOVA with priori planned comparisons was performed to investigate whether there were significant differences in PE responses to SP at different times of perfusion (SP 2, 3& 4), between control and diabetic rats, based on whether or not these rats received guanethidine treatment.

5.3. Results

Guanethidine treatment insignificantly increased the BBF in control rats [(24 ± 0.8 cm²) (95%CI 22.4-25.6)] (111% of non-treated control) when compared to non-treated animals [(21.6 ± 1.3 cm²) (95%CI 19.05-24.1)]. Guanethidine treatment did not change BBF in diabetic rat [(17.4 ± 0.9 cm²) (95%CI 15.6-19.2)] when compared to non-treated diabetic animals [(16.7 ± 1.3 cm²) (95%CI 14.1-19.2)] (Fig 5-1).
In control rats, there was no difference between guanethidine-treated SNP responses \([25.1 \pm 1.3 \text{ cm}^2] \text{(95\% CI 22.6-27.6)}\) and those of non-treated animals \([26.1 \pm 1.8 \text{ cm}^2] \text{(95\% CI 22.6-29.6)}\) (Fig 5-2).
However, in diabetic rats, SNP responses in the treated groups were significantly higher (182% of non-treated diabetic) [(13.5 ± 1 cm²) (95%CI 11.5-15.5)] than their non-treated counterparts [(7.4 ± 0.6 cm²) (95%CI 6.2-8.6)].

Guanethidine treatment did not change the vascular response to SP in control animals [(46.5 ± 3.2 cm²) (95%CI 40.2-52.8)] but improved the response to SP in diabetic rats to 185% of non-treated diabetic (Fig 5-3). This response reached (39.9 ± 5.6 cm²) (95%CI 28.9-50.9), which was statistically significant only from SP in non-treated diabetic animals [(21.6 ± 3.4 cm²) (95%CI 14.9-28.3)].

Fig 5-3: Vascular responses to SP (1μM) in control and diabetic rats before and after guanethidine treatment (50mg/kg, i.p), presented as mean ± SEM (n = 4-10).
* Denotes significance from control rats (P<0.05)
* Denotes significance from diabetic rats (P<0.05)
The response to ES in control rats \([(17.6 \pm 1.3 \text{ cm}^2) (95\%\text{CI } 15-20.1)]\) was increased after treatment with guanethidine \((119\% \text{ of non-treated control}) \([(20.9 \pm 1.1 \text{ cm}^2) (95\%\text{CI } 18.7-23)]\) but did not reach statistical significance (Fig 5-4). However, a far greater improvement in ES response was observed in diabetic rats after guanethidine treatment \((212\% \text{ of non-treated diabetic}) \([(10.8 \pm 2.2 \text{ cm}^2) (95\%\text{CI } 6.5-15.1)]\) when compared to ES in non-treated diabetic rats \([(5.1 \pm 2 \text{ cm}^2) (95\%\text{CI } 1.2-9)]\).

![Graph showing vascular responses to ES](image)

**Fig 5-4:** Vascular responses to ES \((20V, 5Hz, 2\text{msec, for 1 minute})\) in control and diabetic rats before and after guanethidine treatment \((50mg/kg, i.p)\), presented as mean \(\pm\) SEM \((n = 4-8)\).

* Denotes significance from control rats \((P<0.05)\)

+ Denotes significance from guanethidine-treated control rats \((P<0.05)\)

x Denotes significance from diabetic rats \((P<0.05)\)

Basal PE \((R_1)\) in control or diabetic rats was not changed after guanethidine treatment (Appendix B, Table B-3). The PE response to SP \((SP_2, 3 \& 4)\) was calculated as percent above basal \(\pm\) SEM. SEMs were omitted from Fig 5-5 for clarity but quoted in the text. The PE response to SP, in control rats \((36 \pm 19\%, 72 \pm 18\%, 89 \pm 14\%)\), were significantly different to diabetic rats \((9 \pm 23\%, 16 \pm 32\%, 0.1 \pm 23\%)\). Guanethidine treatment did not change the PE response in control rats \((29 \pm 10\%, 44 \pm 14\%, 70 \pm\)
26%). Although this treatment increased the PE response in diabetic rats (28 ± 19%, 39 ± 17%, 23 ± 5%), it did not reach statistical significance (Fig 5-5).

![Graph showing PE response in control and diabetic rats before and after guanethidine treatment.](image)

**Fig 5-5:** PE in control (C) and diabetic (D) rats before and after guanethidine treatment (50mg/kg, i.p), presented as percent above basal (n = 4-10).

### 5.4. Discussion

Sympathetic nerve fibres that innervate skin blood vessels can influence microvascular blood flow via the release of sympathetic neurotransmitters like NA and NPY. A reduction in rat hindquarter blood flow, after 6 days of diabetes, was suggested to be a vasoconstrictor response and not due to impairment in endothelium-mediated vasorelaxation or responsiveness to nitric oxide (NO) (Brands and Fitzgerald, 1998). The results in chapter 3 showed a reduction in the BBF levels and the vascular inflammatory responses in diabetic rats over time. In this chapter, possible contribution of sympathetic efferents to these responses in early diabetes is examined.

The results showed that guanethidine treatment did not alter the BBF in 4 weeks diabetic rats (Fig 5-1), suggesting that the observed reduction in BBF in diabetic rats (77% of control) is not due to sympathetically mediated vasoconstriction. This raised the possibility that in early diabetes, certain endothelial-mediated factors may play a role in reducing BBF in skin microvascular bed. This possibility is further examined in chapter 6. The fact that guanethidine did not alter BBF in control or diabetic rats suggests that
sympathetic nerves play a minor role in regulating basal blood flow under the current experimental conditions (anaesthesia and blister induction).

This study, however, demonstrates that sympathetically mediated vasoconstriction contributes to the reduction in smooth muscle reactivity in 4 weeks diabetic rats (28% of control) (Fig 5-2). The mechanisms underlying this sympathetic effect on smooth muscle reactivity might be related to alterations in the NA level and the sensitivity of alpha-1, alpha-2, and beta-2, adrenoreceptors located on smooth muscle cells. NA acts on both alpha 1- and alpha-2 adrenoreceptors causing smooth muscle contraction. Low NA levels in the skin tissue (Ahlgren and Levine, 1993) and reduced NA release from the spinal cord were previously shown to occur in early diabetes (Bitar et al., 1999; Ohtani et al., 1997). This could lead to the development of adrenoreceptor supersensitivity, causing enhanced contraction in the smooth muscle by increasing intracellular calcium (Kamata et al., 1988). Guanethidine treatment might prevent this contraction by inhibiting NA release and activation of alpha-adrenoreceptors on the smooth muscle. NA also acts on beta-2 adrenoreceptors to mediate vasodilatation. Beta-adrenoreceptor stimulation can lead to smooth muscle relaxation by regulating the intracellular calcium concentration. It was reported that the population size and the affinity of these receptors were reduced in early diabetes (Bitar and DeSouza, 1990; Ramanadham and Tenner, 1987) (chapter 1, section 1.8.3). This might lead to the observed reduction in smooth muscle relaxation in 4 weeks diabetic rats, which is in agreement with a previous study (Kamata et al., 1992). A reduction in NA level, however, might also cause supersensitivity to develop in beta-adrenoreceptors and this may reduce some of the contraction caused by alpha-adrenoreceptors.

Guanethidine treatment did not affect SP response in control rats. However, in diabetic rats, this treatment enhanced the vascular response to SP (185% of non-treated diabetic), which could be due to improvement in smooth muscle reactivity (182% of non-treated diabetic) (Figs 5-2 & 5-3).
The vascular response to ES in control rats showed a slight increase (119% of non-treated control) after guanethidine treatment (Fig 5-4). This suggests that ES technique (preparation of the sciatic nerve) could have resulted in some sympathetic nerve activation that caused the reduction in this response. The vascular response to ES in diabetic rats was significantly enhanced after guanethidine treatment. The enhancement in ES in diabetic rats after this treatment (212% of non-treated diabetic) was greater than that of control, which indicates that sympathetic nerve contribution in diabetic rats is greater under this experimental condition. Part of the enhancement in ES response after guanethidine treatment, in diabetic rats, could be explained by the improvement in SNP response (182% of non-treated control) (post-terminal effect). As mentioned before, adrenoreceptors in diabetic rats might become supersensitive when circulating NA is reduced and this may explain the remaining 30% enhancement in the ES response after the treatment (pre-terminal changes).

NPY and ATP release from sympathetic nerves can vary in proportion depending on the tissue, the species, and on the parameter of nerve stimulation (Burnstock, 1986) and NPY resting plasma levels are low in most species (Pernow, 1988). The relationship between NPY and diabetes has been investigated mainly in the brain tissues (see also chapter 1, section 1.8.3). It was reported that the distribution or relative density of NPY immunoreactive nerve fibres was not changed after 2, 4, 8 or 12 weeks of diabetes in the rat skin (Karanth et al., 1990).

As for ATP, evidence exists that in early diabetes there was no significant change in ATP concentration or the release of ATP between control and diabetic rats (2 and 8 weeks respectively) (Belai et al., 1991; Carlsson and Arnqvist, 1981). It was previously shown that application of low frequency electrical nerve stimulation reduces the chance of NPY / ATP release from sympathetic nerves (Pernow, 1988). Therefore, the roles of NPY and ATP in early diabetes were not examined in this thesis.
In conclusion, this chapter provides evidence for an increase in the contribution of sympathetic efferents in regulating microvascular blood flow in early diabetes. An increase in sympathetically mediated vasoconstrictor effect controlling smooth muscle reactivity was also demonstrated. Finally, it was proposed that adrenoreceptor supersensitivity might play a role in increasing smooth muscle contraction and reducing the inflammatory response to ES of the sciatic nerve.
CHAPTER 6

Diabetes and modulation of microvascular blood flow:

Role of endothelial-derived constrictor factors and

Protein Kinase C
6.1. Introduction

Role of Endothelins (ETs)

Endothelin-1 (ET-1) is a potent vasoconstrictor polypeptide produced by many cell types, upon demand (e.g. in an emergency and/or in a defensive event) and its production is regulated at the level of mRNA transcription and translation (Nakamura et al., 1990). ET-1 has a wide range of biological actions but its physiological importance is still uncertain (see also chapter 1, section 1.8.4 & Table 1-5). Elevated level of glucose has been shown to modulate ET-1 synthesis and receptor expression in cultured endothelial and vascular smooth muscle cells (chapter 1, section 1.8.4.1). Circulating (plasma) levels of ET-1 have been previously reported to increase (2-10 fold) in diabetic patients (Battistini et al., 1993). However, in animals with type I diabetes, there are conflicting reports regarding the plasma level of ET-1 (chapter 1, section 1.8.4.1). This might be explained by the pre-existing duration of diabetes and the vascular bed under examination (Hopfner et al., 1999).

It was shown that high glucose levels could also modulate endothelium-derived relaxing factor (EDRF) production and action (Hopfner and Gopalakrishnan, 1999). The authors suggested that formation of substances such as advanced glycated end-products (AGEs) and free radicals, by damaged endothelium, could be involved in inhibiting EDRF and regulating ET-1 production and action (chapter 1, section 1.8.4.1, Fig 1-10). It was proposed that under pathological conditions, ET-1 might also inhibit EDRF (Barton et al., 1998). Furthermore, insulin appears to be a modulator of ET-1, ET receptors, and ET-1 mediated responses in both in vitro and in vivo settings (Hopfner and Gopalakrishnan, 1999). The role of insulin in diabetes is discussed in chapter 9.

ET-1 mediates its biological responses mainly by binding to ET-A and ET-B receptors (chapter 1, section 1.8.4 & Fig 1-8). Under normal conditions, the in vivo response to ET-1 varies depending on the vascular bed and the expression/density of ET-A and ET-B receptors (Hopfner and Gopalakrishnan, 1999). Various pathological conditions, including diabetes, can modify the density of these receptors (Nayler, 1990). The binding of ET to its receptors activates a variety of signal transduction processes but the activation of phospholipase C (PLC) and the subsequent release of intracellular
calcium appears to be the major signal transduction pathway involved (Reid and La, 1995) (see also chapter 1, section 1.8.4, Fig 1-9). There is conflicting evidence regarding the function of ET in microvascular permeability and it seems that the route of administration and the species involved are important factors (Brain et al., 1989; Lawrence et al., 1995).

The results in chapter 3 and 4 led to the hypothesis that the presence of certain substances may be responsible for the observed reduction in microvascular inflammatory responses in rats with early diabetes. The literature has associated a link between ETs and vascular complications in certain disorders such as diabetes (Battistini et al., 1993; Haak et al., 1992; Takahashi et al., 1990). A few studies have used skin to assess the haemodynamic action of ET-1 in rats (Brain et al., 1989; Chander et al., 1988; Lawrence et al., 1995) but none to date have investigated the role of ET-1 in the modulation of skin microvascular blood flow in early diabetes.

This chapter is designed to determine the quantitative contribution of ET-A and ET-B receptors to the observed reduction in microvascular responses in 4 weeks streptozotocin (STZ)-treated diabetic rats. It was hypothesized that ET-1 may be involved in reducing microvascular blood flow in early diabetes and its effects may be inhibited by early intervention. BQ-123 (a specific ET-A receptor antagonist) and BQ-788 (a specific ET-B receptor antagonist) are used to examine the role of these receptors in a) modulating endothelial and smooth muscle cell mediated vascular responses and b) modulating the inflammatory response to ES of the sciatic nerve in diabetic rats.

**Role of protein kinase C (PKC)**

PKC, a family of kinases, play a major role in intracellular signalling. High extracellular glucose activates PKC but the identity of the isoforms involved and their location in the cell where they operate is unclear (chapter 1, section 1.8.5.4). Evidence exists that a number of vascular alterations in diabetes are caused by activation of PKC, including a decrease in the activity of Na⁺/K⁺-ATPase and an increase in extracellular matrix, cytokines, permeability, contractility, and cell proliferation (Park et al., 1999).
Enhanced PKC activity by hyperglycaemia might be tissue specific. Enhanced PKC activity was previously noted in the heart (Inoguchi et al., 1992; Liu et al., 1999) and glomeruli (Awazu et al., 1991) but may not occur in the brain and peripheral nerves (Koya and King, 1998). Enhancement in PKC activity was attributed to an increase in diacylglycerol (DAG), a major endogenous activator of PKC (Ishii et al., 1998). Recently, it was shown that enhanced ET-1 expression in diabetes was partially due to PKC activity (Park et al., 2000). Under physiological conditions in human endothelial cells, ET-1 induces endothelial NOS (eNOS) gene expression by activating ET-A receptors and PKC regulates this induction (Marsen et al., 1999). This shows that regulation of eNOS at the genomic level occurs via post-transcriptional mechanisms.

The role of PKC in vascular alterations associated with diabetes has been shown (chapter 1, section 1.8.4). However, the role of this enzyme in modulating skin microvascular blood flow in early diabetes has not been previously investigated. In this chapter, the role of PKC in STZ-induced diabetic rat model is examined using bisindolylmaleimide (BIM), a specific PKC inhibitor.

6.2. Method

Ten groups of rats (n = 4-12) were used in this study, five of which were made diabetic with STZ and the other five were used as controls. Control rats were injected with 0.1M sodium citrate buffer solution (CB-treated) (chapter 2, section 2.4.1).
Control and diabetic rats were then prepared for experiment 1 (peptide perfusion) alone or together with experiment 2 (electrical stimulation (ES) of the sciatic nerve). In experiment 1, after the baseline was established, one of the following substances, i.e. BQ-123, BQ-788 (both at 10 μM), or BIM (1μM) was perfused for 10 minutes prior to, and together with, substance P (SP, 1μM, 30 min). This was followed by separate perfusion of Ringer’s solution (20 minutes) and sodium nitroprusside (SNP, 100μM, 10 min) over the blister base respectively. In experiment 2, first SNP was perfused for 10 minutes followed by Ringer’s solution. Then either BQ-123 or BQ-788 was perfused for 10 minutes followed by electrical stimulation of the sciatic nerve. Perfusion of the antagonist was continued during the stimulation period and for 20 min after that. Plasma extravasation (PE) was collected from all peptide perfusion experiments. At the end of each experiment rats were sacrificed with a pentobarbitone overdose.

6.2.1. Expression of data and statistical analyses

Vasodilatation responses were calculated as areas under the response curves. PE responses are presented as percent above basal. All data are expressed as mean ± standard error of the mean (SEM). The absolute values and 95% confidence intervals (95% CI) of these values are quoted in the text.

Two-way Analyses of Variances (ANOVAs) were performed to investigate whether there were significant differences in basal blood flow (BBF) and responses to SNP, SP, and ES, between control and diabetic rats, according to whether rats were exposed to BQ-123 or BQ-788. This test could also show interaction effects between these groups. Where ANOVA indicated a significant main effect, post hoc independent sample t-tests were performed. The same analysis and post hoc comparisons were also performed to investigate whether there were significant differences in BBF and responses to SNP and SP, between control and diabetic rats, based on whether rats were exposed to BIM or not. A two-way repeated measure ANOVA with priori planned comparisons was performed to investigate whether there were significant differences in PE responses between control and diabetic rats before and after BQ-123, BQ-788, and BIM perfusions.
6.3. Results

Effect of endothelin antagonists on BBF, SNP, and SP

The BBF in diabetic rats [(16.7 ± 1.3 cm²) (95% CI 14.1-19.2)] (measured for 20 minutes), was significantly lower than control animals [(25.1 ± 0.9 cm²) (95% CI 23.3-26.9)] (Fig 6-1 & 6-2).

**Fig 6-1:** BBF in control rats before and after BQ-123 and BQ-788 (both at 10μM), presented as mean ± SEM (n = 4-9).

**Fig 6-2:** BBF in diabetic (Diab) rats before and after BQ-123 and BQ-788 (both at 10μM), presented as mean ± SEM (n = 4-10).
Perfusion of BQ-123 or BQ-788 did not significantly change the BBF in control rats (Fig 6-1). However, the BBF in diabetic rats was improved after BQ-123 \((20.8 \pm 2.1 \text{ cm}^2)\) (95% CI 16.7-24.9), reaching 83% of control (Fig 6-2). This response was not altered by BQ-788 perfusion \([(16 \pm 0.6 \text{ cm}^2) \text{ (95\% CI 14.8-17.2)}]\). The improvement in diabetic BBF after BQ-123 perfusion did not reach statistical significance.

In control rats, BQ-123 and BQ-788 perfusions did not have any significant effect on vascular responses to SNP and SP (Figs 6-3 & 6-4).

![Graph](image)

**Fig 6-3:** The vascular response to SNP \((100\mu\text{M})\) in control rats before and after BQ-123 and BQ-788 (both at \(10\mu\text{M}\)), presented as mean ± SEM \((n = 8-12)\).

![Graph](image)

**Fig 6-4:** The vascular responses to SP \((1\mu\text{M})\) in control rats before and after BQ-123 and BQ-788 (both at \(10\mu\text{M}\)), presented as mean ± SEM \((n = 4-9)\).
On the other hand, the SNP response in diabetic rats was equally and significantly improved after both BQ-123 [(17.9 ± 2.1 cm$^2$) (95% CI 13.8-22)] and BQ-788 [(18.8 ± 1.1 cm$^2$) (95% CI 16.6-20.9)] perfusions, reaching approximately 70% of control value [(26.1± 1.8 cm$^2$) (95% CI 22.6-29.6)] (Fig 6-5).

The vascular response to SP in diabetic rats was partially improved after perfusing BQ-123 [(32.4 ± 2.7 cm$^2$) (95%CI 27.1-37.7)]. Although this response reached 63% of the control SP response [(51.4 ± 2.9 cm$^2$) (95%CI 45.7-57.1)], it did not reach statistical significance (Fig 6-6).

**Fig 6-5:** The vascular response to SNP (100μM) in diabetic (Diab) rats before and after BQ-123 and BQ-788 (both at 10μM), presented as mean ± SEM (n = 8-10).
* Denotes significance from diabetic rats (P<0.05)
Fig 6-6: The vascular response to SP (1μM) in diabetic (Diab) rats before and after BQ-123 and BQ-788 (both at 10μM), presented as mean ± SEM (n = 4-10).

* Denotes significance from diabetic rats (P<0.05)

On the other hand, BQ-788 was more potent [(44.4 ± 4.1 cm²) (95%CI 36.4-52.4)] and improved this response to 86% of control.

Effect of endothelin antagonists on ES

In control rats, the response to ES was significantly enhanced after BQ-123 perfusion [(28.5 ± 2.5 cm²) (95% CI 23.6-33.4)], reaching 162% of control response (Fig 6-7). This response was also increased after BQ-788 perfusion [(27.8 ± 5 cm²) (95% CI 18-37.6)] (158% of control) but did not reach statistical significance.

In diabetic rats, BQ-123 perfusion partially improved the vascular response to ES [(9.6 ± 2.2 cm²) (95%CI 5.3-13.9)], reaching approximately 55% of control response [(17.6 ± 1.3 cm²) (95%CI 15.1-20.1)] (Fig 6-8). BQ-788, on the other hand, proved to be more potent than BQ-123 and significantly improved the ES response in diabetic animals to 84% of its control value [(14.8 ± 0.9 cm²) (95%CI 13-16.6)].
**Fig 6-7:** The vascular response to ES (20V, 5Hz, 2msec, for 1 minute) in control rats before and after BQ-123 and BQ-788 (both at 10μM), presented as mean ± SEM (n = 8-12). * Denotes significance from control rats (P<0.05)

**Fig 6-8:** The vascular response to ES (20V, 5Hz, 2msec, for 1 minute) in diabetic (Diab) rats before and after BQ-123 and BQ-788 (both at 10μM), presented as mean ± SEM (n = 8-10). * Denotes significance from diabetic rats (P<0.05)
Effect of PKC inhibitor on BBF, SNP, and SP

BIM perfusion did not change the BBF in control [(26 ± 0.9 cm$^2$) (95% CI 24.2-27.8)] or diabetic rats [(15.6 ± 0.7 cm$^2$) (95% CI 14.2-17)] (Fig 6-9).

**Fig 6-9:** BBFs in control, control after BIM, diabetic (Diab), and diabetic rats after BIM (1μM), presented as mean ± SEM (n = 5). * Denotes significance from control rats (P<0.05)

BIM perfusion did not alter the vasodilator response to SNP in control rats [(26.8 ± 1.8 cm$^2$) (95%CI 23.3-30.3)] but significantly enhanced this response in diabetic rats [(14.7 ± 2 cm$^2$) (95%CI 10.8-18.6)], reaching 56% of control response (Fig 6-10). BIM perfusion did not have an effect on the vascular response to SP in control [(50 ± 3.2 cm$^2$) (95%CI 56.3-43.7)] or diabetic rats [(21.7 ± 1.1 cm$^2$) (95%CI 19.5-23.8)] (Fig 6-11).
Fig 6-10: Vascular responses to SNP (100 μM) in control, control after BIM, diabetic (Diab), and diabetic rat after BIM (1 μM), presented as mean ± SEM (n = 5). * Denotes significance from control rats (P<0.05) x Denotes significance from diabetic rats (P<0.05)

Fig 6-11: Vascular responses to SP (1 μM) in control, control after BIM, diabetic (Diab), and diabetic rats after BIM (1 μM), presented as mean ± SEM (n = 5). * Denotes significance from control rats (P<0.05)

**Effect of endothelin antagonists and PKC inhibitor on PE**

PE in control rats was not altered after BQ-123 or BQ-788 perfusions (Appendix B, Table B-4). However, in diabetic rats, both receptor antagonists reduced basal PE (R₁) but only BQ-123 reduced this value to near normal control level. BQ-123 also improved the ability of SP to induce a PE response.
The PE response to SP (SP₂, ₃ & ₄) was calculated as percent above basal ± SEM. SEMs were omitted from Fig 6-12 for clarity but quoted in the text. BQ-123 perfusion increased the PE response to SP in diabetic rats by 43 ± 15%, 75 ± 13%, and 66 ± 11%. BQ-788 also increased the above response by 28 ± 12%, 74 ± 25%, and 93 ± 27% respectively (Fig 6-12).

BIM perfusion did not change R₁ in diabetic rats (Appendix B, Table B-4). However, it improved the PE response to SP only during the last 20 minutes of the response (5 ± 14%, 27 ± 17%, 34 ± 14%), when compared to non-perfused diabetic animals (9 ± 23%, 16 ± 32%, 0.1 ± 23%). However, these responses did not reach the control value (36 ± 19%, 72 ± 18%, 89 ± 14%) (Fig 6-12).

![Graph](image)

**Fig 6-12:** PE in control, diabetic, and diabetic rats before and after BQ-123, BQ-788 (both at 10μM), and BIM (1μM), presented as percent above basal (n = 4-10).

### 6.4. Discussion

**Related to ETs**

Previous studies have shown that changes in plasma glucose level in the diabetic condition is directly involved in regulation of ET-1 release, modulation of receptor expression, and response to the peptide (chapter 1, section 1.8.4.1, Figs 1-10 & section 1.8.5.3, Fig 1-14). Many studies have proposed that potent vasoconstrictors such as ET-1
could be involved in the development of vascular complications associated with diabetes (chapter 1, section 1.8.4).

In this chapter, the relative contribution of ET receptors to reduced skin microvascular blood flow in 4 weeks diabetic rats was examined, an issue which has not been previously investigated.

In diabetic rats, BBF levels did not change after BQ-788 perfusion but were partially increased after BQ-123 perfusion (83% of control) (Fig 6-2). The latter result suggests that endothelial mediated factors may be involved in reducing BBF in these animals. This notion was further supported in chapter 5 by showing that the reduction in diabetic BBF is not related to sympathetic vasoconstriction.

The reduced vascular response to SNP, a NO donor, in diabetic rats suggests that NO has a lower efficiency to induce vasodilatation (Fig 6-5). This could be due to deactivation of NO or inefficiency in the second messenger (cyclic guanosine monophosphate (cGMP)) pathway. ET could be a possible mediator for both of these actions and the results in this chapter support this proposition. The data showed that both BQ-123, an ET-A receptor antagonist and BQ-788, an ET-B receptor antagonist, significantly improved smooth muscle-dependent vasodilatation in diabetic rats to about 70% of control values (Fig 6-5). It is therefore reasonable to postulate that apart from NO deactivation, ET may either inhibit guanylate cyclase, thus reducing cGMP, or activate PKC (Barton et al., 1998; Khalil et al., 1996). The possible role of free radicals in deactivating NO donated by SNP cannot be excluded.

There is evidence that both ET receptors (ET-A & ET-B) exist in the rat skin (Lawrence et al., 1995). With respect to their activities, a number of studies in vivo demonstrated that both receptors were capable of mediating vascular smooth muscle contraction (Soe et al., 1994) but the relative contribution of each receptor is dependent on both the species and the vascular bed under study. In this study, both BQ-123 and BQ-788 perfusions increased the vascular response to SNP in 4 weeks diabetic rats (Fig 6-5). The increase in SNP response after BQ-123, in 4 weeks diabetic rats, suggests that ET-A receptors may be up regulated, contributing to the reduction in SNP responses. A previous study is
supportive of this proposition (Deng et al., 1999). On the other hand, it was suggested that increase in ET-1 could down-regulate ET-B receptors (Masao et al., 1999). Reduction in vasodilator responses mediated by ET-B receptors may exacerbate the observed vasoconstriction mediated by ET-A receptors in diabetic rats.

The current data suggests equal involvement of both ET-A and ET-B receptors in inhibiting smooth muscle-dependent vasodilation in diabetic rats at the level of skin microvasculature. In contrast, ET-B receptor activation plays a greater role in inhibiting the vascular responses to SP and ES of the sciatic nerve in these animals. Both SP and ES responses in diabetic rats reached approximately 85% of their own control responses after BQ-788 perfusion, compared to 63% and 55% of their own controls after BQ-123 (Figs 6-6 & 6-8). This might be related to an alteration in ET-B receptor activity after 4 weeks of diabetes. It should be noted here that whether SP is perfused exogenously over the blister base or released endogenously upon ES of the sciatic nerve, it induces the release of NO from endothelial cells via activating neurokinin-1 (NK-1) receptors (Andrews et al., 1989). In this experiment, ET may inhibit both the action and release of NO from endothelial cells. The greater involvement of ET-B receptors in the vascular responses to SP and ES raised the possibility that activation of ET-B receptors in 4 weeks diabetic rats can play a significant role in inhibiting NO release from endothelial cells. In diabetes, there is a relationship between hyperglycaemia and / or a low concentration of insulin and an increase in ET levels, which in turn could be involved in inhibition of NO release from endothelial cells (chapter 1, sections 1.8.4.1).
ET may also indirectly damage endothelium over time, leading to a further increase in ET-1 production (Huszka et al., 1997; Takahashi et al., 1990; Vermes et al., 1993). Hence, it is possible that a feedback mechanism is involved and an increase in ET-1 may further inhibit NO release and cause an increase in production of ET-1 in turn. This was supported by a previous study showing that, in STZ-rats, changes in response to ET-1 and its plasma concentrations are duration dependent and occur in parallel with changes in endothelial function (Hopfner et al., 1999).

The other possibility is that a reduction or lack of insulin may affect the production and release of NO in early diabetes leading to an increase in ET-1 production, which again can inhibit NO release by a possible feedback mechanism (chapter 1, section 1.8.5.3). Previous studies demonstrating the relationship between insulin, NO, and ET under physiological conditions support this hypothesis. One of these studies showed that insulin could affect ET levels by direct stimulation of intact endothelial cells (Hu et al., 1993). In diabetes, damage to endothelial cells may reduce the production of ET by this direct action. Others demonstrated that insulin could also increase the release of NO (Steinberg et al., 1994) under physiological conditions leading to a decrease in ET production (Kourembanas et al., 1993). In diabetes, this action of insulin might be altered.

A previous study, using an atherosclerosis model, suggested that ET-1 may inhibit NO by interacting with ET-A receptors (Barton et al., 1998). In the current study, however, the results raise the possibility that in 4 weeks diabetic rats, ET-1 may inhibit NO release via an ET-B mediated mechanism (chapter 1, section 1.8.4 & Fig 1-8). The greater effectiveness of BQ-788 in regaining 85% of the inflammatory response to SP and ES as opposed to regaining 70% of the response to SNP raised the possibility that ET-B receptors may be involved in inhibiting NO release.

In diabetic rats, the response to SP increased from 42% to 63% of control after BQ-123 perfusion (Figs 6-6). This increase could be totally due to improvement in smooth muscle reactivity (68% of control) (Fig 6-5). The increase in diabetic SP response after BQ-788 (86% of control) is partially due to improvement in smooth muscle reactivity in these rats (72% of control). The remaining 14% increase in SP response in diabetic rats may be related to a mechanism involving NO release.
In control rats, both ET receptor antagonists did not alter BBF (Fig 6-1) or responses to SNP (Fig 6-3) or SP (Fig 6-4). However, a significant increase in response to ES (by 62%) after BQ-123 perfusion was previously observed without altering resting blood flow (Khalil et al., 1996), supporting the current results (Figs 6-1 & 6-7). The data, therefore, suggest that under control conditions, preparation of the sciatic nerve for ES experiment involves the release of ETs. That BQ-788 perfusion in diabetic rats increased the vascular response to ES by 88%, versus 62% in control rats, suggests a greater involvement of ET-B receptors in inhibiting the ES response in diabetic rats. ET-B antagonist did not alter any of the inflammatory responses in control animals, suggesting that its involvement in these responses in diabetic rats could not be related to the experimental procedure used.

The results showed BQ-123 and BQ-788 perfusions to be equally potent in normalizing basal PE (R1) in diabetic rats and improving the ability of SP to induce a PE response (Fig 6-12) (Appendix B, Table B-4). The normalization of R1, in particular, suggests that permeability changes in endothelial cells in diabetes are a function of ET-1 acting on both ET-A and ET-B receptors and are reversible up to 4 weeks after induction of diabetes (experimental period). That the PE response to SP was also increased after perfusion of BQ-123 and BQ-788 suggests that the effect of ET is not permanent, as blocking both ET receptors resulted in restoration of endothelial cell permeability to near normal levels (when calculated as percent above basal).

**Related to PKC**

Alterations in PKC activity have been associated with abnormalities in the eye, kidney and, nerves of diabetic animals and patients. Therefore, the role of PKC in modulating skin microvascular blood flow in early diabetes was investigated.

Perfusion of BIM over the blister base of control or diabetic rats did not have any effect on BBF (Fig 6-9). The lack of BIM effect on BBF in 4 weeks diabetic rats suggests that PKC does not play a role in modulating skin BBF in these animals.
BIM perfusion did not have an effect on the vasodilator response to SNP in control rats but improved this response in 4 weeks diabetic rats from 28% to 56% of control (Fig 6-10). This improvement in smooth muscle reactivity supports the hypothesis that free radicals or ET may alter the activity of PKC in early diabetes and contribute to vascular dysfunction (Ha and Kim, 1999; Konishi et al., 1997). The current results suggest that reduction in smooth muscle reactivity in diabetic rats may be partly related to a temporary and reversible effect of ET and PKC on skin microvasculature.

In diabetes there is an established relationship between ET-1, PKC activity, and changes in intracellular calcium concentration. It was suggested that activation of PKC by hyperglycaemia could also enhance ET-1 (Park et al., 2000), which can lead to alterations in the level of intracellular calcium (Masao et al., 1999). Hence, it is possible that increase in PKC activity in 4 weeks diabetic rats could have increased extracellular calcium influx and caused vascular contraction (Abebe and MacLeod, 1990; Legan, 1989) (see also chapter 1, section 1.5.4.1). This effect might have been reduced by BIM and resulted in improvement in SNP response (Fig 6-10).

It was previously reported that enhanced PKC activity in diabetes might be tissue specific. BIM enhanced the SNP response in diabetic rats to 56% of control (Fig 6-10). The lack of improvement in microvascular response to SP after BIM perfusion was somewhat unexpected, particularly in view of the significant improvement in smooth muscle reactivity (Fig 6-11). An independent smooth muscle reactivity response clearly showed that PKC is involved. However, this effect was masked when the response to SP was examined, indicating that the involvement of other factors predominate under the diabetic condition. The exact role of PKC in reducing microvascular blood flow in early diabetes cannot be determined from the experiments undertaken in this chapter and therefore requires further examination.

PKC is also known to be capable of regulating vascular permeability under the diabetic condition or in the presence of high glucose (Wolf et al., 1991) (chapter 1, section 1.8.5.4). The current results showed that $R_1$ in diabetic rats after BIM perfusion was not different from those of non-treated diabetic rats (Fig 6-12). The improvement in the PE
response to SP in these rats after BIM perfusion did not reach statistical significance. This indicates that the temporary effects of PKC do not contribute to disruption in endothelial cell function.

In conclusion, evidence was provided for the involvement of ET in modulating peripheral endothelial and smooth muscle-dependent vasodilatation in 4 weeks diabetic rats. The data suggests that ET plays a role in endothelial cell permeability changes in diabetes. It was demonstrated that PKC does not contribute to disruption in endothelial cell function in 4 weeks diabetic rats. It was also shown that there are subtle differences in the relative contribution of the two ET receptor subtypes (ET-A & ET-B) to early changes in diabetic vascular responses. It was demonstrated that local pharmacological manipulation at the level of skin microcirculation, using ET receptor antagonists (BQ-123 & BQ-788), could normalize these early changes, which could be clinically significant.

CHAPTER 7

Diabetes and modulation of microvascular blood flow:
Role of reactive oxygen species (ROS)
7.1. Introduction

Reactive oxygen species (ROS) are generated naturally in living organisms and they have potential to produce high levels of tissue damage (chapter 1, section 1.5.3 & Fig 1-4). Endothelial-generated ROS (Bonnardel-Phu and Vicaut, 2000a) could modify vascular endothelium by direct and indirect means. These include interference with nitric oxide (NO) availability (Jourd'heuil et al., 1997; Munzel et al., 1997), membrane lipid peroxidation (Hayakawa and Raij, 1999), and formation of advanced glycated end-products (AGEs) (Stehouwer et al., 1997; Schiekofer et al., 2000) (see also chapter 1, section 1.5.3).

ROS are important in physiological activities of smooth muscles and in the pathogenesis of various diseases where the function of smooth muscle is altered (Bauer et al., 1999). Previous studies showed that ROS can contribute to cell dysfunction by interfering with calcium homeostasis (Dreher and Junod, 1995; Suzuki et al., 1991). For example, it was demonstrated that hydroxyl radicals are capable of potentiating the voltage-dependent influx of calcium (Chang et al., 1993). Lipid peroxidation by-products, on the other hand, may interfere with calcium homeostasis by inhibiting key enzymes involved in calcium mobilization (Wells et al., 1997). A different study showed that superoxide anions could contribute to impaired endothelial function by modifying calcium channels (Chang et al., 1993).

The body’s natural antioxidants (e.g. superoxide dismutase (SOD), catalase, glutathione redox enzymes) are capable of neutralizing oxygen free radicals and similar agents (Thomas, 1995). An acute increase in plasma glucose concentration can enhance free radical production (Mullarkey et al., 1990; Traverso et al., 1998) and attenuate antioxidant enzyme activities (Kamata and Kobayashi, 1996; Mak et al., 1996). It was suggested that alterations in free radical / antioxidant balance might be responsible for altered endothelial function and other complications of diabetes (Giugliano et al., 1996; Stehouwer et al., 1997). Also, in diabetes, the efficiency of the body’s antioxidant system, may not only be related to the duration of this disease but also to some post-translational modifications (Kakkar et al., 1996). Hyperglycaemia or insulin deficiency
was also suggested to be partially responsible for an increase in endothelin-1 (ET-1) production in diabetic rats, via a ROS-mediated mechanism (Chen et al., 2000).

N-acetylcysteine (NAC), a powerful hydroxyl radical scavenger (Aruoma et al., 1989), has been widely used in *in vitro* and *in vivo* studies (chapter 1, section 1.5.3). Pretreatment with NAC was shown to protect sensory nerves (Sagara et al., 1996) and prevent endothelial dysfunction (Pieper and Siebeneich, 1998) in streptozotocin (STZ)-induced diabetic rats. Tirilazad mesylate (Freedox), a clinically proven antioxidant, prevents lipid peroxidation and was shown to be cytoprotective by preserving post-injury calcium homeostasis (Saniova, 1997).

No studies to date have investigated the role of free radicals / ROS in modulating skin microvascular blood flow in early diabetes. This chapter examines the quantitative contribution of superoxide anions and hydroxyl radicals and the effect of lipid peroxidation to the documented (see results in chapter 3) reduced microvascular responses in 4 weeks diabetic rats. It was hypothesized that free radicals may be involved in reducing microvascular blood flow in early diabetes and their effects may be inhibited by early intervention. The role of ROS in modulating endothelial (inflammatory) and smooth muscle vascular responses are examined by using three antioxidants: SOD, NAC, and tirilazad mesylate. In addition, the proposal by Chen et al 2000 that hyperglycaemia or insulin deficiency could increase ET-1 production in diabetic rats via a ROS-mediated mechanism, raised the possibility of interaction between ET and free radicals. This possibility is also examined using the combined treatment of BQ-123, an ET-1 antagonist, and SOD, a superoxide anion scavenger.

7.2. Method

A total of 18 groups of rats (n = 4-10) were used in this study, 8 controls and 10 diabetic rats. Control rats were injected with 0.1M sodium citrate buffer solution (CB-treated) (chapter 2, section 2.4.1). Among diabetic rats, 2 groups were pre-treated with tirilazad mesylate (5mg/kg, i.p., every 2nd day) and 2 groups were left untreated.

In peptide perfusion experiments, once the base line was established, first substance P (SP, 1μm, 30 min), then sodium nitroprusside (SNP, 100μM, 10 min), and finally CGRP
(1μm, 10 min) were perfused over the blister base. In ES experiments, after the base line was established, SNP was perfused followed by Ringer’s solution and stimulation of the sciatic nerve (20V, 5Hz, 2msec for 1 minute).

The peptide perfusion and ES experiments were performed on 2 control groups, 2 diabetic groups, and 2 tirilazad mesylate-treated diabetic groups (see diagram below). Other groups of rats were first equally divided between control and diabetics. Then in separate experiments, after the base line was established, SOD (100U/ml), NAC (100μM) or combined BQ-123 (10μM) + SOD (100U/ml) was perfused over the blister base of rats for 10 minutes prior to, and then together with, the inflammatory stimulus (i.e. ES or SP & SNP perfusion). Plasma extravasation (PE) was collected from all perfusion experiments. At the end of each experiment, rats were sacrificed with pentobarbitone overdose.

7.2.1. Expression of data and statistical analyses

Results from vasodilatation responses were calculated as areas under the response curves. PE responses are presented as percent above basal. All data are expressed as mean ± standard error of the mean (SEM). The absolute values and 95% confidence intervals (95% CI) of these values are quoted in the text.

Two-way Analyses of Variances (ANOVA) were performed to investigate whether there were significant differences in basal blood flow (BBF) and responses to SNP, SP, and ES, between control and diabetic rats, according to whether the rats were exposed to or...
treated with any of the following drugs (SOD, NAC, Tirilazad mesylate, BQ-123 + SOD). This test also shows interaction effects between these groups. Where ANOVA indicated a significant main effect, post hoc comparisons were conducted using the Tukey HSD test. A two-way repeated measure ANOVA with priori planned comparisons was performed to investigate whether there were significant differences in PE responses, between control and diabetic rats, according to whether the rats were exposed to or treated with any of the following drugs (SOD, NAC, Tirilazad mesylate, BQ-123 + SOD). To examine other results involving control and diabetic rats, separate independent sample t-tests were performed.

7.3. Results

The BBF in control rats [(25.1 ± 0.9 cm²) (95% CI 23.3-26.9)] did not significantly change after perfusing SOD, NAC or the combined SOD + BQ-123 (Fig 7-1). The results in chapter 6 also showed no change in control BBF after perfusing BQ-123 alone (Chapter 6, Fig 6-1).

In diabetic rats, some improvements in BBF were observed following NAC perfusion [(19.9 ± 1.9 cm²) (95% CI 16.1-23.6)], tirilazad mesylate treatment [(18.3 ± 1.3 cm²) (95% CI 15.7-20.8)], and the combined BQ-123 + SOD perfusion [(19.3 ± 1.4 cm²) (95% CI 16.6-22)] (between 73-79% of control) but not after perfusing SOD [(16.6 ± 1.2 cm²)]
(95% CI 14.2-18.9)] (Fig 7-2). In chapter 6, a partial increase in diabetic BBF was also shown after BQ-123 perfusion [(20.8 ± 2.1 cm²) (95% CI 16.7-24.9)] (83% of control) (Chapter 6, Fig 6-2). The observed increase in diabetic BBF after perfusion or treatment with the above drugs did not reach statistical significance.

\[ \text{Area under the response curve (cm}^2\text{)} \]

**Fig 7-2:** BBFs in diabetic (Diab) rats, diabetic rats after SOD (100U/ml), NAC (100μM), combined [BQ-123 (10μM) + SOD (100U/ml)], and diabetic rats treated with Freedox (Tirilazad mesylate) (5mg/kg/i.p), presented as mean ± SEM (n = 4-10).

In control rats, the vascular response to SNP did not significantly change after perfusing SOD, NAC, the combined BQ-123 + SOD, and BQ-123 (Fig 7-3 & Chapter 6, Fig 6-3).
Fig 7-3: The vascular response to SNP (100μM) in control rats and control rats after SOD (100U/ml), NAC (100μM), and combined [BQ-123 (10μM) + SOD (100U/ml)], presented as mean ± SEM (n = 4-10).

In diabetic rats, the response to SNP [(7.4 ± 0.6 cm²) (95% CI 6.2-8.6)] was significantly improved after SOD and NAC perfusions and treatment with tirilazad mesylate (Fig 7-4).

Fig 7-4: The vascular response to SNP (100μM) in diabetic (Diab) rats, diabetic rats after SOD (100U/ml), NAC (100μM), combined [BQ-123 (10μM) + SOD (100U/ml)], and diabetic rats treated with Freedox (Tirilazad mesylate) (5mg/kg/i.p), presented as mean ± SEM (n = 4-10).

* Denotes significance from diabetic rats (P<0.05)
+ Denotes significance from diabetic rats after SOD (P<0.05)
# Denotes significance from diabetic rats after NAC (P<0.05)
They reached 76%, 81%, and 90% of control response [(26.1 ± 1.8 cm²) (95% CI 22.6-29.6)] respectively. SNP responses were (19.9 ± 3.3 cm²) (95% CI 13.4-26.4) after SOD perfusion, (21.2 ± 1.2 cm²) (95% CI 18.8-23.6) after NAC perfusion, and (23.5 ± 1.7 cm²) (95% CI 20.2-26.8) after tirilazad mesylate treatment. The combined BQ-123 + SOD perfusion normalized this response [(29.1 ± 1.7 cm²) (95% CI 25.8-32.4)] in diabetic rats. This response was also significantly improved after perfusing BQ-123 alone in these animals [(17.9 ± 2.1 cm²) (95% CI 13.8-22)] (Chapter 6, Fig 6-5).

In control rats, the vascular response to SP did not significantly change after perfusing SOD, NAC, combined BQ-123 + SOD, and BQ-123 (Fig 7-5 & Chapter 6, Fig 6-4). However, in diabetic rats, the vascular response to SP [(21.6 ± 3.4 cm²) (95% CI 14.9-28.3)] showed a slight improvement after SOD perfusion (from 42% to 50% of control), reaching (25.9 ± 2.6 cm²) (95% CI 20.8-31) (Fig 7-6).

![Graph](attachment:image.png)

**Fig 7-5:** The vascular response to SP (1μM) in control rats and control rats after SOD (100U/ml), NAC (100μM), and combined BQ-123 (10μM) + SOD (100U/ml), presented as mean ± SEM (n = 4-10).
On the other hand, the combined SOD + BQ-123 perfusion managed to normalize this response [(49.9 ± 4.5 cm²) (95% CI 41.1-58.7)]. The vascular response to SP in diabetic rats was partially increased by BQ-123 perfusion alone [(32.4 ± 2.7 cm²) (95%CI 27.1-37.7)], reaching 63% of control SP (Chapter 6, Fig 6-6). Other substances significantly improved the SP response in diabetic rats but tirilazad mesylate treatment showed more potency [(45.1 ± 2.3 cm²) (95% CI 40.6-49.6)] than NAC [(40.7 ± 1.8 cm²) (95% CI 37.2-44.2)]. These responses reached 88% and 79% of control SP [(51.4 ± 2.9 cm²) (95% CI 45.7-57.1)] respectively (Fig 7-6).

The vascular response to CGRP in diabetic rats [(10.4 ± 1.3 cm²) (95% CI 7.8-12.9)] was significantly reduced, reaching 42% of control response [(24.8 ± 4.6 cm²) (95% CI 15.8-33.8)]. This response was normalized [(29.2 ± 4.6 cm²) (95% CI 20.2-38.2)] after tirilazad mesylate treatment (Fig 7-7).
The vascular response to ES in control rats \([(17.6 \pm 1.3 \text{ cm}^2) (95\% \text{ CI 15-20.1})]\) was enhanced by all antioxidants, reaching \((22.2 \pm 1.9 \text{ cm}^2) (95\% \text{ CI 18.5-25.9})\) after SOD and \((19.6 \pm 2.9 \text{ cm}^2) (95\% \text{ CI 13.9-25.3})\) after NAC perfusions (126\% & 111\% of control respectively). However, the increase in this response after perfusing the combined BQ-123 + SOD perfusion \([(26.4 \pm 3.1 \text{ cm}^2) (95\% \text{ CI 20.3-32.5})]\) and BQ-123 alone \([(28.5 \pm 2.5 \text{ cm}^2) (95\% \text{ CI 23.6-33.4})]\) reached statistical significance (150\% & 162\% of control response respectively) (Fig 7-8 & Chapter 6, Fig 6-7).

The ES response in diabetic rats \([(5.1 \pm 2 \text{ cm}^2) (95\% \text{ CI 1.2-9})]\) was partially improved after perfusing BQ-123 \([(9.6 \pm 2.2 \text{ cm}^2) (95\% \text{ CI 5.3-13.9}) (55\% \text{ of control ES})]\) and SOD \([(12.1 \pm 2.8 \text{ cm}^2) (95\% \text{ CI 6.6-17.8}) (70\% \text{ of control ES})]\) (Chapter 6, Fig 6-8 & Fig 7-9). However, neither of these responses reached statistical significance. The ES response in diabetic rats was normalized after perfusing NAC \([(22.6 \pm 2.7 \text{ cm}^2) (95\% \text{ CI 17.3-27.9})]\), combined BQ-123 + SOD \([(24.6 \pm 3.3 \text{ cm}^2) (95\% \text{ CI 18.1-31.1})]\), and the treatment with tirilazad mesylate \([(21.7 \pm 2.5 \text{ cm}^2) (95\% \text{ CI 16.8-26.6})]\).
**Fig 7-8:** The vascular response to ES (20V, 5 Hz, 2 msec for 1 minute) in control rats and control rats after SOD (100U/ml), NAC (100μM), and combined [BQ-123 (10μM) + SOD (100U/ml)], presented as mean ± SEM (n = 5-10). * Denotes significance from control rats (P<0.05)

**Fig 7-9:** The vascular response to ES (20V, 5 Hz, 2 msec for 1 minute) in diabetic (Diab) rats, diabetic rats after SOD (100U/ml), NAC (100μM), combined [BQ-123 (10μM) + SOD (100U/ml), and diabetic rats treated with Freedox (Tirilazad mesylate) (5mg/kg/i.p), presented as mean ± SEM (n = 5-10). * Denotes significance from diabetic rats (P<0.05)

All the substances used in this study improved basal PE (R₁) in diabetic rats but only the increase in R₁ after perfusing SOD, BQ-123, and the combined SOD + BQ-123 was statistically significant (Appendix B, Table B-5).
The PE response to SP (SP$_2$, 3 & 4) was calculated as percent above basal ± SEM. SEMs were omitted from Fig 7-10 for clarity but quoted in the text. SOD, NAC and tirilazad mesylate improved the PE response in diabetic rats upon SP perfusion. The PE response to SP, after NAC perfusion (10 ± 17%, 49 ± 11%, 54 ± 5%) was similar to those in tirilazad mesylate-treated rats (19 ± 13%, 50 ± 8%, 58 ± 6%) and both were greater than those after SOD perfusion (11 ± 6%, 36 ± 5%, 40 ± 3%) (Fig 7-10). However, BQ-123 and the combined BQ-123 + SOD proved to be more potent and normalized the PE response to SP in diabetic rats (9 ± 23%, 16 ± 32%, 0.1 ± 23%). These responses were (43 ± 15%, 75 ± 13%, 66 ± 11%) and (23 ± 30%, 74 ± 25%, 90 ± 32%) respectively when compared to control (36 ± 19%, 72 ± 18%, 89 ± 14%).

![Figure 7-10](image)

*Fig 7-10:* PE in control rats, diabetic rats, diabetic rats after SOD (100U/ml), NAC (100μM), combined [BQ-123 (10μM) + SOD (100U/ml)], and diabetic rats pre-treated with Freedox (Tirilazad mesylate) (5mg/kg/i.p), presented as percent above basal (n = 4-10).

### 7.4. Discussion

Numerous studies have shown that there is an increase in production and release of free radicals with diabetes and it is believed that they play a role in diabetes-induced vascular complications (chapter 1, section 1.5.3). It was shown that an increase in free radicals could contribute to peripheral nerve and endothelial cell dysfunction (chapter 1, section 1.5.3). Antioxidants have been used extensively in experimental diabetes to reduce or...
reverse the effect of free radicals. This chapter examines the role of ROS in modulating skin microvascular responses in the 4 weeks STZ-diabetic rat model.

Results in chapter 6, showed an improvement in the vasodilatation responses to SNP, SP, and ES in diabetic rats (68%, 63% & 55% of own controls respectively) after perfusing BQ-123 alone. The current study demonstrated an improvement in these responses after perfusing SOD (76%, 50% & 70% of control). It was reported that free radicals increase ET-1 levels in 4 weeks diabetic kidney (Chen et al., 2000). Hence, the combined perfusion of BQ-123 + SOD was used to examine the possible interaction between ET and free radicals in modulating microvascular responses in diabetic rat skin. It should be noted that since BQ-788 and NAC, at the concentrations tested, individually improved SP vasodilation responses in diabetic rats to approximately 80-90% of the control, the combined treatment of these two drugs was not pursued (Fig 7-6 & chapter 6, Fig 6-6).

An increase in diabetic BBF was observed after perfusing NAC, BQ-123 + SOD, and tirilazad mesylate treatment but not after SOD perfusion alone (Fig 7-2). The increase in BBF after NAC perfusion (79% of control) suggests possible involvement of other free radicals other than superoxide anions, such as hydroxyl radicals and hydrogen peroxide in contributing to early changes in microvascular blood flow in diabetes. NAC, a powerful scavenger of hydroxyl radicals, reacts slowly with hydrogen peroxide and does not react with superoxide anions (Aruoma et al., 1989). Lack of increase in diabetic BBF after perfusing SOD is supportive of this view.

In diabetic rats, enhancement in BBF after perfusing the combined BQ-123 + SOD (77% of control) was similar to those after BQ-123 perfusion alone (83% of control) but greater than SOD alone (66% of control) (see also chapter 6). This raised the possibility that ET-1 might have contributed more to the reduction in BBF in 4 weeks diabetic rats than superoxide anions, since the blood flow was improved after blocking ET-A receptors by BQ-123. Based on the results from the Chen et al (2000) study, endogenously or exogenously derived ROS can also enhance ET-1 production in 4 weeks diabetic rats. This may explain the greater increase in diabetic BBF after perfusing the combined BQ-123 + SOD compared to an increase in BBF after SOD perfusion alone. The results
involving tirilazad mesylate treatment suggest that oxidative damage may occur in early diabetes (4 weeks) (Figs 7-2, 7-4, 7-6 & 7-9), which could be inhibited by treatment with tirilazad mesylate leading to an increase in microvascular blood flow.

The vascular response to SNP, a NO donor, was not altered by SOD, NAC, BQ-123 + SOD perfusions in control rats (Fig 7-3). However, these agents and tirilazad mesylate treatment significantly improved SNP responses in diabetic rats to 76%, 82%, 111% and 90% of control respectively (Fig 7-4). This suggests that the reduction in SNP response in diabetic rats (28% of control) might be related to deactivation of NO by free radicals or ETs (Gryglewski et al., 1986).

In diabetic rats, improvement in SNP responses after NAC perfusion and tirilazad mesylate treatment suggests several possible inhibitory roles for free radicals. They may deactivate NO or interfere with the secondary messenger pathway, preventing vasodilatation, or induce contraction of the smooth muscle directly. An example of the former situation could be when hydroxyl radicals oxidize low-density lipoprotein. The product (oxLDL) can selectively inhibit vascular smooth muscle relaxation by decreasing cyclic nucleotides (Galle et al., 1992) and the normalization of the response to CGRP after tirilazad mesylate supports this possibility (Fig 7-7). Another possibility is the direct involvement of hydroxyl radicals in smooth muscle contraction by altering membrane conductance and / or interfering with calcium homeostasis and dependent processes. There is evidence supporting the proposition that hydroxyl radicals and oxLDL can increase cytosolic calcium, possibly through both membrane channels and reticular release (Sasaki and Okabe, 1993; Wells et al., 1997).

In diabetic rats, improvement in the SNP response after SOD perfusion also raised the possibility of NO deactivation by superoxide anions (Diederich et al., 1994; Gryglewski et al., 1986). Superoxide anions also play an important role in lipid peroxidation, a reaction that can interfere with calcium homeostasis (Wells et al., 1997). High levels of superoxide anions can lead to formation of hydroxyl radicals, which are capable of potentiating the voltage-dependent influx of calcium (Chang et al., 1993). This could explain the improvement in the SNP response after NAC perfusion in diabetic rats.
When the SNP response after SOD perfusion was compared to that after perfusing BQ-123 (68% of control), in diabetic rats, it was apparent that superoxide anions and ET-1 similarly contribute to the inhibition of this response. The current results also showed that the combined effect of BQ-123 + SOD in improving vascular responses in diabetic rats is greater than the effect of SOD or BQ-123 alone, which is suggestive of an additive action. Since the response to CGRP, in diabetic rats, was also normalized by antioxidant (Tirilazad mesylate) treatment, it is most probable that deactivation of cyclic nucleotides by ET-1 and superoxide anions play a major role in vascular alterations in early diabetes.

In control rats, results showed no change in SP responses after SOD, NAC, and BQ-123 + SOD perfusions (Fig 7-5) and this is in agreement with a previous study (Khalil et al., 1999). In diabetic rats, however, these agents improved vascular responses to SP (50% & 79% of control response) but only perfusion of the combined BQ-123 + SOD normalized it (Fig 7-6).

Previous studies in our laboratory showed that tirilazad mesylate treatment does not affect responses in normal young animals (Khodr et al., 2001). This treatment however, proved to be the second best agent in improving SP responses in diabetic rats, reaching 88% of control. This could be mainly due to the effect of preventing oxidative damage (Hayakawa and Raij, 1999). It is possible that part of the improvement in SP responses in diabetic rats after tirilazad mesylate treatment is related to preventing NO deactivation.

The results also showed greater improvement in the diabetic inflammatory response to SP after perfusing NAC (79% of control) compared to SOD (50% of control) (Fig 7-6). This suggests that hydroxyl radicals could play a major role in endothelial-mediated vascular responses measured under the current experimental conditions (Pieper and Siebeneich, 1998).

It should be noted that SOD is a large molecule and will only act extracellularly when perfused over the blister base. Therefore, intracellular superoxide anions would not be scavenged with this treatment regime. This may be a reason for the relatively small effects of SOD observed in this study.

Nerve dysfunction in diabetes is associated with increased oxidative stress and the results in this chapter showed improvement in the response to ES in diabetic rats using
antioxidants (Fig 7-9). SOD perfusion increased the ES response in control rats by 26% and in diabetic rats by 40% (from 29% to 69% of control value) (Figs 7-8 & 7-9). It is, therefore, reasonable to propose that at least 26% of the increase in ES response in diabetic rats could be related to scavenging free radicals that are released independent of the diabetic condition (i.e. due to experimental conditions).

Unlike SOD, NAC perfusion did not alter the ES response in control rats but significantly enhanced this response in diabetic rats by 343%. This indicates that the involvement of hydroxyl radicals in inhibiting the ES response in diabetic rats is mainly related to diabetes rather than to the preparation of the nerve for the ES technique or blister induction.

In diabetic rats, the improvement in vascular responses to ES after tirilazad mesylate treatment (123% of control) was similar to that after NAC perfusion (128% of control). This suggests that part of the inhibitory action of hydroxyl radicals on the ES response in these animals might be caused by oxidative damage. It should be noted that since NAC was only perfused for a short period of time, the full potential of this drug in this experiment cannot be extrapolated and therefore a true comparison of the effects of NAC and tirilazad mesylate cannot be made. Lipid peroxidation caused by ROS is known to damage the cell membrane and / or alter its conductance (Saniova, 1997). Tirilazad mesylate can exert its protective property by inhibiting the lipid peroxidation process and preserving the vitamin E content in the cell membrane (Saniova, 1997). Vitamin E has been shown to have neuroprotective properties in diabetes (Cameron and Cotter, 1999).

The normalization of microvascular responses to SNP and SP, in diabetic rats, after perfusing BQ-123 + SOD suggests that ET and superoxide anions may have an additive effect in reducing these responses. In control rats, the response to ES was enhanced after the combined BQ-123 + SOD perfusion (150% of control). The data in chapter 6 showed that BQ-123 perfusion also increased the ES response in control rats (162% of control) without altering BBF. This suggests that under this condition, the technique used to prepare the sciatic nerve (in ES experiments) results in the release of ET, which acts on ET-A receptors to reduce the vascular response. The current results indicate that the ES technique may also be responsible for the release of free radicals such as superoxide
anions. Therefore, superoxide anions and ET (acting on ET-A receptors) released by this technique per se, could have substantiated the effect of the combined BQ-123 and SOD on the ES response in diabetic rats (enhanced by 382%).

In diabetes, ROS were shown to be responsible for an increase in microvascular permeability (Bonnardel-Phu and Vicaut, 2000b). In this study, evidence was provided that ROS increases vascular permeability in STZ-treated rats as early as 4 weeks and this could be inhibited by treatment with antioxidants. Four weeks after induction of diabetes, the basal PE was increased by 135%. This response was also improved after NAC perfusion and tirilazad mesylate treatment (both by approximately 70%). However, while basal PE after SOD perfusion was significantly improved in diabetic rats (by 30%), this was only normalized after the combined perfusion of BQ-123 + SOD. The latter result suggests that permeability changes in endothelial cells are reversible up to 4 weeks of diabetes (experimental period). In chapter 6, the data showed that basal PE was also normalized after BQ-123 perfusion alone in diabetic rats. This suggests that ETs play a greater role than free radicals in increasing vascular permeability. The ability of SP to induce a PE response was also normalized after BQ-123 + SOD perfusion. This suggests that the effect of ET, via ET-A receptors, is not permanent, since blocking this receptor resulted in restoration of endothelial cell permeability to the normal level. Among other antioxidants, tirilazad mesylate treatment showed the greatest potency in improving the PE response, followed by NAC and SOD perfusions. This could be related to the ability of tirilazad mesylate to protect the cell membrane by reducing fluidity (Saniova, 1997).

In conclusion, it was demonstrated that free radicals are involved in modulating peripheral endothelial and smooth muscle-dependent vasodilatation in 4 weeks diabetic rats. In addition, the actions of ROS and ETs in inhibiting microvascular blood flow are additive. The data from this section and those obtained in chapter 6 collectively suggest that ETs play a greater role than free radicals in altering endothelial cell permeability in diabetes. There are subtle differences in the relative contributions of different free radicals and local pharmacological manipulation at the level of the peripheral microcirculation could normalize diabetic vascular responses. The clinical significance of this study is that it raises the possibility that changes in microvascular blood flow and
endothelial cell permeability that occur during early diabetes can be reversed using free radical scavengers and ET antagonists.

CHAPTER 8

Diabetes and modulation of microvascular blood flow: Role of advanced glycated end-products (AGEs) (short- and long-term effects)
8.1. Introduction

The glycation process is a natural biochemical event occurring between aldose sugars and proteins and lipids (chapter 1, section 1.5.2, Fig 1-2). This process occurs at a slow rate under normal ambient sugar concentrations but is enhanced in the diabetic condition (Lalla et al., 1998; Nawroth et al., 1999). AGE-modified proteins are normally either repaired or replaced and degraded *in vivo* by interaction with RAGE, a cell surface receptor for AGES, which is found on many cells (Thornalley, 1998).

Vascular and neuronal abnormalities have been linked to glycated products (Schiekofer et al., 2000). For example, it was postulated that formation of AGES could contribute to the development of a number of diabetic complications (Chibber et al., 1997; Makita et al., 1995; Wautier and Guillausseau, 1998). AGEs formation on the matrix component of the vessel wall can cause structural damage (Bierhaus et al., 1998). Functionally, AGE-RAGE interaction can lead to the generation of a pro-inflammatory environment, causing vascular damage and functional impairment (Lalla et al., 1998; Nawroth et al., 1999). AGEs also induce the expression of endothelin-1 (ET-1), altering endothelial function (Bierhaus et al., 1998; Quehenberger et al., 1995). It has been hypothesized that the interaction of glycated macromolecules with RAGE might inhibit transmembrane calcium signalling in the presence of high glucose (Mene et al., 1999). Also, evidence exists that both soluble RAGE (sRAGE) and the antibody against RAGE (anti-RAGE IgG) can block AGE-RAGE interaction and ultimately prevent related vascular complications (Wautier et al., 1996).

Glycated products can generate free radicals either themselves or by interacting with RAGE and this can lead to endothelial dysfunction (Bucala et al., 1991). In diabetes, AGE-RAGE interaction also causes depletion of the cellular antioxidant defence mechanism (Kashiwagi et al., 1996; Mak et al., 1996).

Aminoguanidine (AG), a hydrazine compound, inhibits ongoing AGE formation but is unable to remove irreversibly bound AGES from connective tissues and matrix compounds that have occurred over time. AG was shown to retard or prevent the development of AGE-related diabetic complications in streptozotocin (STZ)-treated rats (Vlassara et al., 1992). AG, *in vivo*, acts as an antioxidant, quenching hydroxyl radicals
and lipid peroxidation in cells, and preventing oxidant-induced apoptosis (Giardino et al., 1998).

No studies to date have examined the role of AGEs in modulating skin microvascular blood flow in early diabetes. Therefore, in this chapter, short- and long-term effects of amadori and AGEs (early and late products of glycation process) (see chapter 1, section 1.5.2, Fig 1-2 & Appendix C) in modulating skin microvascular blood flow in 4 weeks diabetic rats are examined. It was hypothesized that AGEs may be involved in reducing microvascular blood flow in early diabetes and its effect may be inhibited by early intervention. Microvascular responses in diabetic rats are examined after short-term exposure of their microvasculature to amadori and AGEs and after long-term pre-treatment with AG to prevent the formation of AGEs.

8.2. Method

A total of 14 groups of rats (n = 4-10) were used, of which 9 groups were made diabetic with STZ as described in chapter 2, section 2.4.3. Control rats were injected with 0.1M sodium citrate buffer solution (CB-treated) (chapter 2, section 2.4.1). Four groups of diabetic rats were equally divided and two sets were treated with AG (25mg/kg, i.p.) for a duration of 2 weeks (AG (2W)) and the others were treated for 4 weeks (AG (4W)). The AG treatment was either commenced on day 14 for the duration of 2 weeks (late intervention) or on day 3 (after diabetes was confirmed) for the duration of 4 weeks (early intervention).
In the peptide perfusion protocol, first substance P (SP, 1μM, 30 min), then sodium nitroprusside (SNP, 100μM, 10 min), and finally calcitonin gene-related peptide (CGRP, 1μM, 10 min) were perfused over the blister base. In electrical stimulation (ES) experiments, first SNP (100μM, 10 min) was perfused over the blister base and then the sciatic nerve was stimulated (20V, 5Hz, 2 msec for 1 minute).

Peptide perfusion and ES experiments were performed on 2 control groups, 2 diabetic groups, and 4 AG-treated groups of rats (control and diabetic). In the amadori (1mg/ml) and AGEs (10mg/ml) groups (2 control and 2 diabetic), after establishing the baseline, either of these substances were perfused for 5 minutes followed by 30 minutes incubation where the peristaltic pump was stopped. This was to allow longer exposure of the microvasculature to these substances. Then a further 10 minutes perfusion of these substances was performed. Immediately after that, SP was perfused followed by SNP as described in chapter 2, section 2.6. For the remaining groups (1 control and 1 diabetic), anti-RAGE IgG (100μg/ml) was perfused for 10 minutes prior to and together with SP, followed by Ringer’s solution and SNP. Plasma extravasation (PE) was collected from all perfusion experiments. At the end of each experiment, rats were sacrificed with pentobarbitone overdose.

8.2.1. Expression of data and statistical analyses

Results from vasodilatation responses were calculated as areas under the response curves. PE responses are presented as percent above basal. All data are expressed as mean ± standard error of the mean (SEM). The absolute values and 95% confidence intervals (95% CI) of these values are quoted in the text.

Separate two-way Analyses of Variances (ANOVAs) were performed to investigate whether there were significant differences in basal blood flow (BBF) and responses to SNP, SP, CGRP, and ES, between control and diabetic rats, according to whether the rats were exposed to or treated with any of the following drugs (amadori, AGEs, anti-RAGE IgG, AG (2W), AG (4W)). This test also shows interaction effects between these groups. Where ANOVA indicated a significant main effect, post hoc comparisons were conducted using the Tukey HSD test. Separate two-way repeated measure ANOVA with
priori planned comparisons was performed to investigate whether there were significant differences in PE responses, between control and diabetic rats, according to whether the rats were exposed to or treated with any of the following drugs (amadori, AGEs, anti-RAGE IgG, AG (2W), AG (4W)).

### 8.3. Results

BBFs in control [(25.1 ± 0.9 cm²) (95% CI 23.3-26.9)] or diabetic rats [(16.7 ± 1.3 cm²) (95% CI 14.1-19.2)] did not significantly change after amadori, AGEs, and anti-RAGE IgG perfusions or AG treatments (Figs 8-1 & 8-2).

![Graph showing BBFs in control rats and control rats after amadori (1mg/ml), AGEs (10mg/ml), and anti-RAGE IgG (100μg/ml), presented as mean ± SEM (n = 4-8).](image-url)
Short-term exposure to amadori and AGEs, in control rats, increased vascular responses to SNP and SP. Amadori significantly enhanced the vasodilator responses to SNP and SP, reaching 150% and 173% of own control responses respectively (Table 8-1, Figs 8-3 & 8-4). Although exposure to AGEs also increased these responses in control rats (143% & 162% of own control responses respectively), only the response to SP reached statistical significance. Perfusion of anti-RAGE IgG did not have any effect on SNP or SP responses in control rats.

<table>
<thead>
<tr>
<th>Modulator</th>
<th>Control SNP (cm²)</th>
<th>Control SP (cm²)</th>
<th>Diabetic SNP (cm²)</th>
<th>Diabetic SP (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>(26.1 ± 1.8)</td>
<td>(51.4 ± 2.9)</td>
<td>(7.4 ± 0.6)</td>
<td>(21.6 ± 3.4)</td>
</tr>
<tr>
<td></td>
<td>(95% CI 23-30)</td>
<td>(95% CI 46-57)</td>
<td>(95% CI 6-9)</td>
<td>(95% CI 15-28)</td>
</tr>
<tr>
<td>Amadori</td>
<td>(39.4 ± 4.8)</td>
<td>(89.1 ± 9.4)</td>
<td>(14.3 ± 1.6)</td>
<td>(38.2 ± 5.3)</td>
</tr>
<tr>
<td></td>
<td>(95% CI 30-49)</td>
<td>(95% CI 71-107.5)</td>
<td>(95% CI 11-17)</td>
<td>(95% CI 28-49)</td>
</tr>
<tr>
<td>AGEs</td>
<td>(37.3 ± 5.8)</td>
<td>(83.5 ± 8.8)</td>
<td>(8 ± 2)</td>
<td>(20.05 ± 2.5)</td>
</tr>
<tr>
<td></td>
<td>(95% CI 26-49)</td>
<td>(95% CI 66-101)</td>
<td>(95% CI 4-12)</td>
<td>(95% CI 15-25)</td>
</tr>
<tr>
<td>Anti-RAGE</td>
<td>(26.4 ± 6.3)</td>
<td>(43.1 ± 2)</td>
<td>(29 ± 1.8)</td>
<td>(37 ± 2.6)</td>
</tr>
<tr>
<td></td>
<td>(95% CI 14-39)</td>
<td>(95% CI 39-47)</td>
<td>(95% CI 25.5-32.5)</td>
<td>(95% CI 32-42)</td>
</tr>
</tbody>
</table>

Table 8-1: Vascular responses to SP (1µM) and SNP (100µM) in control and diabetic rats before and after amadori (1mg/ml), AGEs (10mg/ml), and anti-RAGE IgG (100µg/ml) (n = 4-10). Data are presented as mean ± SEM and their 95% CIs.
In diabetic rats, exposure to amadori caused a significant improvement in vasodilation responses to both SNP and SP, reaching 55% and 74% of their own control values (Table 8-1, Figs 8-5 & 8-6). In diabetic rats, AGEs did not have an effect on either SNP or SP
responses, whereas anti-RAGE IgG normalized SNP and improved SP responses to 72% of control response.

**Fig 8-5:** The vascular response to SNP (100μM) in diabetic (Diab) rats, diabetic rats after amadori (1mg/ml), AGEs (10mg/ml), anti-RAGE IgG (100μg/ml), and diabetic rats treated with AG (25mg/kg, i.p) (2 or 4 weeks), presented as mean ± SEM (n = 4-10).

+ Denotes significance from diabetic rats (P<0.05)

X Denotes significance from diabetic rats after amadori (P<0.05)

# Denotes significance from diabetic rats after AGEs (P<0.05)

**Fig 8-6:** The vascular response to SP (1μM) in diabetic (Diab) rats, diabetic rats after amadori (1mg/ml), AGEs (10mg/ml), anti-RAGE IgG (100μg/ml), and diabetic rats treated with AG (25mg/kg, i.p) (2 or 4 weeks), presented as mean ± SEM (n = 4-10).

+ Denotes significance from diabetic rats (P<0.05)

X Denotes significance from diabetic rats after amadori (P<0.05)

# Denotes significance from diabetic rats after AGEs (P<0.05)
Treatment of diabetic rats with AG, after 2 weeks and 4 weeks (late and early onset treatments), significantly improved vasodilatation responses to SNP (both about 80% of control) and SP (both about 70% of control) (Table 8-2, Figs 8-5 & 8-6).

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>Non-treated (4 weeks)</th>
<th>AG-treated (2 weeks)</th>
<th>AG-treated (4 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP</td>
<td>(7.4 ± 0.6 cm²)</td>
<td>(21.4 ± 2.5 cm²)</td>
<td>(20.9 ± 2.6 cm²)</td>
</tr>
<tr>
<td></td>
<td>(95% CI 6-9)</td>
<td>(95% CI 16.5-26)</td>
<td>(95% CI 16-26)</td>
</tr>
<tr>
<td>SP</td>
<td>(21.6 ± 3.4 cm²)</td>
<td>(37.4 ± 3.2 cm²)</td>
<td>(37.6 ± 1.9 cm²)</td>
</tr>
<tr>
<td></td>
<td>(95% CI 15-28)</td>
<td>(95% CI 31-44)</td>
<td>(95% CI 34-41)</td>
</tr>
<tr>
<td>CGRP</td>
<td>(10.4 ± 1.3 cm²)</td>
<td>(22.1 ± 1.5 cm²)</td>
<td>(21.6 ± 3 cm²)</td>
</tr>
<tr>
<td></td>
<td>(95% CI 8-13)</td>
<td>(95% CI 19-25)</td>
<td>(95% CI 16-27.5)</td>
</tr>
<tr>
<td>ES</td>
<td>(5.1 ± 2 cm²)</td>
<td>(12.4 ± 1.3 cm²)</td>
<td>(17.6 ± 2.6 cm²)</td>
</tr>
<tr>
<td></td>
<td>(95% CI 1-9)</td>
<td>(95% CI 10-15)</td>
<td>(95% CI 12.5-23)</td>
</tr>
</tbody>
</table>

**Table 8-2:** *Vascular responses to SNP (100µM), SP (1µM), CGRP (1µM), and ES (20V, 5Hz, 2msec, for 1 minute) in diabetic rats treated with AG (25mg/kg, i.p) (2 or 4 weeks) (n = 4-10). Data are presented as mean ± SEM and their 95% CIs.

The vascular response to CGRP in diabetic rats was improved to nearly 90% of control [(24.8 ± 4.6 cm²) (95% CI 15.8-33.8)] by both AG treatments (Fig 8-7). The vascular response to ES, on the other hand, was improved to 70% of its own control [(17.6 ± 1.3 cm²) (95% CI 15-20.1)] after late onset AG treatment (2 weeks) but normalized after early onset treatment with AG (4 weeks) (Fig 8-8).
**Fig 8-7:** The vascular response to CGRP (1μM) in control, diabetic (Diab), and diabetic rats treated with AG (25mg/kg, i.p) (2 or 4 weeks), presented as mean ± SEM (n = 4-10).

* Denotes significance from control rats (P<0.05)
+ Denotes significance from diabetic rats (P<0.05)

**Fig 8-8:** The vascular response to ES (20V, 5Hz, 2msec for 1 minute) in control, diabetic (Diab), and diabetic rats treated with AG (25mg/kg, i.p) (2 or 4 weeks), presented as mean ± SEM (n = 4-8).

* Denotes significance from control rats (P<0.05)
+ Denotes significance from diabetic rats (P<0.05)

The basal PE (R₁) in diabetic rats was improved after all perfusions but did not reach statistical significance (Appendix B, Table B-6). There was no change in R₁ after AGEs perfusion. On the other hand, R₁ was significantly improved after AG treatments,
decreasing from 235% of control basal value before treatment to 170% after late onset (2 weeks) and 142% after early onset (4 weeks) treatments.

The PE response to SP (SP₂, ₃ & ₄) was calculated as percent above basal ± SEM. SEMs were omitted from Fig 8-9 for clarity but quoted in the text. The most effective treatment was anti-RAGE IgG perfusion, where the PE response to SP showed the greatest increase above basal (64 ± 28%, 98 ± 27%, 86 ± 15%) (Fig 8-9). The second-most effective treatment was early onset treatment with AG, enhancing this response to 52 ± 22%, 86 ± 15%, and 82 ± 17% above basal. These responses were similar to PE response in control rats (36 ± 19%, 72 ± 18%, 89 ± 14%). This indicates that both anti-RAGE IgG perfusion and early onset AG treatment could significantly improve vascular permeability in diabetic rats. Amadori perfusion and late onset AG treatment also enhanced the PE response in diabetic rats but the former perfusion was more effective (30 ± 17%, 45 ± 15%, 48 ± 20%) than the latter treatment (5 ± 16%, 32 ± 18%, 42 ± 22%). The PE response in diabetic rats (9 ± 23%, 16 ± 32%, 0.1 ± 23%) was not altered after AGEs perfusion (13 ± 10%, 20 ± 9%, 4 ± 15%).

**Fig 8-9:** PE in control rats, diabetic rats, diabetic rats after amadori (1mg/ml), AGEs (10mg/ml, anti-RAGE IgG (100µg/ml), and diabetic rats treated with AG (25mg/kg, i.p) (2 or 4 weeks), presented as percent above basal (n = 4-10).
8.4. Discussion

AGEs formation and deposition are much enhanced in diabetes and their presence has been linked to secondary complications, especially microvascular disease (chapter 1, section 1.5.2). The mechanisms through which AGEs exert their effects are complicated and involve many factors, including the duration of the disease. In this chapter, the role of short- and long-term effects of early and late glycated products in modulating inflammatory responses in 4 weeks STZ-induced diabetic rats was examined.

The results showed no change in BBFs in control and diabetic rats after exposure to amadori, AGEs, anti-RAGE IgG or treatments with AG (Figs 8-1 & 8-2). This suggests that in the diabetic condition exogenous or endogenous glycated products do not play a major role in modulating vascular tone.

In control rats, the vascular response to SP was significantly increased after amadori and AGEs perfusions (173% & 162% of control response) (Fig 8-4). This could be partially due to a concomitant increase in smooth muscle reactivity after perfusing these substances in control rats (150% & 143% of control respectively) (Fig 8-3). The release of hydrogen peroxide by amadori (Elgawish et al., 1996; Mossine et al., 1999) may also play a role in the observed increase in the SP response in control rats in the presence of amadori products. Based on the results from a previous study (Boyer et al., 1995), it is possible that hydrogen peroxide may induce endothelial vasodilation independent of calcium and via a specific signal-reponseive phospholipase A2. In addition, a pro-inflammatory environment generated by AGE-RAGE interaction (Lalla et al., 1998) could have contributed to the increase in these responses in control rats. Overall, it appears that control inflammatory responses are enhanced in the presence of amadori and AGEs. It should be mentioned that in control animals, since the antioxidant system is fully functional, superoxide anions generated by amadori (Bucala et al., 1991) could be removed from the system.

In diabetic rats, short-term exposure of the microvasculature to amadori significantly enhanced the vascular response to SNP (55% of control) and SP (74% of control) but no
changes in these responses was observed after perfusing AGEs (Figs 8-5 & 8-6). The increase in these responses after amadori suggests that amadori is capable of modifying vascular responses in diabetic rats. This modification, however, may depend on the duration of diabetes. In early diabetes, more amadori than AGEs may be present producing more superoxide anions and hydrogen peroxide than other free radicals.

A previous study, *in vitro*, showed that smooth muscle cells were damaged after long-term incubation (2 weeks) with early glycated products (Sakata et al., 1998). The authors suggested that the generation of hydrogen peroxide was responsible for this damage. The current results showed an enhancement in the SNP response in diabetic rats after 45 minutes incubation and perfusion of amadori. This suggests that short-term exposure to these products might not have generated hydrogen peroxide in high enough concentration to cause extensive damage to smooth muscle cells.

The lack of AGEs effect on SP and SNP responses in diabetic rats raises the possibility that endogenous AGEs are already involved in modulating these responses and therefore exogenous AGEs, used in this study, may not be capable of further modification. Studies by Rumble et al (Rumble et al., 1997) and Hill et al (Hill and Ege, 1994) support this proposition by showing the presence of AGEs in early diabetes.

Exposure of the microvasculature to anti-RAGE IgG did not change SNP or SP responses in control rats. However, it improved the SP response to 72% of control and normalized the SNP response in diabetic rats. This result provides further support for the proposition that endogenous AGEs are involved in the modulation of microvascular inflammatory responses in diabetic rats. Furthermore, RAGE receptors may play a role in modulating microvascular blood flow in 4 weeks diabetic rats. It is possible that anti-RAGE IgG prevented endogenous AGE-RAGE interaction in diabetic rats and reduced the deleterious effects of endogenous AGEs in the system. For example, anti-RAGE IgG may have improved vasodilatation, in diabetic rats, by preventing NO inhibition directly and indirectly (Bucala et al., 1991; Ceriello, 1999; Galle et al., 1998; Kashiwagi et al., 1996) (see also chapter 1, section 1.5.2).

It should be noted that, in this study, since only a single dose of anti-RAGE IgG was used (100μM/ml, 40 min perfusion over the blister base), this could not reflect the true
contribution of endogenous AGEs to the vascular responses in 4 weeks STZ-treated diabetic rats. Endogenous AGEs true contribution can only be extrapolated from a dose-response effect of anti-RAGE IgG on the vascular response, an experiment that was not performed in the current study. The alternative approach was to examine the long-term role of endogenous AGEs using long-term treatment with AG (late and early onset treatments) to prevent AGEs formation.

A previous study reported that AG prevented vascular defects (hyperpermeability and unresponsiveness to vasodilatory agents) induced by (short-term) AGEs in control rats (Vlassara et al., 1992). In diabetic animals, on the other hand, AG treatment decreased AGEs accumulation in large arteries and improved abnormalities of the diabetic peripheral nerves (Hujiberts et al., 1993; Kihara et al., 1991).

In diabetic rats, late and early onset treatment with AG (2 and 4 weeks), significantly improved vascular responses to SP (both 70% of control) and CGRP (both about 90% of control) (Table 8-2, Figs 8-6 & 8-7). These results could be partially explained by the similar significant enhancement in their smooth muscle-dependent vasodilations after both treatments (both about 80% of control) (Fig 8-5). It is possible that in diabetic rats, AG inhibited AGEs formation and hence protected NO from inactivation by these products. One mechanism through which AG may have exerted this protective effect is by inhibiting lipid peroxidation (Giardino et al., 1998), a process that can cause oxidative stress. The other possible mechanism may be related to inhibition of transmembrane calcium signalling by AGE-RAGE interaction by AG (Mene et al., 1999).

As shown in the results, SP and CGRP responses in diabetic rats were similarly improved regardless of the onset and duration of AG treatments. Early onset AG treatment for 4 weeks did not result in a full recovery of any of the post-terminal vascular responses. This suggests that other factors, apart from AGEs, might be responsible for some of the changes in vascular responses in 4 weeks STZ-treated diabetic rats. For example, the production of substances by AG that have similar action and potency to free radicals may be involved and other studies are supportive of this proposition (Ou and Wolff, 1993; Skamarauskas et al., 1996).

Tilton and co-workers (1993) showed that AG is a potent inhibitor of nitric oxide synthase (NOS) activity. This raises the possibility that AG treatment may inhibit NO
synthesis in diabetic rats. Previous studies, using both healthy and 4 weeks diabetic rats, showed a significant reduction in the vasodilator responses to BK and SP after perfusion of a NOS inhibitor, \(\text{N}^\text{G}\)-nitro-L-arginine-methyl ester (L-NAME) (100\(\mu\)g/ml) (Khalil and Helme, 1992; Ralevic et al., 1992) (see also results in chapter 4). In diabetic rats, vascular responses to BK and SP were also significantly reduced (see results in chapter 4). The current results showed partial enhancement of microvascular responses in diabetic rats after acute AG treatment. These results raised the possibility that an inhibitory action of AG on NOS may have partially masked the effect of AG to increase these responses in diabetic animals through the prevention of AGEs formation.

Improvement in diabetic vascular responses to ES, after both AG treatments (70% of control after late onset and normalization after early onset treatment), could be related to the ability of AG to preserve sensory nerve function in these rats (Fig 8-8). This was supported by Ryle et al (1997) who showed that, in early diabetic rats, low levels of AGEs were present in the sciatic nerve and spinal cord. Defects in axonal transport were also reported in the nerves of short-term diabetic rats (Tomlinson et al., 1988). In addition, a significant increase in tubulin glycation was observed in the rat sciatic nerve after 4 weeks of diabetes (Cullum et al., 1991). Cullum et al suggested that AG may have interfered with the tubulin glycation process and as a result prevented deficits to occur in nerve axonal transport. Results showed a further 30% improvement in the ES response after early onset AG-treated rats, when compared to late onset treated animals, which is independent of the smooth muscle reactivity. This raises the possibility that sensory nerves are sensitive to the damaging effects of AGEs and that early onset AG treatment could protect these nerves from such damage. This is supported by Soulis-Liparota et al (1996), who demonstrated that the duration of AG treatment plays an important role in protecting diabetic tissues against damage.

In chapter 3, it was demonstrated that the vascular permeability of post-capillary venules, in 4 weeks diabetic rats, was altered (Appendix B, Table B-1). Also, there was an increase in basal perfusate (\(R_1\)) and poor SP response in diabetic vessels. In the current study, both \(R_1\) and SP\(_2, 3, \& 4\) were normalized in 4 weeks diabetic rats after anti-RAGE IgG perfusion and early onset AG treatment. Although \(R_1\) was improved more after
amadori perfusion than late onset treatment with AG, the PE response to SP was similar after perfusion of both substances (Appendix B, Table B-6 & Fig 8-9). Anti-RAGE perfusion restored the PE response in 4 weeks diabetic rats. Results after AG treatments and anti-RAGE IgG perfusion suggests that permeability changes in endothelial cells is temporary and reversible up to 4 weeks of diabetes (experimental period) (Vlassara et al., 1992; Wautier et al., 1996). Anti-RAGE IgG may restore diabetic endothelial cell function back to its original form and early onset AG treatment may protect diabetic endothelial cells from functional and structural disturbances exerted by AGEs over the 4 week period of diabetes.

In conclusion, in this chapter evidence was provided that short-term exposure of the microvasculature to amadori products and AGEs could modulate microvascular function in control rats. In addition, amadori enhanced, while exogenous AGEs did not alter, inflammatory vascular responses in early diabetes. It was proposed that early products of the glycation process, namely amadori, could provide a respite for the microvasculature and hence, improve the inflammatory responses in diabetic rats. It was demonstrated that AGEs receptors are involved in modulating the inflammatory responses in diabetic rats. The improvement in vascular responses in early diabetic rats after short-term exposure to anti-RAGE IgG is encouraging. Hence, further investigation is required to establish the possible clinical use of this antibody. In addition, it was suggested that short-term AG treatment can improve the inflammatory responses in 4 weeks diabetic rats and that the duration and onset of treatment can play an important role with reference to the extent of preventing neurovascular damage. The time frame beyond which anti-RAGE IgG and AG treatment may not be capable of reversing diabetic vascular changes also needs to be established.

CHAPTER 9

Hyperglycaemia and changes in microvascular blood flow: Role of insulin
9.1. Introduction

Previous studies in animal models and patients with diabetes have demonstrated that strict glycaemic control, using both short- and long-term insulin treatment, can assist in slowing the progression of diabetes-induced complications (Amthor et al., 1994; Havas, 1999; Tack et al., 1998). Insulin deficiency, in type I diabetes, often leads to neuronal deficits. It was shown that early insulin therapy could prevent alterations in vasoreactivity and endothelial cell function (chapter 1, section 1.8.5.3). It was suggested that these beneficial effects might be related to glycaemic control (Biessels et al., 1996).

Insulin can regulate intracellular calcium levels by influencing the activity of calcium channels and the enzymes involved in vascular smooth muscle cells but high intracellular calcium was shown to impair insulin sensitivity (Cleland et al., 1998; Zemel, 1995). Vasodilatation induced by insulin in skeletal muscle is dependent on Na⁺/K⁺-ATPase at the level of the endothelium (Tack et al., 1996). It was suggested that, in diabetes, alteration in the activity of this enzyme may affect the function of peripheral sensory nerves (Greene and Lattimer, 1983; King et al., 1994; Ver et al., 1997).

Under physiological conditions, insulin is also a modulator of Endothelin-1 (ET-1), ET receptors, ET-1 mediated responses, and a stimulator of nitric oxide (NO) activity (chapter 1, section 1.8.5.3, Fig 1-14). Insulin deficiency or hyperglycaemia could also be partly responsible for the enhanced ET-1 production induced by reactive oxygen species (ROS) (Chen et al., 2000). Previous studies demonstrated that, in diabetes, insulin inhibited lipid peroxidation (Rungby et al., 1992) and advanced glycation process (Turk et al., 1999). Hence, interaction of insulin with ROS, ET-1, NO, and glycation products may be important in the modulation of blood flow in the diabetic condition.

In this chapter, the role of insulin in the modulation of skin microvascular blood flow in early diabetes is investigated. The aim was to examine the relationship between time of commencement and duration of insulin treatment and development of skin microvascular alterations in 4 weeks diabetic rats. It was hypothesized that hyperglycaemia may be involved in reducing microvascular blood flow in early diabetes and its effect may be inhibited by early intervention.
9.2. Method

A total of ten groups of rats (n = 4-10) were used for this study, 2 control groups, 2 diabetic groups (2 weeks), and 6 diabetic groups (4 weeks) post-onset. Control rats were injected with 0.1M sodium citrate buffer solution (CB-treated) (see chapter 2, section 2.4.1). Four diabetic groups (4 weeks) were treated with insulin (2-6IU/100g/day, s.c.) for 2 different periods of time. Half of them were treated for 4 weeks, commencing soon after diabetes onset (early intervention) and the other half were treated for only 2 weeks, commencing at week 2 (late intervention) and continuing until the day of the experiment. The protocol for insulin treatment is described in detail in chapter 2, section 2.5. The remaining two diabetic groups (4 weeks) were not treated (NT).

In experiment 1 (peptide perfusion), first substance P (SP, 1μM, 30 min), then sodium nitroprusside (SNP, 100μM, 10 min), and finally calcitonin gene-related peptide (CGRP, 1μM, 10 min) were perfused over the blister base. In experiment 2 (electrical stimulation (ES) of the sciatic nerve), first SNP (100μM, 10 min) was perfused over the blister base and then the sciatic nerve was stimulated (20V, 5Hz, 2 msec for 1 minute). Plasma extravasation (PE) was collected from all perfusion experiments. At the end of each experiment, rats were sacrificed with pentobarbitone overdose.
9.2.1. Expression of data and statistical analyses

Results from vasodilatation responses were calculated as areas under the response curves. PE responses are presented as percent above basal. All data are expressed as mean ± standard error of the mean (SEM). The absolute values and 95% confidence intervals (95% CI) of these values are quoted in the text.

A two-way Analysis of Variance (ANOVA) was performed to investigate whether there were significant differences in weight gain, basal blood flow (BBF), and responses to SNP, SP, CGRP, and ES, between control, diabetic, and diabetic rats treated with insulin (late and early intervention). Where a significant main effect was detected, post hoc comparisons were conducted using the Tukey HSD test. A two-way repeated measure ANOVA with priori planned comparisons was performed to investigate whether there were significant differences in PE responses between control, diabetic, and diabetic rats treated with insulin (late and early intervention).

9.3. Results

The glucose level in diabetic rats was kept close to the normal range throughout the experimental period, except for the late onset insulin-treated groups that were hyperglycaemic for the first 2 weeks after the disease onset. The average urine glucose level for insulin treated (2 & 4 weeks) groups of diabetic rats is shown in Appendix D. The blood glucose of insulin-treated diabetic rats on the day of the experiment was between 3.0-4.8 mmol/lit.

The BBF in diabetic rats [(16.7 ± 1.3 cm²) (95%CI 14.1-19.2)] was increased after 4 weeks insulin treatment [(21.8 ± 2 cm²) (95%CI 17.9-25.7)], reaching 87% of control response [(25.1 ± 0.9 cm²) (95%CI 23.3-26.9)] (Fig 9-1).
**Fig 9-1:** BBFs in control rats, diabetic (Diab) (2 & 4 weeks) rats, and diabetic rats (4 weeks) treated with insulin (2-6IU/100g/day) (late and early interventions), presented as mean ± SEM (n = 4-10). * Denotes significance from control rats (P< 0.05)

Rats with 4 weeks diabetes showed a significant reduction in weight [(202 ± 6.7g) (95%CI 189-215)] compared to control rats of the same age (approximately 10 weeks) [(398 ± 6.1g) (95%CI 386-410)]. Both insulin treatments increased the average weight of diabetic rats. They increased to (238 ± 6.6 g) (95%CI 225-251) after late insulin intervention and to (300 ± 7.1g) (95%CI 286-313.9) after early insulin intervention, with the latter being more effective (Fig 9-2).
The vascular response to SNP in rats was significantly decreased after 2 weeks [(11.6 ± 1.1 cm²) (95%CI 9.4-13.7)] and 4 weeks of diabetes [(7.4 ± 0.6 cm²) (95%CI 6.2-8.6)] (44% & 28% of control respectively) (Fig 9-3).

**Fig 9-2:** Weigh in control rats, diabetic (Diab) (2 & 4 weeks) rats, and diabetic rats (4 weeks) treated with insulin (2-6IU/100g/day) (late and early interventions), presented as mean ± SEM (n = 4-7).

* Denotes significance from control rats (P<0.05)
# Denotes significance from diabetic rats (both 2 and 4 weeks) (P<0.05)

**Fig 9-3:** Vascular responses to SNP (100μM) in control rats, diabetic (Diab) (2 & 4 weeks) rats, and diabetic rats (4 weeks) treated with insulin (2-6IU/100g/day) (late and early interventions) (n = 4-10).

* Denotes significance from control rats (P<0.05)
+ Denotes significance from diabetic rats (4 weeks) (P<0.05)
This response was significantly improved after rats received late onset insulin treatment [(15.1 ± 0.9 cm²) (95%CI 13.3-16.9)], reaching 58% of control value [(26.1 ± 1.8 cm²) (95%CI 22.6-29.6)]. However, the response to SNP was normalized in diabetic rats that received early onset insulin treatment [(25.4 ± 1.9 cm²) (95%CI 21.7-29.1)]. The vascular response to SP in rats also showed a significant reduction after 2 weeks [(26.2 ± 4.9 cm²) (95%CI 16.6-35.8)] and 4 weeks of diabetes [(21.6 ± 3.4 cm²) (95%CI 14.9-28.3)].

In diabetic rats, late insulin intervention did not improve the response to SP [(19.1 ± 1.5 cm²) (95%CI 16.2-22)] but early insulin intervention normalized it [(57 ± 4.3 cm²) (95%CI 48.6-65.4)] (Fig 9-4).

![Graph showing vascular responses to SP (1μM) in control rats, diabetic (Diab) (2 & 4 weeks) rats, and diabetic rats (4 weeks) treated with insulin (2-6IU/100g/day) (late and early interventions), presented as mean ± SEM (n = 4-10). * Denotes significance from control rats (P<0.05) + Denotes significance from diabetic rats (4 weeks) (P<0.05)]](Fig 9-4)
The CGRP response was reduced in rats after 2 weeks of diabetes \([13.5 \pm 3.1 \text{ cm}^2] (95\% \text{CI 7.4-19.6})\], reaching 54\% of the control value \([24.8 \pm 4.6 \text{ cm}^2] (95\% \text{CI 15.8-33.8})\] (Fig 9-5).

Late onset insulin treatment did not improve this response in diabetic rats \([14.2 \pm 1.5 \text{ cm}^2] (95\% \text{CI 11.3-17.5})\], when compared to non-treated animals \([10.4 \pm 1.3 \text{ cm}^2] (95\% \text{CI 7.8-12.9})\] (Fig 9-5). This lack of improvement in CGRP response was similar to the SP response in diabetic rats after late onset insulin treatment. Once again, early onset insulin treatment normalized the response to CGRP in diabetic rats \([25.5 \pm 4.2 \text{ cm}^2] (95\% \text{CI 17.3-33.7})\] (Fig 9-5).

The response to ES was more reduced in 4 weeks diabetic rats \([5.1 \pm 2 \text{ cm}^2] (95\% \text{CI 1.2-9})\] than 2 weeks diabetic animals \([8.6 \pm 1.4 \text{ cm}^2] (95\% \text{CI 5.8-11.3})\]. Late onset insulin treatment significantly improved the ES response in diabetic rats \([14.3 \pm 1 \text{ cm}^2] (95\% \text{CI 12.3-16.3})\], reaching 81\% of control \([17.6 \pm 1.3 \text{ cm}^2] (95\% \text{CI 15-20.1})\], whereas early onset insulin treatment normalized it \([23.7 \pm 2.2 \text{ cm}^2] (95\% \text{CI 19.4-28})\] (Fig 9-6).
Fig 9-6: Vascular responses to ES (20V, 5 Hz, 2 msec, for 1 minute) in control rats, diabetic (Diab) (2 & 4 weeks) rats, and diabetic rats (4 weeks) treated with insulin (2-6IU/100g/day)(late and early interventions), presented as mean ± SEM (n = 4-8).

* Denotes significance from control rats (P<0.05)
+ Denotes significance from diabetic rats (4 weeks) (P<0.05)
X Denotes significance from diabetic rats (2 weeks) (P<0.05)

The results show that basal PE level (R1) was improved after both insulin treatments but early intervention was more effective (Appendix B, Table B-7). Before any insulin treatment, R1 in 4 weeks diabetic rats was 235% of control. This was decreased to 192% after 2 weeks and 119% after 4 weeks of insulin treatment.

The PE response to SP (SP 2, 3 & 4) was calculated as percent above basal ± SEM. SEMs were omitted from Fig 9-7 for clarity but quoted in the text. The PE response to SP in diabetic rats (9 ± 23%, 16 ± 32%, 0.1 ± 23%) was improved after late onset insulin treatment (23 ± 15%, 27 ± 14%, 41 ± 10%) (Fig 9-7). However, this response was normalized (53 ± 6%, 103 ± 16%, 117 ± 18%) and became relatively higher than control (36 ± 19%, 72 ± 18%, 89 ± 14%) by early onset insulin treatment.
Fig 9-7: PE in control rats, diabetic (Diab)(2 &4 weeks) rats, and diabetic rats (4 weeks) treated with insulin (2-6IU/100g/day) (late and early interventions), presented as percent above basal ± SEM (n = 4-10).

9.4. Discussion

Evidence is accumulating to indicate a link between the adequate control of hyperglycaemia in diabetes and the prevention of development and progression of diabetic vascular complications (chapter 1, section 1.8.5.3). The role of hyperglycaemia in diabetic animals has been examined previously in different tissues (i.e. sciatic nerve and aorta) using a daily insulin treatment regimen (Bennett et al., 1998; Diemel et al., 1992; Turk et al., 1999). Recently, Bennett et al 1999 investigated the role of insulin in cutaneous microvascular responses in diabetic rats (Bennett et al., 1998; Diemel et al., 1992; Turk et al., 1999). There are several differences between Bennett’s study and this study. Two major differences are that in Bennett’s study a lower dose of STZ (50mg/kg, i.p.) was used to induce diabetes in rats and the insulin treatment (4IU/day, using s.c. insulin implants) was commenced 4 weeks after induction of diabetes.

In this study, a higher dose of STZ (75mg/kg) was used to induce diabetes in rats and insulin treatment was introduced from day 1 or two weeks later at a concentration of 2-6IU/100g/day. This study was designed to examine the role of insulin treatment in the modulation of skin microvascular blood flow in early (4 weeks) diabetes.
Late onset insulin treatment (2 weeks) did not improve the BBF in diabetic rats but early onset treatment with insulin (4 weeks) improved BBF from 66% to 87% of control (Fig 9-1). The latter result raises the possibility that early onset insulin treatment may increase NO production and / or prevents its deactivation. A previous study supports this proposition by showing that insulin modulates endothelial nitric oxide synthase (eNOS) expression in microvessels (Kuboki et al., 2000) (see also chapter 1, section 1.8.5.3, Fig 1-14). This treatment may also prevent NO deactivation by ROS. It was shown that adequate control of blood glucose level with insulin treatment is capable of preventing ROS production (Turk et al., 1999) and ROS are known to be able to inactivate both NO and insulin activity (Paolisso and Giugliano, 1996). In addition, a previous study suggested that hyperglycaemia or insulin deficiency might also be partly responsible for the enhanced ET-1 production induced by ROS (Chen et al., 2000) (see also chapter 1, section 1.8.4.1, Fig 1-10).

The difference in weight gain between early and late onset insulin treatment groups reflects poor glycaemic control in the group of animals that received the later treatment (Fig 9-2, Appendix D).

It was demonstrated that late insulin intervention improved the smooth muscle reactivity in diabetic rats to 58% of control response, whereas early onset treatment normalized it (Fig 9-3). An increase in SNP response after both treatments could be partially due to an increase in cyclic guanosine monophosphate (cGMP) via NO mediated stimulation of guanylate cyclase, which is supported by previous studies (Kuboki et al., 2000; Trovati et al., 1996). Other possible mechanisms may be involved in increasing this response. For example, two important factors, namely ROS and advanced glycated end-products (AGEs), were shown to be involved in reducing smooth muscle reactivity in diabetic rats by inactivating NO (Gryglewski et al., 1986; Suzuki et al., 1991). Early onset insulin treatment may prevent the undesired effect of these factors by inhibiting ROS formation and retarding the advanced glycation process (Rungby et al., 1992; Turk et al., 1999) before it occurs. In addition, insulin treatment (<1 hour) might prevent ET-1-induced vasoconstriction by attenuating intracellular calcium. There is evidence showing that insulin reduces both voltage- and receptor- mediated calcium influx and stimulates both
the transcription and activity of Ca\(^{2+}\)-ATPase in vascular smooth muscle cells (Hopfner and Gopalakrishnan, 1999; Zemel, 1995). Early onset insulin treatment may either inhibit excess production of ET-1 or down-regulate endothelin-A (ET-A) receptors on these cells by enhancing subsequent intracellular calcium responses to ET-1 (chapter 1, section 1.8.4.1, Fig 1-11). An increase in messenger ribonucleic acid (mRNA) encoding Na\(^+\)/K\(^+\)-ATPase was observed after short-term insulin treatment (1-6 hrs) of cultured smooth muscle cells (Tirupattur et al., 1993). This may also explain the increase in the SNP response in diabetic rats after insulin treatment in this study.

The absence of any improvement in the vascular response to SP in diabetic rats after late onset insulin treatment (37% of control) was somewhat unexpected, suggesting that 2 weeks diabetes may have caused irreversible endothelial changes in these animals (Fig 9-4). In chapters 6, 7, and 8, however, it was demonstrated that ET-B receptor antagonist (BQ-788), Tirilazad mesylate (Freedox), the combined ET-A receptor antagonist (BQ-123) + superoxide dismutase (SOD), and early onset treatment with aminoguanidine (AG) (4 weeks) can fully reverse the changes in microvascular responses induced by diabetes. This suggests that substances such as free radicals, excess ETs, and AGEs are produced during the first two weeks of diabetes and may well be involved in causing vascular alterations. Hence, it is possible that once these factors are involved, correction of hyperglycaemia per se might not be the most beneficial approach to solve this problem. The best approach is to prevent the action of these factors or to prevent their involvement from the beginning. The current results suggest that early onset insulin treatment can protect microvessels against the effects of these agents and prevent microvascular alterations in early diabetes.

Normalization of SP response after 4 weeks of insulin treatment (Fig 9-4) supports the notion that early onset insulin treatment could prevent microvascular alterations in diabetic rats by inhibiting the effects of ETs, free radicals, and AGEs. For example, protection of smooth muscle cells against the effects of these factors could partially explain the improvement in the SNP response in diabetic rats after 4 weeks treatment with insulin. Protection of the vascular endothelium against the effect of ETs, free radicals, and AGEs and preventing NO deactivation could be other outcomes of this
treatment. The increase in SP response above normal in 4 weeks insulin-treated rats (110% of control) might be due to an enhancement in NO synthesis and cGMP level by insulin treatment (Trovati et al., 1996).

The vascular responses to CGRP in diabetic rats after both early and late onset insulin treatments showed a similar trend to those of SP (Fig 9-5). The normalization of the CGRP response in diabetic rats after early onset insulin treatment, once again, could be partially due to the normalization of the smooth muscle reactivity and partially due to an increase in cyclic adenosine monophosphate (cAMP) by direct insulin action. There is evidence showing that insulin increases the level of cAMP in smooth muscle cells (Trovati et al., 1996).

The vascular response to ES in diabetic rats was improved to 81% of control after late insulin intervention but was normalized after early insulin intervention (Fig 9-6). Part of the improvement in ES response after either insulin treatments could be related to the improvement in the smooth muscle reactivity in these animals. The greater ES response after 4 weeks insulin treatment may be due to prevention of nerve ischaemia over time partially by effective glycaemic control and this is supported by previous studies (Biessels et al., 1996; Stevens et al., 1994).

In chapter 7, the ES response in control rats after perfusing SOD was increased to 126% of control. It was suggested that the 26% increase in response above control could be related to the ES technique and possible release of superoxide anions (Bassirat and Khalil, 2000). In this study, the ES technique could also have caused the release of free radicals such as superoxide anions. However, the early onset insulin treatment may protect the antioxidant system in diabetic rats and encourage efficient removal of free radicals and hence improvement in the ES response (135% of control).

Insulin plays a role in regulating vascular permeability in diabetic rats. The PE results showed that 2 weeks treatment with insulin improved the R₁ in diabetic rats and this response was normalized only after 4 weeks insulin treatment (Appendix B, Table B-7). This suggests that effective insulin treatment could restore PE towards normal in diabetic...
rats, which is supported by a previous study (Bennett et al., 1998). The PE response to SP (SP 2, 3 & 4) in late onset insulin treated diabetic rats showed some improvements (Fig 9-7). This suggests that after 2 weeks of diabetes some other factors such as ETs, free radicals, and AGEs have control over endothelial function and that correcting for hyperglycaemia per se is not sufficient to restore normal endothelial function. In support of this proposition, the PE response to SP in early onset insulin treated diabetic rats was improved to well above normal levels [(53%, 103%, 117%) compared to those in control rats (36%, 72%, 89%)]. This improvement in response may be due to excess NO release as a result of insulin’s direct effect on sensory nerves (Diemel et al., 1992).

Reversal of STZ effects through maintenance of near normoglycaemia levels with insulin treatment also suggests that STZ effects are not due to a direct neurotoxic action of STZ further support the results in chapter 3 (Part 1).

In conclusion, this study provided evidence that early onset insulin treatment could have an important role in preventing skin microvascular damage by diabetes. The possibility was raised that adequate control of hyperglycaemia at the early stages of diabetes may reduce the long-term impact on the microvasculature. It was also demonstrated that alteration in diabetic vascular permeability is reversible up to 4 weeks after the disease onset (experimental period).
CHAPTER 10

Wound healing in diabetic rats
10.1. Introduction

The process of wound healing has been described in detail in chapter 1, section 1.9. Unmyelinated afferent nerves with polymodal nociceptors play an important role in initiating the wound repair process (chapter 1, section 1.9.2.1). They mediate both early and late components of the neurogenic inflammatory responses (vasodilatation (VD), plasma extravasation (PE), and immune responses) (Brain, 1997; Eglezos et al., 1991; Khalil and Helme, 1989; Khalil and Helme, 1990b). Among sensory neuropeptides that mediate these effects, substance P (SP) and calcitonin gene-related peptide (CGRP) are held to be the most important. Previous studies in wound healing have proposed a trophic role for SP and CGRP (chapter 1, sections 1.9.2.1 & 1.9.2.2). Sensory peptides are also important for the chemotactic activity of immune cells in skin wounds and for the subsequent availability of certain growth factors essential for healing (Richards et al., 1997).

Poor wound healing in diabetes has been documented (chapter 1, section 1.9.3). Previous studies have suggested that fibroblast proliferation could be affected by lack of growth factors (Doxey et al., 1995), dysfunctional immune cells (Rosenberg, 1990), and high glucose levels (Hehenberger et al., 1999) This in turn could partially explain delayed wound healing in 4 weeks diabetic rats (Ramamurthy et al., 1998).

Nitric oxide (NO) has been shown to play an important role in inflammation and / or wound healing (Willerson, 1995). In 2 weeks diabetic rat skin, the level of wound-induced NO was decreased and this was paralleled with impaired wound reparative collagen deposition (Schaffer et al., 1997).

The plasma level of endothelin-1 (ET-1) in diabetic rats and in normal rats with skin wounds is increased (Makino and Kamata, 1998; Matzuzaki, 1993) and a role for endothelins (ETs) in wound repair processes has been previously reported (Goligorsky et al., 1999).

Insulin-like growth factor (IGF)-1 is a stimulator of glycosaminoglycan (GAG) biosynthesis (Cechowska-Pasko et al., 1999) and is involved in wound healing in diabetic rats (Tsuboi et al., 1995). It was shown that the reduction in IGF-1 and GAG levels in diabetic rat skin could be due to insulin deficiency (Cechowska-Pasko et al., 1996a).
Free radicals have been linked with delayed wound healing in diabetes (Hallberg et al., 1996). It has been suggested that insulin could improve repair processes by increasing the activity of some antioxidants and production of hydrogen peroxide in rat macrophages (Pereira et al., 1995), increasing NO production (Steinberg et al., 1994), and stimulation of NO and ET activities (Cardillo et al., 1999).

It was suggested that aminoguanidine (AG) could prevent the inhibitory effect of diabetes on angiogenesis and granulation tissue formation (Teixeira et al., 1999). In diabetes, advanced glycated end-products (AGEs) could disregulate cytokine production and increase tissue destruction (Lalla et al., 1998). After tissue has been damaged, AG may prevent changes induced by the glycation process and AGEs, which include alterations to macrophage phenotype and the impairment of cellular and molecular communications (Iacopino, 1995).

Previous studies have focused mainly on histological and morphological changes associated with poor wound healing, while this study is focusing on the mechanisms underlying changes in wound repair process in early diabetes. Chapter 10 was designed based on the information obtained in previous chapters. So far, results from the previous chapters showed that certain treatment protocols could prevent the deleterious effects of hyperglycaemia, free radicals, AGEs, and ETs on skin microvasculature in 4 weeks diabetic rats. In the previous chapters insulin, Tirilazad mesylate (Freedox), AG, and the combination of ET-A receptor antagonist (BQ-123) and a superoxide anion scavenger (superoxide dismutase (SOD) were shown to be effective in improving microvascular blood flow in diabetic rats. This current chapter examines the effect of those treatments on tissue repair processes involved in full-thickness skin wounds in early diabetes. It was hypothesized that since microvascular blood flow was improved by these treatments, wound repair will be also improved in these animals.
10.2. Method

6 groups of rats were used (n = 5-6), one control, one diabetic, and 4 drug treated diabetic groups. Control rats were treated with 0.1M sodium citrate buffer solution (CB-treated) at the same time as other groups were treated with streptozotocin (STZ) (chapter 2, sections 2.4.1 & 2.4.3).

Diabetes was induced in 5 groups of rats and after three days, when the onset of the disease was confirmed, drug treatments were commenced in 3 of those groups (groups 3, 4, & 5). The control group and one of the diabetic groups (groups 1 & 2) received no further treatments (NT). On day 28, a thermal wound was induced on the back of the neck of all rats (see chapter 2, section 2.11.1). These wounds were monitored in all groups until they were fully contracted (healed). Rats in groups 3, 4, and 5 continued to receive their treatments (started on day 3) until wounds were healed. Rats in group 6 received five days treatment starting on day 28 when the wound was induced. The last treatment (5th) for this group was on day 32 (see chapter 2, section 2.11.2).

Group 1: Control, no further treatment (NT)
Group 2: Diabetic, no further treatment (NT)
Group 3: Diabetic, treated with insulin
Group 4: Diabetic, treated with AG
Group 5: Diabetic, treated with Tirilazad mesylate (Freedox)
Group 6: Diabetic, treated with PEG-SOD (100U/ml) + BQ-123 (100μM)

Induction of thermal wound and measurement of healing are described in chapter 2, sections 2.11.1 and 2.11.3.
10.2.1. Expression of data and statistical analyses

Wound sizes were traced and then calculated as areas (in cm$^2$) using a digital planimeter. These values are expressed in the text as mean ± standard error of the mean (SEM).

For statistical analysis, first a general two-way (group x day) repeated measures Analysis of Variance (ANOVA) with a priori planned contrasts was performed to investigate whether there were any significant group (control and diabetic) or treatment (insulin, AG, Tirilazad mesylate, BQ-123 + SOD) differences. Then results were divided into 3 sets of data, based on the information related to different stages of wound healing. The first stage of the tissue repair process, the inflammatory stage, could occur during the first 2 days after wound induction. This is followed by the proliferation and wound contraction stage, between days 2-7. Finally, events leading to tissue remodelling and full wound closure could occur between days 7-17.

For the first (days 1 & 2 of all groups) and second sets of data (days 2 to 7 of all groups) a repeated measure ANOVA was performed. Where a significant main effect was detected, post hoc comparisons were conducted using the Tukey HSD test. For the third set of data (days 7 to 18 of all groups), first the average of every two days (days 7-9, 9-11, etc) were calculated and then a repeated measure ANOVA was performed. Where a significant main effect was detected, again, the Tukey HSD test was used to perform post hoc comparisons. The average of every two days was calculated for the third set of data, as it was necessary to reduce the total number of results so they can be analyzed by the statistical computer software used in this study.

In addition, to examine the overall rate of healing between groups, a one-way ANOVA was performed. Where a significant main effect was detected, post hoc comparisons were performed using the Tukey HSD test. The department of Maths and Statistics at the University of Melbourne was consulted for the best way of analyzing these data and the following results were obtained and presented based on their advice.
10.3. Results

General observations
The time required for complete healing was monitored in 6 groups of rats: control, diabetic (NT), and 4 diabetic treated groups. Due to the complexity of the statistical analyses of the results, graphical representation of the significant differences was not shown on the graphs in this chapter. Therefore, in each results section, the first paragraph describes the statistically significant differences between treated, non-treated, and control groups, based on the time required for full wound closure. Significant differences between each treatment on individual days are also described in the results. Figure 10-1 represents the general pattern of wound healing in these animals.

None of the treatments alter blood glucose level except in group 3 where insulin treatment was used to control hyperglycaemia. In group 3 the average blood glucose level was around 2.5mmol/lit and in other groups it was above 27mmol/lit during the experimental period (approximately 7 weeks). In control rats (group 1) the number of
days for complete wound closure (14 ± 0.4 days) was significantly shorter than non-treated diabetic rats (group 2) (18 ± 0.2 days) (Fig 10-1). In insulin-treated diabetic rats (group 3) the healing time was similar to AG-treated diabetic animals (group 4) (16 ± 0.3 & 16 ± 0.2 days respectively) and these were not significantly different to group 2. In tirilazad mesylate-treated diabetic rats (group 5) wounds were fully closed after 13 ± 0.2 days, which was similar to those in BQ-123 + SOD-treated diabetic rats (group 6) (13 ± 0.3 days). The time required for full wound contraction in both groups 5 and 6 was significantly reduced compared to group 2.

On day 1, there was no significant difference between average wound sizes of all groups. All rats showed an early increase in wound sizes, which reached its maximum by day 2, followed by a general sharp reduction in size after that day. All treated rats showed similarity in their wound sizes especially on day 7. Based on the knowledge related to different stages of wound healing, the results were divided into 3 separate sets of data as mentioned earlier.

**Wound healing in normal and non-treated diabetic rats (Groups 1 & 2)**

The time required for full wound closure in control rats (14 ± 0.4 days) was significantly shorter than non-treated diabetic animals (18 ± 0.2 days) (Fig 10-2).
Fig 10-2: Wound healing profile in control and diabetic (Diab) rats (both non-treated), presented as mean ± SEM (n = 6).

On day 2, the average wound size in control rats [(4.95 ± 0.1 cm²) (95% CI 4.75-5.15)] was significantly bigger than non-treated diabetic rats [(4.05 ± 0.3 cm²) (95% CI 3.5-4.6)].

Generally, control rats showed steeper healing profiles when compared to non-treated diabetic rats. In group 1, overall wounds were reduced more in size (by 2.5 cm²) between days 2 and 7 compared to group 2 (by 1.3 cm²). However, the average wound sizes on individual days were not significantly different, except on day 2.

After day 6, wounds in control rats continued to decrease until full closure was achieved (on day 14). The average wound size in control rats was significantly reduced (by 40%) between day 7 [(2.5 ± 0.05 cm²) (95% CI 2.4-2.6)] and day 9 [(1.45 ± 0.1 cm²) (95% CI 1.25-1.65)]. Wound contraction in non-treated diabetic rats was slow between days 2-6 and after day 14.
Wound healing in insulin-treated diabetic rats (group 3)

The time required for complete wound closure in insulin-treated diabetic rats (16 ± 0.3 days) was not significantly different from non-treated diabetic (18 ± 0.2 days) or control animals (14 ± 0.4 days) (Fig 10-3).

![Graph showing wound healing profile]

**Fig 10-3:** Wound healing profile in control, diabetic (Diab), and insulin (2-6IU/100g)-treated diabetic rats, presented as mean ± SEM (n = 5).

In group 3, an increase in average wound size from day 1 [(4.1 ± 0.3 cm²) (95% CI 3.5-4.7)] to day 2 [(4.5 ± 0.2 cm²) (95% CI 4.1-4.9)] was not significant. This increase in size was more in control (by 0.55 cm²) and less in non-treated diabetic animals (by 0.15 cm²).

Between days 2-7, wounds in diabetic animals treated with insulin were reduced more in size (by 2.1cm²) than those in group 2 (non-treated diabetic rats) (by 1.3cm²) but less than those in group 1 (control) (by 2.5 cm²). The average wound size in insulin-treated rats on day 3 [(3.1 ± 0.3 cm²) (95% CI 2.3-3.7)] and 4 [(3.2 ± 0.2 cm²) (95% CI 2.8-3.6)] were smaller than their counterparts in non-treated diabetic rats [(3.8 ± 0.2 cm²) (95% CI 3.2-4.4) and (3.4 ± 0.2 cm²) (95% CI 3-3.8) respectively]. These results did not reach statistical significance. However, on days 3 and 4, the wounds in insulin-treated rats were significantly smaller than controls.
There was a significant reduction in wound size in group 3 animals between days 9 \((2 \pm 0.1 \text{ cm}^2) (95\% 1.8-2.2)\) and 11 \((1 \pm 0.1 \text{ cm}^2) (95\% \text{ CI } 0.8-1.2)\). The time for full wound contraction was reduced in insulin-treated animals from day 12 onwards.

**Wound healing in AG-treated diabetic rats (group 4)**

The time required for complete wound closure in AG-treated diabetic rats \((16 \pm 0.2 \text{ days})\) was similar to the insulin-treated group (group 3) \((16 \pm 0.3 \text{ days})\) and was not significantly different from control \((14 \pm 0.4 \text{ days})\) or non-treated diabetic group \((18 \pm 0.2 \text{ days})\) (Fig 10-4).

![Graph showing wound healing profile](image)

**Fig 10-4:** Wound healing profile in control, diabetic (Diab), and AG (25mg/kg)-treated diabetic rats, presented as mean ± SEM \((n = 6)\).

The increase in wound size in group 4 animals, between days 1 and 2 (by 0.1 cm²), was similar to non-treated diabetic rats (by 0.15 cm²).

The reduction in wound size, between days 2 and 7, in AG-treated rats (by 1.9 cm²) was also greater and faster than the reduction in group 2 (non-treated diabetic rats) (by 1.3 cm²). Compared to non-treated diabetic rats, group 4 animals showed smaller wound sizes on days 3 \([(3.5 \pm 0.2 \text{ cm}^2) (95\% \text{ CI } 3.1-3.9)]\) and 4 \([(3.2 \pm 0.3 \text{ cm}^2) (95\% \text{ CI } 2.6-]
3.8), which again did not reach statistical significance. These wounds, on the other hand, were significantly smaller than those in control animals on days 3 and 4.

The profile of healing in group 4 animals, between days 5-12, resembled control rats. The time required for full wound contraction was also reduced in these rats after day 12.

**Wound healing in Tirilazad mesylate (Freedox)-treated diabetic rats (group 5)**

The time required for full wound contraction in tirilazad mesylate-treated diabetic rats (13 ± 0.2 days) was shorter than control (14 ± 0.2 days), insulin-treated, and AG-treated diabetic rats. This required time was significantly shorter in tirilazad mesylate-treated group when compared to non-treated diabetic group (18 ± 0.2 days) (Fig 10-5).

![Wound healing profile in control, diabetic (Diab), and Freedox (Tirilazad mesylate) (5 mg/kg)-treated diabetic rats, presented as mean ± SEM (n = 6).](image)

**Fig 10-5:** Wound healing profile in control, diabetic (Diab), and Freedox (Tirilazad mesylate) (5 mg/kg)-treated diabetic rats, presented as mean ± SEM (n = 6).

On day 2, there was no significant difference between average wound size in this group [(4.2 ± 0.2 cm²) (95% CI 3.8-4.6)] and group 1 (control animals) [(4.4 ± 0.1 cm²) (95% CI 4.2-4.6)] and group 2 (non-treated diabetic rats) [(3.9 ± 0.1 cm²) (95% CI 3.7-4.1)]. However, from day 1 to day 2, wounds in group 5 animals increased more in size (by 0.4 cm²) than group 2 rats (by 0.15 cm²).
Between days 2-7, wounds in diabetic animals treated with tirilazad mesylate were reduced more in size (by 2.25cm²) than those in group 2 (non-treated diabetic rats) (by 1.3cm²). In group 5, wounds on day 3 [(3.4 ± 0.2 cm²) (95% CI 3-3.8)] and 4 [(3.1 ± 0.2 cm²) (95% CI 2.7-3.5)] were similar in size to those in group 2 but were significantly smaller than those in group 1 rats.

The profile of healing in group 5 animals, between days 5-9, resembled control animals. Tirilazad mesylate-treated diabetic rats showed a significant reduction in wound size between day 7 [(2.35 ± 0.2 cm²) (95% CI 2-2.7)] and day 9 [(1.4 ± 0.1 cm²) (95% CI 1.2-1.6)]. The time required for full wound contraction in these rats was also reduced after day 11.

Wound healing in BQ-123 and SOD-treated diabetic rats (group 6)

The time required for full wound contraction in (BQ-123 + SOD)-treated diabetic rats (13 ± 0.3 days) was similar to tirilazad mesylate-treated animals (group 5) (13 ± 0.2 days) and shorter than other groups. However, compared to that in non-treated diabetic rats (group 2) (18 ± 0.2 days) this required time was significance reduced (Fig 10-6).

![Graph showing wound healing profile](image)

**Fig 10-6:** Wound healing profile in control, diabetic (Diab), and combined (BQ-123 + SOD) (100μM + 100U/ml)- treated diabetic rats, presented as mean ± SEM (n = 6).
Wounds in group 6 animals increased the most in size (by 0.75 cm²) from day 1 [(4 ± 0.2 cm²) (95% CI 3.6-4.4)] to day 2 [(4.75 ± 0.1 cm²) (95% CI 4.55-4.95)], compared to other groups and reached statistical significance. The wound size on day 2 in group 6, however, was not significantly different to those in group 1 (control animals) [(4.95 ± 0.1 cm²) (95% CI 4.75-5.15)] and group 2 (non-treated diabetic rats) (4.05 ± 0.3 cm²) (95% CI 3.5-4.6)].

Between days 2-7, the extent of wound contraction in BQ123 + SOD-treated diabetic rats was similar to control animals. Wounds were reduced by 2.35 cm² in size in group 6, during this period, which was similar to controls (by 2.5 cm²). In group 6 rats, the average wound size on days 3 [(3.3 ± 0.3 cm²) (95% CI 2.7-3.9)] and 4 [(2.85 ± 0.2 cm²) (95% CI 2.45-3.2)] were again smaller than those in non-treated diabetic animals but still significantly different from those in control rats. The profile of healing in BQ123 + SOD-treated diabetic rats, between days 5-9, resembled control animals.

Group 6 animals also showed a significant reduction in their wound size between days 7-11 (by 1.9 cm²). On day 9, wounds were significantly smaller [(1.46 ± 0.1 cm²) (95% CI 1.3-1.65)] than those on day 7 [(2.42 ± 0.1 cm²) (95% CI 2.2-2.6)]. However, by day 11, wounds were even more reduced in size reaching only (0.55 ± 0.1 cm²) (95% CI 0.35-0.75). Group 6 also showed a significantly reduction in wound size after day 11.
Brief comparison of wound healing between diabetic treated groups (groups 3, 4, 5, 6)

The number of days required for full wound closure in groups 5 (Tirilazad mesylate-treated rats) and 6 ((BQ-123 + SOD)-treated rats) was greater than 3 (insulin-treated animals) and 4 (AG-treated rats) (Fig 10-7).

![Graph showing wound healing profile](image)

**Fig 10-7:** Wound healing profile in diabetic (Diab) rats treated with insulin, AG, Freedox (Tirilazad mesylate), and combined (BQ-123 + SOD) (same concentrations as before), presented as mean ± SEM (n = 5-6).

From day 1 to day 2, group 6 animals showed the most increase in wound size (by 0.75 cm²). This was followed by groups 5 and 3, both showing 0.4 cm² increase in their wound sizes, and finally group 4 with the least wound expansion (by 0.1 cm²). Between days 2-7, wounds in group 6 animals contracted the most (by 2.35 cm²) and in shorter period of time than any other treated diabetic group. Overall, the extent of wound contraction in group 5 was similar to group 3 and 4. Wounds were reduced in group 5 by 2.25 cm² during this period, which was similar to those in group 3 (by 2.1 cm²) and 4 (by 1.9 cm²).

Between days 7-9, groups 6 and 5 both showed a significant reduction in wound size (by 1 cm² and 0.94 cm² respectively), which resembled control in size (by 1 cm²) and the time
required for full wound contraction. Wounds on days 9-11 were also significantly reduced in groups 3 (by 1 cm$^2$) and 6 (by 0.9 cm$^2$). The wound contraction was significantly less after day 12 in groups 3 and 4, and after day 11 in groups 5 and 6.

10.4. Discussion

Wound repair is a complex process that involves inflammation, granulation, and tissue remodeling. Inflammation is a protective response, which initiates the process of tissue repair and intact unmyelinated primary afferent sensory nerves play a major role in this process (chapter 1, section 1.9.2). It has been documented that diabetic individuals have poor wound healing, among other complications (Nathan, 1993; Schaffer et al., 1997).

The focus of this study was to examine the mechanisms underlying poor tissue repair in early diabetes, an area that has not been previously explored. In earlier chapters, the involvement of hyperglycaemia, AGEs, reactive oxygen species (ROS), and ET in reducing inflammatory responses in early diabetes was established. It was demonstrated that treatment with insulin, AG, tirilazad mesylate, and perfusion of the combined BQ-123 + SOD over the blister base can improve these responses in 4 weeks diabetic rats. In this chapter the role of hyperglycaemia, AGEs, ROS, and ET in the repair of full-thickness skin wounds in diabetic condition was examined using the above agents. The changes in wound size were monitored over time until full wound closure occurred.

Full-thickness wounds, including the thermal burn wound, are known to heal by re-epithelialization and contraction (Champion et al., 1970). It should be noted that full-thickness burn wounds in humans could be complicated by excessive connective tissue remodeling and permanent scar formation (Wood and Bladon, 1985). In this chapter, wound size and time for complete wound closure were measured following a standard thermal injury (chapter 2, section 2.11). The change in wound size, as monitored by the open wound area, was used as the outcome measure of wound healing. It is believed that these measurements, documenting the change in surface wound area over time, could be representative of two aspects of wound healing, namely, re-epithelialization and contraction. However, because quantitative histological and immunohistochemical techniques were not applied in this study, it is conceded that assessment of wound size as
reported here might not exactly reflect the true extent of wound re-epithelialization and contraction (Khalil and Helme, 1990b).

The results demonstrated that, in general, diabetic animals do not show a significant wound expansion on day 2 (Fig 10-1). Within hours of skin injury, there is an acute (non-proliferative) inflammation characterized by vasodilatation, oedema, and neutrophil and mononuclear cell extravascular accumulation (coupled with death and dissolution of previously injured cells and extracellular fibrils) (Wood and Bladon, 1985). Shortly after, a band of polymorphonuclear cells forms while epidermal cells (within 24hrs) start to migrate from the margin of the wound towards the injured area. Delayed wound expansion in diabetic rats may be due to a delayed response to injury and impaired functioning of the immune cells in diabetes, which is supported by previous studies on polymorphonuclear leucocytes (PMNs) and fibroblasts (Hehenberger et al., 1999; Rosenberg, 1990).

In diabetes, impaired sensory C-fibres could alter the production and / or release of inflammatory mediators (e.g. SP, CGRP) (Garrett et al., 1996; Nemeth et al., 1999a; Rittenhouse et al., 1996a). It was previously reported that in diabetic rats, inflammatory responses are reduced (Nemeth et al., 1999b) and the results in chapter 3 are in agreement with this report. Hence, it is possible that the delay in response to injury in diabetic rats is related to a reduction in inflammatory responses and sensory neuropeptides. This is supported by previous studies showing that sensory neuropeptides are important in vasodilatation (Khalil and Helme, 1990b), plasma extravasation (Khalil and Helme, 1989), skin fibroblasts proliferation (Nilsson et al., 1985), neutrophil migration, and modulation of immune response (Eglezos et al., 1991). The delayed response to injury in diabetes may also be related to the role of SP in facilitating the arrival of important cells to the injured site by increasing vessel permeability and dilatation (Holzer, 1998). A reduction in vascular permeability (Mathison and Davison, 1993), due to a reduced NO synthesis in early diabetic wounds (Schaffer et al., 1997), might inhibit the arrival of important cells and / or substances into the surrounds of the injured site (Shuler et al., 1995). A reduction in blood flow, due to a vasoconstrictive environment created by reduced NO, and the loss of the effects of the endogenous inhibitors of platelet
aggregation and vasoconstriction at the site of endothelial injury (Willerson, 1995) may also contribute to this delay.

The results in this chapter showed a general delay in wound contraction in non-treated diabetic rats when compared to control animals (Fig 10-1), which is in agreement with previous studies (Franzen and Roberg, 1995; Ramamurthy et al., 1998). The data also showed that all wounds in non-treated diabetic rats, between days 2 and 7, contracted by only 1.3 cm² when compared to a contraction of 2.5 cm² in control rats. This period of time could represent the proliferative phase of the repair process. At the re-epithelialization stage, epidermal mitotic activity is increased, just beyond the edge of the wounded zone, producing new cells until the wound is covered with epidermis. New blood vessels also form within the tissue with the aid of growth factors and correct communication between the cells involved. It is possible that in diabetes the reduction in growth factors, such as basic fibroblast growth factor (bFGF), and changes in post terminal-effects mediated by neuropeptides, such as SP, could diminish re-epithelialization and neovascularization and hence reduce wound contraction. This proposition is supported by a previous study showing acceleration in wound healing by bFGF in diabetic mice (Okumura et al., 1996), via a mechanism that involves enhancing angiogenesis and granulation tissue formation.

It has also been suggested that a decrease in neovascularization and less organization at the late inflammatory phase of wound healing in diabetic mice could be due to alterations in leucocyte infiltration and interleukin (IL)-6 levels (Fahey et al., 1991). It is also known that SP can stimulate cytokine production by oesinophils and macrophages (Lotz et al., 1988) as well as being required for the chemotactic activity of monocytes, macrophages, and T-lymphocytes in skin wounds (Richards et al., 1997). These studies raised the possibility that alterations in SP content might not only reduce the production of growth factors required for this stage of wound healing but also interfere with macrophage function. This possibility is supported by the study of Fahey et al 1991.

Changes in SP mediated events in diabetes involving platelet-derived growth factor (PDGF) could lead to a delay in neovascularization. This is supported by previous studies.
showing an initial (first 5 days) increase in PDGF to be absent in wounded tissue of diabetic rats due to a decrease in its cellular production (Doxey et al., 1995). Reduction in NO in early diabetic wounds (Schaffer et al., 1997) could interfere with angiogenesis by effecting endothelial cell migration (Murohara et al., 1999). This may explain how changes in post-terminal events mediated by SP in early diabetes could interfere with angiogenesis. The results in chapter 4 suggested that NO synthesis (by NOS) is not altered in 4 weeks diabetic rats but the release of certain substances during this disease condition may compromise its activity. The reduction in NO activity could ultimately play a role in impaired wound healing in diabetes.

After day 7, wound contraction in diabetic rats was generally slower than control animals. A greater reduction in wound size was observed in control rats between days 7-9 (Fig 10-2). The time for full wound contraction was also significantly reduced in diabetic rats after day 14. The final stage of healing is the remodelling phase, during which the dermis responds to injury with the production of collagen and matrix proteins. In the current study, slow healing and wound contraction after day 7 in diabetic rats suggests that the remodelling phase of the healing process, including production of collagen and matrix proteins, might be compromised in these animals. This proposition is in agreement with previous studies (Ramamurthy et al., 1998; Sato et al., 1999).

Growth factors have a profound influence on extracellular matrix formation (Greenhalgh, 1996). Hence, it is possible that the reduction in the levels of sensory peptides in these animals may indirectly influence all stages of matrix formation. Impaired healing in 4 weeks diabetic rats may also be related to reduced IGF-1 biosynthesis in the skin (Blakytny et al., 2000).

NO is involved in collagen deposition and it was reported that this stage of healing is compromised in diabetes (Schaffer et al., 1997). The results in chapter 4 suggest that, in early diabetes, the activity of NO is affected. These raise the possibility that slow healing and wound contraction in diabetic rats, after day 7, might be related to impaired collagen deposition, due to inactivation of NO by the released substances in the diabetic condition.
**Role of insulin**

From day 1 to 2, wounds in group 3 (insulin-treated animals) increased more in size (by 0.4 cm²) than group 2 (non-treated diabetics) (by 0.15 cm²). A significant wound contraction (by 2.1 cm²) was also observed in insulin-treated diabetic rats between days 2-7 (Fig 10-3). This suggests that insulin treatment could play a role in improving the inflammatory phase of the tissue repair process (e.g. VD & PE). The results in chapter 9, showing normalization of the vascular responses to SP, CGRP, ES and the basal PE response (R1) in early onset insulin treated diabetic rats, is supportive of this notion.

The vasodilation ability of insulin has been previously documented (Baron, 1999). It was also shown that insulin could increase NO production (Steinberg et al., 1994) by regulating endothelial nitric oxide synthase (eNOS) gene expression (Kuboki et al., 2000). Insulin could also cause vasodilation by increasing the production of hydrogen peroxide in rat macrophages (Pereira et al., 1995). Hydrogen peroxide was previously shown to have vasodilator ability (Boyer et al., 1995). Furthermore, a daily insulin treatment regime similar to that used in this study was shown to be able to prevent alterations in vasoreactivity and endothelial cell function in 4 weeks diabetic rats (Stevens et al., 1993). All these effects could create a vasorelaxant environment at the site of injury in diabetic rats. Insulin treatment can restore neurogenic oedema towards normal (Bennett et al., 1998) and this may improve the transport of inflammatory / immune cells to the injured site and improve the response to tissue injury in diabetic rats.

The number of days required for complete wound closure in insulin-treated diabetic rats (group 3) (16 ± 0.3 days) was shorter than non-treated diabetic rats (group 2) (18 ± 0.2 days) (Fig 10-3). Also, the level of blood glucose measured daily in 4 weeks diabetic rats, during the experimental period, indicated that the insulin treatment managed to keep the blood glucose level close to the normal range (2-5 mmol/lit) in these animals. One possible explanation for increased wound contraction in these animals could be related to the level of glucose. Insulin treatment and a near normal level of glucose in rats may improve fibroblast proliferation (Hehenberger et al., 1999) by influencing cell surface integrins (Wertheimer et al., 1998). There is evidence showing that an increase in NO by insulin (Steinberg et al., 1994) could improve angiogenesis by supporting endothelial cell migration via an integrin-dependent mechanism. Insulin was also shown to be able to
increase the activity of the antioxidant system (Cu-Zn-SOD, catalase, glutathione peroxidase) in diabetic rats (Pereira et al., 1995). An increase in endogenous antioxidants may protect NO against deactivation and, together with an increase in NO production, could improve angiogenesis in insulin-treated animals. The results in chapter 7 showing an improvement in SP response in 4 weeks diabetic rats with antioxidants (e.g. SOD, N-acetylcysteine (NAC), and Tirilazad mesylate) supports the possible involvement of ROS in deactivating NO. Both an increase in fibroblast proliferation and an improvement in angiogenesis may explain the increase in wound contraction on days 2-7 in insulin-treated diabetic rats.

Between days 7-11, full wound contraction in group 3 animals (insulin-treated animals) occurred in a shorter period of time than non-treated diabetic rats but slightly longer than control animals. There was also a similarity between the extent of wound contraction in groups 3 and 6 (combined BQ-123 + SOD-treated animals) between days 9-11 (Figs 10-3 & 10-6). This may be partially related to insulin capable of improving the efficiency of the antioxidant system in diabetic rats and hence protecting NO against deactivation by ROS (Pereira et al., 1995).

It is known that collagen is the only protein susceptible to fragmentation by superoxide anions (Monboisse and Borel, 1992). Therefore, in group 3, part of the improvement in wound repair after day 9 might be related to an increased activity of SOD by insulin (Pereira et al., 1995). An increase in antioxidant activity in general may also reduce levels of hydroxyl radicals, as they are known to denature collagen (Arisawa et al., 1996). Insulin was previously shown to improve matrix formation (Pierre et al., 1998) and reparative collagen deposition in diabetic animals (Schaffer et al., 1997). Insulin may have caused this improvement by increasing GAG biosynthesis (Cechowska-Pasko et al., 1999) and reducing its susceptibility to degradation (Cechowska-Pasko et al., 1996a) by an increase in wound collagenase (Ramamurthy et al., 1998). This may contribute to the faster wound contraction in group 3 (16 days) compared to non-treated animals (18 days) (Fig 10-3).

Overall, the current results suggest that one of the major roles of insulin in improving microvascular blood flow and wound contraction in diabetic rats could be related to the reduction in glucose concentration in these rats.
**Role of AG**

The number of days required for full wound contraction in AG-treated diabetic rats (group 4) was reduced (16 ± 0.2 days) when compared to non-treated diabetic rats (group 2) (18 ± 0.2 days) (Fig 10-4). From day 1 to 2, the increase in wound size in group 4 (by 0.1 cm²) was similar to group 2 animals (by 0.15 cm²). This suggests that other factors, besides AGEs, may be involved, which could exert a greater effect on the inflammatory phase of the tissue repair process in diabetic rats.

Between days 2-7, the reduction in wound size in group 4 (AG-treated animals) (by 1.9 cm²) was greater than those in non-treated diabetics (by 1.3 cm²). Structurally, AGEs are known to decrease vessel elasticity, increase its thickness, increase its rigidity, and narrow the vessel lumen (Bierhaus et al., 1998), which could affect blood flow in diabetic rats. AG may reduce these effects and contribute to improvement in blood flow in diabetic rats by inhibiting AGEs formation. Unmyelinated afferent sensory nerves and their peptides play an important role in initiating the wound repair process (chapter 1, section 1.9.2). Previous studies reported an increase in sciatic nerve tubulin glycation in 2 weeks diabetic rats (Cullum et al., 1991) and a reduction in axonal nerve transport (Tomlinson et al., 1988). AG may preserve sensory nerve function by interfering with the glycation process (involving tubulins) and in turn may influence blood flow and angiogenesis (Teixeira et al., 1999). In chapter 8, evidence was provided that the reduced inflammatory responses (e.g. SP, ES, PE) in 4 weeks diabetic rats can be normalized by early onset AG treatment.

AGEs are also known to affect monocyte and macrophage phenotypes. Monocytes / macrophages are chemotactic to AGEs and are involved in removal of AGEs from the system and replacement of vascular matrix macromolecules that cross-link by exposure to glucose, hence participating in normal tissue turnover (Nawroth et al., 1999). AGEs formation in diabetes may alter the macrophage / monocyte phenotype by disregulating their cytokine production, resulting in increased inflammatory tissue destruction (Grossi and Genco, 1998). Growth factors are known to influence all stages of the healing process, hence any alterations in their levels could cause delayed wound healing (Greenhalgh, 1996). Platelet glycation might be another reason for delayed wound contraction at the early stages of the repair process. In addition, factors released by
platelets have been shown to be capable of stimulating fibroblast contraction (Goto et al., 1998).

Inhibition of AGEs by AG may have also improved the proliferative effect of NO (Hogan et al., 1992) and its vasodilator activity (Bucala et al., 1991). Results in chapter 8 showed a significant improvement in SP responses in 4 weeks diabetic rats after treatment with AG, which supports the above studies.

It was suggested that AG can create a vasorelaxant environment by quenching hydroxyl radicals and inhibiting ET-1 induced by AGEs (Bierhaus et al., 1998). Free radicals produced by AGEs (Taniguchi et al., 1996) might also encourage the ET-1 effect in this situation by deactivating NO activity. Evidence was presented in chapters 6 and 7 that ET-1 and hydroxyl radicals are involved in reducing microvascular blood flow in 4 weeks diabetic rats. Therefore, one can speculate that a possible underlying mechanism for impaired tissue healing in early diabetic rats may involve free radicals and ET-1. AG treatment could reduce the effect of these agents and improve microvascular blood flow and ultimately tissue repair processes, by preventing AGEs formation.

Another study suggested that impaired wound healing in diabetic organisms are due, at least in part, to the enhanced interaction of AGEs with cellular receptors for AGE (RAGE) (Lalla et al., 1998). The data in chapter 8 showed that short-term exposure of diabetic (4 weeks) microvasculature to anti-RAGE IgG could significantly improve (e.g. response to SP) or normalize vasodilatation (e.g. response to SNP). AGE-RAGE interaction not only generates free radicals but also causes a reduction in the cellular antioxidant defence mechanism (Kashiwagi et al., 1996; Mak et al., 1996; Mullarkey et al., 1990). Both of these effects could lead to NO inactivity and in turn, affect blood flow and angiogenesis in diabetic rats (Teixeira et al., 1999). Prevention of AGEs formation by AG may explain the accelerated wound contraction between days 2-7 in 4 weeks diabetic rats treated with AG.

Wound contraction in AG-treated rats between days 5-12 was faster than non-diabetic rats. Alteration in the macrophage / monocyte phenotypes could enhance inflammatory tissue destruction and connective tissue degradation (Grossi and Genco, 1998). Prevention of AGEs formation by AG might reduce this effect and explain the observed accelerated wound contraction in AG-treated rats between those days. AG could also
accelerate protein turnover by making glycated molecules more susceptible to proteolysis (Skamarauskas et al., 1996) and increases granulation tissue formation, a process that is inhibited in diabetes (Teixeira et al., 1999).

ROS can inhibit NO in diabetes (Bucala et al., 1991) and could be generated by AGEs (Taniguchi et al., 1996). Both reduction in NO and presence of ROS in diabetes have been implicated in impairment in collagen deposition (Arisawa et al., 1996; Schaffer et al., 1997). Results in chapter 8 showed that short-term perfusion of anti-RAGE IgG and long-term treatment with AG could significantly improve vasodilatation in early diabetes. Therefore, the possibility was raised that this improvement might be related to the reduction of some deleterious effects of endogenous AGEs. These include inhibition of NO, generation of free radicals, and depletion of antioxidant defence system (Bucala et al., 1991; Ceriello, 1999; Galle et al., 1998; Kashiwagi et al., 1996).

Overall, it can be speculated that the effectiveness of the AG treatment in reducing the time required for full wound closure (16 ± 0.2 days) could be related to inhibiting AGEs formation and protecting the microvasculature against their deleterious effects.

**Role of Tirilazad mesylate (Freedox)**

In tirilazad mesylate-treated animals (group 5) the number of days required for full wound closure (13 ± 0.2 days) was shorter than controls (group 1) (14 ± 0.2 days) (Fig 10-5). In the first two days, wound expansion in group 5 (by 0.4 cm²) showed similarity to those in group 1 (by 0.55 cm²). Between days 2-7, the extent of wound contraction in group 5 was similar to group 1. This might be the result of an improvement in blood flow, endothelial cell migration, and wound reparative collagen deposition induced by tirilazad mesylate treatment. It is known that tirilazad mesylate preserves vitamin E (an antioxidant) content in cell membranes and prevents ROS production in diabetes (Aragno et al., 2000). Both of these could protect cells against damage (cytoprotective property). Furthermore, tirilazad mesylate could improve inflammatory responses by scavenging free radical intermediates to protect NO against deactivation (Hayakawa and Raij, 1999; Munzel et al., 1997; Saniova, 1997). The results in chapter 7 provided further support for this possible role of tirilazad mesylate in 4 weeks diabetic rats.
The similarity between the vascular response to ES in diabetic rats after tirilazad mesylate treatment and after NAC perfusion (results in chapter 7) suggest that part of the inhibitory action of hydroxyl radicals on the ES response could be prevented by tirilazad mesylate treatment. The neuroprotective action of tirilazad mesylate (Cameron and Cotter, 1999) might play a role in improving microvascular blood flow in diabetic rats with skin wounds. It was shown that preventing alterations in sensory nerve function could improve neutrophil migration (Eglezos et al., 1991) and skin fibroblast and endothelial cell proliferation (Ziche et al., 1990). This may explain significant differences in average wound size on days 2 and 7 between tirilazad mesylate-treated diabetic rats and non-treated diabetic rats.

The profile of healing in group 5, between days 5-9, resembled control animals. The significant reduction in wound size that was observed in group 5, between days 7-9, (by 0.94 cm²) was also similar to those in control rats (by 1 cm²). A previous study showed that the reduction in NO levels in 2 weeks diabetic rats skin was paralleled with impaired wound reparative collagen deposition (Schafer et al., 1997). Furthermore, it was suggested that tirilazad mesylate might protect NO against ROS and products of lipid peroxidation (Munzel et al., 1997; Saniova, 1997). This action of tirilazad mesylate might affect collagen deposition in the treated diabetic rats and contribute to an increase in their wound contraction. Tirilazad mesylate might have also protected collagen against the deleterious effects of ROS. This is supported by earlier studies demonstrating collagen denaturation by hydroxyl radicals (Arisawa et al., 1996) and collagen susceptibility to fragmentation by superoxide anions (Monboisse and Borel, 1992). In addition, it has been proposed that tirilazad mesylate inhibits the reaction between products of lipid peroxidation produced in diabetes and GAG (Muller et al., 1986; Pieper et al., 1997a) and improves protein synthesis (e.g. extracellular matrix components). These effects of tirilazad mesylate might also play a role in improving tissue repair in diabetic animals.

Overall, the current results suggest that the major role of tirilazad mesylate in improving microvascular blood flow and wound contraction in diabetic rats could be mainly related to its natural protective property, which is inhibiting oxidative damage.
**Role of BQ-123+SOD**

Results in chapters 6 and 7 showed that the local perfusion of either BQ-123 or SOD over the blister base could improve microvascular blood flow in 4 weeks diabetic rats. However, only the combined perfusion of BQ-123 + SOD normalized all microvascular responses in these animals. It was demonstrated that perfusion of combined BQ-123 + SOD over the blister base caused a greater improvement in microvascular blood flow than tirilazad mesylate treatment (chapter 7).

In this study, the number of days required for full wound closure in group 6 (BQ-123 + SOD-treated rats) (13 ± 0.3 days) was similar to group 5 (tirilazad mesylate-treated rats) (13 ± 0.2 days) (Figs 10-5 & 10-6). These results raise the possibility that both ROS and ETs could play a major in tissue repair processes.

From days 1-2, the combined BQ-123 + SOD treatment normalized wound expansion in diabetic rats. Between days 2-7, wounds in group 6 reduced the most in size (by 2.35 cm²) compared to all other treated groups. This result suggests that the proliferative stage of healing in 4 weeks diabetic rats may be improved. A possible explanation for these effects is best described by examining the roles of individual substances used in this combined treatment. For example, an increase in ET-1 was shown to down-regulate endothelin-B (ET-B) receptors via intracellular calcium immobilization in diabetes (Masao et al., 1999). BQ-123 can block ET-A receptors, thus inhibiting calcium immobilization and vasoconstriction (Goto et al., 1998). Therefore, it is possible that BQ-123 not only prevented vasoconstriction by inhibiting ET-1 activity but also increased vasodilation by blocking ET-A receptors and encouraging NO release by activating ET-B receptors (Fozard and Part, 1992). Evidence was provided in chapter 6 for the involvement of both ET-A and ET-B receptors in diabetes and the alteration in their activity after 4 weeks duration.

It is known that the level of ROS is increased in diabetes and the efficiency of the antioxidant defence system is compromised (Kamata and Kobayashi, 1996; Mak et al., 1996). In group 6, SOD might prevent the chain reaction that leads to further free radical formation in these rats, allowing the antioxidant system to function more efficiently to prevent further tissue damage. This is supported by a previous study showing SOD can neutralize superoxide anions as well as inhibiting formation of other free radicals.
(hydroxyl radical, peroxynitrite) (Thomas, 1995). The effect of BQ-123 + SOD on NO activity might accelerate wound closure by one or all of the following mechanisms: increasing vasodilatation (Ralevic et al., 1992); reducing activity of vasoconstrictors (Stohs, 1995); improving vascular permeability (Shuler et al., 1995); stimulating transmigration of endothelial cells (Goligorsky et al., 1999), stimulating angiogenesis (Murohara et al., 1999). In chapter 6, the involvement of ETs (e.g. ET-1) and free radicals (e.g. superoxide anions) in modulating inflammatory responses (VD & PE) in 4 weeks diabetic rats was demonstrated. Hence, it is possible that ET-1 and superoxide anions inhibit NO activity, altering microvascular blood flow in 4 weeks diabetic rats with skin wounds. The study by Cameron et al (1991) suggested that SOD could affect nerve conduction velocity (Cameron et al., 1991), which may also play a positive role by improving diabetic microvascular blood flow and skin wound repair in the current study. The profile of healing in group 6, between days 5-9, resembled the control group (Fig 10-6). Between days 7-9, the reduction in wound size in group 6 (by 1 cm²) resembled the control group (by 1 cm²) and tirilazad mesylate-treated animals (group 5) (by 0.94 cm²). Between days 9-11, a significant reduction in wound size was also observed in group 6 animals (by 0.9 cm²), which was greater than group 5 (by 0.8 cm²) and far greater than controls (by 0.45 cm²). These results might be explained by the joint effects of combined BQ-123 + SOD in increasing matrix production and reducing its degradation. A previous study showed that ET-1 is involved in extracellular matrix turnover through ET-A receptors (Gutierrez et al., 1996). This raised the possibility that BQ-123, by blocking ET-1 activity, might have improved proteoglycan metabolism. ET-1 is also capable of increasing collagen production (Gutierrez et al., 1996) but this could be inhibited by products of lipid peroxidation (Muller et al., 1986). Free radicals are known to cause lipid peroxidation (Stohs, 1995), generating products that can react with proteins and GAG, thus reducing enzyme activity and protein synthesis (Muller et al., 1986). SOD could play an important role in protecting intracellular matrix components against degradation by free radicals (Monboisse and Borel, 1992). Hence, a possible role for SOD in improving reparative wound collagen deposition by preventing NO deactivation cannot be ignored (Schaffer et al., 1997). Since in the diabetic condition the level of free radicals and ETs and the activity of antioxidants
are altered (Chapter 1, sections 1.5.3. & 1.8.4.1), the above factors might negatively affect the final stage of the tissue repair process and contribute to delayed wound contraction in this study.

Between days 2-7, the similarity in the extent of wound contraction between group 6 and group 5 suggests that ET-1 and free radicals might equally inhibit the proliferative stage of the tissue repair process in 4 weeks diabetic rats. However, between days 7-11, the extent of wound contraction in group 6 was greater than group 5 suggesting that ETs could exert a greater inhibitory effect on the repair process, especially at the later stages.

In conclusion, this study showed that all treatments used have contributed, to varying degrees, to different stages of wound repair and to the overall improvement in repair processes in diabetic rats. The most effective treatments were tirilazad mesylate and the combined BQ-123 + SOD while AG and insulin were the least effective treatments. Insulin and AG improved tissue repair processes that were mainly observed towards the proliferative stage, with only insulin playing a role in the tissue remodelling stage. tirilazad mesylate and the combined BQ-123 + SOD treatments affected both the proliferative and tissue remodelling stages of the repair process. Furthermore, pharmacological manipulation at the level of the microvasculature (e.g. local injection of BQ-123 + SOD) and systemic treatments (e.g. insulin, AG, and Tirilazad mesylate) were shown to improve tissue repair processes in early diabetes. This work raised the exciting possibility that early intervention could prevent microvascular alterations and this could be of clinical importance. It also indicates that further research is needed to determine the impact of early intervention on decelerating late diabetic complications.

CHAPTER 11

General discussion
11.1. General overview and Methodology

Delayed tissue repair is a serious complication of long-term diabetes mellitus. The effect of this disease condition on skin microvasculature has not been widely studied and no clear evidence exists regarding the mechanism(s) involved in the modulation of skin blood flow and tissue repair processes during early diabetes. The research undertaken within this thesis aimed to investigate the effect of early diabetes in modulating microvascular blood flow, directly and indirectly, via modulating sensory nerve activity. The effect of these changes on tissue repair processes in the skin of 4 weeks streptozotocin (STZ)-induced diabetic rats was also examined.

The neurovascular system, comprising of unmyelinated primary afferent sensory neurones and the microvasculature innervated by these nerves both play major roles in modulating the inflammatory and tissue repair processes. Sensory nerve terminals respond to injury via the release of sensory neuropeptides, which mediate inflammation and tissue repair. Diabetes is known to impact on the function of the sensory nerves and skin microvasculature, leading to a reduction in the inflammatory responses (Gamse and Jancso, 1985; Mathison and Davison, 1993) and delay in tissue repair (Fahey et al., 1991; Schaffer et al., 1997).

The primary aim of this research was to examine the involvement of a number of factors, namely, hyperglycaemia, endothelins (ETs), free radicals, and advanced glycated end-products (AGEs) in the changes induced during early diabetes in the skin microvascular blood flow and, ultimately, tissue repair processes. The literature has suggested that these factors play a role in the development of long-term diabetic vascular complications (chapter 1, sections 1.5.2, 1.5.3 & 1.8.4).

Chapter 11 provides an overview of the methodology employed and the methodological limitations encountered, together with the findings from the studies undertaken in this thesis. In addition, directions for future research, including the use of other animal models of diabetes, are proposed.
Chapter 1 presented a review of the literature describing diabetes mellitus and its complications, inflammation, processes of tissue repair, and the relationship between diabetes and repair processes. The mechanisms involved in long-term diabetes complications have also been described in chapter 1. The focus of chapter 1 was to present a review of the current knowledge regarding alterations in the microvasculature and tissue repair processes in the diabetic condition. Review of the literature revealed that information regarding changes in skin microvascular function and the effect of these changes on tissue repair processes, especially in the early stages of diabetes, is very limited and requires further research. Any new information concerning this issue could be of clinical importance and could play a role in devising early intervention approaches to prevent microvascular alterations and decelerate late diabetic complications.

Based on this review, two hypotheses were raised and examined in this study:

First, that factors implicated in long-term diabetic vascular damage could play a role in altering skin microvascular function in early diabetes (4 weeks streptozotocin (STZ)-induced diabetic rats).

Second, that preventing the deleterious effects of these factors could improve skin microvascular blood flow and skin repair processes in early diabetes.

Specifically, the thesis examined the involvement of hyperglycaemia, ETs, free radicals, and AGEs in modulating skin microvascular blood flow and their effects on tissue repair processes in early diabetes.

The second chapter described the general methodology including:

- The induction of diabetes with STZ and alloxan (in Sprague-Dawley rats)
- Methods used to induce an inflammatory response
- Methods used to measure two specific parameters of this response, namely vasodilatation (VD) (using laser Doppler flowmetry) and plasma extravasation (PE)
- Procedures to electrically stimulate sensory C-fibres
- Procedures to induce a full-thickness wound
11.2. Neuronal and local tissue factors modulating microvascular blood flow in early diabetes

According to the literature, the presence of high glucose levels in vivo can alter a number of biochemical processes (Hopfner and Gopalakrishnan, 1999). This in turn may affect the function of sensory nerves, the balance of peptides released from these nerves, and the microvasculature (Cosentino et al., 1997; Guillon et al., 1998; Zochodne et al., 1996). The first empirical study, described in chapter 3 (parts 1 & 2), investigated the effect of diabetes on sensory nerve function as determined by the ability of these nerves to mount an inflammatory response. One of the aims of this chapter was to examine changes in the inflammatory response with time (i.e. to establish a working time frame). Inflammatory responses were induced in STZ-treated animals either chemically, using substance P (SP, 1 μM) and calcitonin gene-related peptide (CGRP, 1 μM) (post-terminal events) (Chapter 3, part 1) or electrically, via selectively stimulating C-fibres (combined pre- and post-terminal events) (chapter 3, part 2). Sodium nitroprusside (SNP, 100 μM), a direct smooth muscle vasodilator, was used to assess changes in smooth muscle reactivity.

Results in (chapter 3) part 1 demonstrated an overall reduction in neurogenic inflammatory responses (VD & PE) over 10 weeks of diabetes, reaching minimum responses 4 weeks after induction of diabetes. In diabetic rats, the basal blood flow (BBF) was reduced, reaching 66% of control BBF after 4 weeks. A significant reduction in vasodilator responses to SP and CGRP (both 42% of own control responses), in 4 weeks diabetic rats, was partly attributed to the significant reduction in smooth muscle reactivity (28% of control response). The reduction in SNP, a nitric oxide (NO) donor, suggests that NO is unable to induce vasodilation. A number of possibilities were raised:

- Deactivation of NO (e.g. by free radicals) (see chapter 7 for further discussion)
- Alterations to the efficiency of the second messenger system by certain factors (e.g. free radicals). Based on the results, the possibility was raised that in early diabetes the cyclic adenosine monophosphate (cAMP) may function more efficiently to compensate for the poor performance by the cyclic guanosine monophosphate (cGMP) system (chapter 1, section 1.5.3)
• Alteration in the intracellular calcium level in smooth muscle due to dysfunction of 
Na\(^+\) / K\(^+\)-ATPase, Ca\(^{2+}\)-stimulated ATPase, Na\(^+\)/Ca\(^{2+}\)-exchanger and Ca\(^{2+}\)-pump in 
the sarcoplasmic membrane (chapter 1, section 1.5.3)
• Reduction in vasodilator effect (e.g. by free radicals and ETs (chapters 6 & 7)

In chapter 3, part 1, the effect of diabetes (4 weeks) on the microvasculature at both 
superficial and deep compartments of the rat skin was examined. For this purpose, two 
different laser Doppler flowmeter (LDF) machines were used, Perimed (PLDF) and Moor 
(MLDF), each with different laser light penetration capabilities (chapter 2, section 2.9). 
Results showed that microvascular blood flow at both superficial and deeper 
compartments of the skin is reduced after 4 weeks of diabetes. The reduction in the 
response to SP was greater using the MLDF compared to the PLDF, which suggests an 
additional involvement of deeper vessels. Furthermore, this data showed that a reduction 
in the microvascular blood flow can be detected after 2 weeks of diabetes and is subject 
to further reduction with time.

The sensory-independent effects of diabetes on the microvasculature were also examined 
in chapter 3, part 1, using sensory denervated (capsaicin pre-treated) animals (with and 
without diabetes) (Chapter 2, section 2.8). Results showed that, in control rats, capsaicin 
treatment alone significantly reduced the inflammatory response to SP (53.8% of control 
response) but in capsaicin pre-treated diabetic rats this response was even further reduced 
(17.6% of control response). The reduction in SP response in capsaicin-treated diabetic 
rats was attributed to the effect of diabetes on the remaining functional sensory C-fibres 
that might not have been destroyed by neonatal capsaicin treatment.

Recent studies have suggested that STZ toxicity might be involved in neuronal cell 
damage (Adeghate and Parvez, 2000; Prickaerts et al., 2000). Therefore, in (chapter 3) 
part 1, the hyperglycaemic effect of diabetes on the microvasculature, independent of 
possible neurotoxic effects of STZ, was also examined via the use of alloxan-induced 
diabetic rats. The suitability of alloxan was based on the knowledge that it can destroy 
pancreatic beta cells in a similar manner to STZ (Thulesen et al., 1997; Yamamoto et al., 
1981) (chapter 1, section 1.6.2 & chapter 2, section 2.4). The changes in microvascular
responses in STZ-treated rats were similar to those in alloxan-treated rats. This suggests that the reduction in these responses in STZ-treated rats is mainly due to the hyperglycaemic effect rather than a possible independent neurotoxic effect of STZ.

In diabetic rats, PE was also increased up to week 4 after induction of diabetes (Appendix B, Table B-1). These results suggest alterations in the permeability of post-capillary venules in 4 weeks diabetic rats.

In chapter 3, part 2, changes in the sensory nerve function with duration of diabetes was examined by selective electrical stimulation (ES) of the unmyelinated C-fibres of the sciatic nerve using specific parameters (20V, 5Hz, 2 msec for 1 minute). Once again, a similar significant reduction in the ES and SNP responses was observed in diabetic rats over time (10 weeks), reaching a maximum at week 4 (both 28% of own control responses). Since the maximum reduction in all inflammatory responses (SP, CGRP, ES) were observed at 4 weeks after induction of diabetes, this time period was chosen as the working time frame for all subsequent experiments in this study.

There are reports showing that in the diabetic condition, sensory neuropeptide content and transport in the sciatic nerve are reduced and the release of these peptides from diabetic tissue is also diminished (chapter 1, section 1.7.3). It was, therefore, important to establish how much of the reduction in inflammatory responses, observed in early diabetic rats, is caused by changes in sensory nerves at the pre-terminal level.

The results suggested that, at 4 weeks of diabetes, the reduction in the inflammatory response to ES is due to a reduction in smooth muscle reactivity (both 28% of own control responses) rather than possible damage to C-fibres affecting its function. Capsaicin and STZ treatments significantly reduced ES responses to 55% and 28.9% of control respectively. However, only a small further reduction in this response was observed when capsaicin pre-treated animals were made diabetic (from 55% to 33.5% of control response). The latter result suggested that the direct effect of diabetes on sensory nerves under the current experimental condition might be minimal since most of these fibres were already destroyed by capsaicin pre-treatment.

The ES response in alloxan-treated rats was less reduced when compared to STZ-treated rats (46% compared to 28.9% of own control responses). This could be due to the
difference in severity of diabetes between these animals (chapter 2, section 2.4.5 & chapter 3, part 2 discussion).

Overall, the results in chapter 3 support the notion that there are disturbances in endothelial cells and smooth muscle cell function in skin microvasculature in early diabetes. It was also demonstrated that post-terminal changes in microvascular function are more apparent than actual pre-terminal changes at this early stage of diabetes.

The second empirical study, described in chapter 4, investigated the role of parasympathetic nerves and local tissue factors in the modulation of microvascular blood flow in early diabetes. To investigate the former, changes in the microvascular response to acetylcholine (ACh), which is released by parasympathetic nerves, was examined in 4 weeks diabetic rats. The function of ACh receptors (nicotinic and muscarinic) was also examined in these animals using two different receptor agonists, nicotine and muscarine chloride. These receptors are located on sensory nerves and endothelial cells respectively.

BBFs in control and diabetic rats in chapter 4 were similar to those in chapter 3. The results showed a significant and similar reduction in the vasodilator responses to ACh, nicotine, and muscarine chloride in diabetic rats (27%, 36% & 34% of own control responses respectively). The similarity in the reduction of these responses suggested the involvement of the following possible mechanisms:

- Deactivation of NO (chapter 7)
- Generation of endothelium-vasoconstrictor prostanoids and alteration in the function of muscarinic receptors (chapter 1, section 1.8.5.1)
- Damage to endothelium and alteration in the function of muscarinic receptors (chapter 1, section 1.8.5.1)

The vasodilator responses to ACh in 4 weeks diabetic rats were measured using both the MLDF and the PLDF. The data suggested that vessels at both superficial and the deeper level of the skin were affected by early diabetes, supporting the results in chapter 3. Since MLDF recorded a greater reduction in the response compared to PLDF, again the possibility of additional involvement of deeper vessels was raised (chapter 4, Table 4-1).
Bradykinin (BK) is an important local tissue factor produced during inflammation and tissue injury. In chapter 4, the role of BK in the modulation of microvascular blood flow in early diabetes was examined. Previous studies have shown that NO, a potent vasodilator, can modulate the action of important peptides such as SP, ACh, BK (Khalil and Helme, 1992; Ralevic et al., 1992; Ralevic et al., 1995b). Therefore, the role of NO in modulating microvascular blood flow in early diabetes was also examined in this chapter, using a NO synthase (NOS) inhibitor, N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME). The aim was to assess the relative contribution of NO to BK and SP responses in 4 weeks diabetic rats.

Results from this part of the study showed a reduction in SP and BK responses (42% & 48% of own control responses) in diabetic rats, which could be partially explained by a decrease in smooth muscle reactivity (30% of control response). In control rats, perfusion of L-NAME significantly decreased the vascular response to BK and SP (65% & 40% of own control responses). In diabetic rats, however, L-NAME similarly reduced SP and BK responses (15% & 20% of own controls). The response to SP in diabetic rats, in the presence or absence of L-NAME, suggested that in these rats NO synthesis is not altered. Therefore, changes in endothelial-mediated responses in diabetic rats could be either related to the concentration of released sensory neurotransmitters or the fate of NO once it is released.

No significant change was observed in diabetic PE after nicotine, muscarine chloride, or BK suggesting that other factors may be involved in altering microvascular permeability in diabetic rats.

Overall, the results in chapter 4 indicated the presence of certain factors in the diabetic condition that could contribute to the reduced microvascular blood flows at the early stages of the disease. The roles of these factors were examined in chapters 6, 7, 8, and 9.

The third empirical study, described in chapter 5, was devoted to investigation of sympathetic nerve involvement in modulating microvascular blood flow in early diabetes. The changes in microvascular responses to vasoactive substances were examined in
diabetic rats treated with a short-term sympathetic neurone blocker, guanethidine monosulphate (Chapter 2, section 2.10).

The BBF in control rats was slightly increased after treatment with guanethidine (111% of control response) but this treatment did not change the BBF in diabetic rats. The former result suggests that sympathetic nerves play a minor role in regulating basal tone under inflammatory conditions. The latter result raised the possibility that certain endothelial-mediated factors may be involved in reducing BBF in early diabetes.

In control rats, no significant change was observed in vascular responses to SNP, SP, or ES after guanethidine treatment. However, in diabetic rats, a significant enhancement was observed in all these responses after this treatment (182%, 185% & 212% of own non-treated diabetics respectively). This suggests that sympathetic efferents play a major role in reducing the above responses in 4 weeks diabetic rats. The reported alterations in the sensitivity of adrenoreceptors in the diabetic condition have been proposed as a possible underlying mechanism.

No significant changes in PE were detected in control or diabetic rats after guanethidine treatment. The latter result suggests that sympathetic efferents are not involved in microvascular permeability changes in early diabetes.

The roles of neuropeptide Y (NPY) and adenosine triphosphate (ATP), both released by the sympathetic nerves, were not examined in this chapter due to a number of factors:

- The chance of NPY and ATP release by low frequency ES used in this study was limited (Pernow, 1988).
- The resting level of NPY is low in most species and the density of its immunoreactive fibres in the skin is unchanged up to 12 weeks after diabetes onset (Karanth et al., 1990; Pernow, 1988).
- In early diabetes (2 & 8 weeks), there is no significant change in ATP concentration or the release of ATP between control and diabetic rats (Belai et al., 1991; Carlsson and Aronqvist, 1981).

Overall, the findings in this chapter demonstrated that the contribution of sympathetic efferents in regulating microvascular blood flow is increased in early diabetes.
11.3. Endothelial-mediated factors modulating microvascular blood flow in early diabetes

Endothelin-1 (ET-1) is a potent vasoconstrictor that is produced by many cells during inflammation and wound repair. Numerous studies have provided evidence that there are alterations in ET-1 levels, ET receptor (e.g. ET-A, ET-B) activities, and vascular responses to this peptide in diabetic animals and humans (chapter 1, section 1.8.4).

The fourth empirical study, described in chapter 6, investigated the role of this potent vasoconstrictor on the modulation of skin microvascular blood flow in early diabetes. The aim was to examine the relative contribution of two ET receptors, ET-A and ET-B, to the observed reduction in microvascular responses in 4 weeks diabetic rats (see results in chapter 3). For this purpose, BQ-123 and BQ-788, ET-A and ET-B receptor antagonists were used.

Results showed an increase in BBF in diabetic rats after BQ-123 perfusion (from 66% to 83% of control response), supporting the results in chapter 5 that endothelial-mediated factors rather than sympathetically-derived neurotransmitters might be involved in reducing diabetic microvascular responses.

The significant improvement in SNP response in 4 weeks diabetic rats (from 28% to 70% of control response), after perfusing BQ-123 and BQ-788, supported a role for ET-1 in reducing smooth muscle reactivity and the following possible mechanisms of action were proposed:

- Inhibition of NO by ET-1 (see results in chapter 6)
- Alterations in the efficiency of the (cGMP) pathway by ET-1-induced enhancement in protein kinase C (PKC) activity (chapter 1, section 1.8.4)

The above results also suggested that, at the level of the microvasculature, ET-A and ET-B receptors were equally involved in inhibiting smooth muscle reactivity.

The ET receptor antagonists used in this study also enhanced the vascular responses to SP and ES in diabetic rats. Perfusion of BQ-123 and BQ-788 improved both responses to SP (63% & 86% of own control responses) and ES (55% & 84% of own control responses)
respectively. These results suggested that ET-B receptors are more involved in inhibiting the above responses than ET-A receptors. The possibility that ET may affect NO activity and/or its release from endothelial cells was raised. It was suggested that damage to endothelial cells by elevated glucose might lead to an increase in ET-1 release and indirect inhibition of NO (chapter 1, section 1.8.4.1, Fig 1-10).

In control rats, PE was not altered after perfusing BQ-123 and BQ-788. However, these agents showed equal potency in improving PE in diabetic rats, suggesting that both ET-A and ET-B receptors are involved in microvascular permeability changes in early diabetes.

The role of PKC in vascular alterations associated with diabetes and a relationship between enhanced ET-1 synthesis, diabetes, and PKC activity have been demonstrated (chapter 1, section 1.8.5.4). However, the role of this enzyme in modulating skin microvascular blood flow in early diabetes has not been previously investigated. The second aim of chapter 6 was to examine the role of this enzyme in 4 weeks diabetic rats, using a specific PKC inhibitor, bisindolylmaleimide (BIM). BIM did not improve the SP response in diabetic rats but significantly enhanced the response to SNP (56% of control response). The enhancement in SNP response, after perfusing BIM in these animals, supports the hypothesis that PKC might be involved in altering the level of intracellular calcium and hence contribute to the reduction in endothelial-mediated vascular responses in 4 weeks diabetic rats. The lack of improvement in the SP response was unexpected, raising the possibility that a pre-terminal event might have masked the involvement of PKC in the modulation of post-terminal events.

Overall, this chapter provided evidence that ETs are involved in altering microvascular blood flow and endothelial cell permeability in early diabetes. It was shown that there are subtle differences in the relative contribution of the two ET receptor subtypes (ET-A & ET-B) to changes in vascular responses in early diabetes. It was also demonstrated that local pharmacological manipulation at the level of peripheral microcirculation, using ET receptor antagonists (BQ-123 & BQ-788), could normalize these early changes. The
exact role of PKC in the modulation of skin blood flow in early diabetes requires further investigation, since the results in this part of the study were inconclusive.

Reactive oxygen species (ROS) are capable of tissue damage and previous studies have shown their involvement in long-term vascular complications associated with diabetes (chapter 1, section 1.5.3). The fifth empirical study, described in chapter 7, investigated the role of ROS in modulating skin microvascular blood flow in early diabetes. The aim was to assess the quantitative contribution of superoxide anions and hydroxyl radicals and the effect of oxidative damage on the observed reduction in microvascular responses in 4 weeks diabetic rats (see results in chapter 3). For this purpose, three antioxidants were used namely, superoxide dismutase (SOD), N-acetylcysteine (NAC), and tirilazad mesylate (Freedox). In order to examine the possible interaction effects between free radicals and ETs, a combination of SOD with an ET receptor antagonist (BQ-123) was also used in this study.

In control rats, perfusion of SOD, NAC, BQ-123 + SOD, and treatment with tirilazad mesylate did not change BBF. In 4 weeks diabetic rats, however, some improvement in BBF was observed using NAC, BQ-123 + SOD, and tirilazad mesylate (from 66% to 79%, 77% & 73% of control BBF respectively) but not SOD alone. These results suggest that hydroxyl radicals and ET-1 contribute more to the reduction in microvascular blood flow in diabetic rats than superoxide anions (chapter 7, Fig 7-2).

These agents did not change the SNP response in control animals. A significant enhancement in smooth muscle reactivity, however, was observed in diabetic rats using SOD, NAC, and tirilazad mesylate (76%, 81% & 90% of control response respectively). This response was normalized after perfusion of the combined BQ-123 + SOD. These improvements suggest that, in early diabetes, ROS may play a role in deactivating NO or reducing the efficiency of its action rather than changing the level of NO production.

Once again, these agents did not significantly change the vascular response to SP in control rats. In diabetic rats, however, the SP response was slightly enhanced after perfusing SOD (from 42% to 50% of control response) but significantly improved after perfusing NAC and tirilazad mesylate-treatment (79% & 88% of control response
respectively). This response was again normalized after perfusing the combined BQ-123 + SOD. Treatment with tirilazad mesylate also normalized the vascular response to CGRP in diabetic rats. The improvement in these responses was suggestive of a greater involvement of hydroxyl radicals, than other ROS, and ET in reducing endothelial-mediated responses in diabetic rats under the current experimental condition.

The following are possible mechanisms that might underlie the effects of free radicals in 4 weeks rats with early diabetes:

- Chemical reaction between superoxide anions and NO could generate peroxynitrite and ultimately lead to the formation of hydroxyl radicals. This could result in deactivation of NO and / or reduction in NO concentration (chapter 1, section 1.5.3).
- Free radicals might interfere with the secondary messenger system pathway (decreasing cyclic nucleotides), thus preventing vasodilatation or inducing smooth muscle contraction by interfering with calcium homeostasis and dependent processes (chapter 1, section 1.5.3)
- The results involving SOD and BQ-123 + SOD in diabetic rats suggested that ETs were more involved than superoxide anions in reducing smooth muscle reactivity. It appears that these agents exert their effect by deactivating NO. Enhanced ET-1 production by ROS (e.g. superoxide anions, hydrogen peroxide) in early diabetic rats and its inhibition by SOD and catalase, was previously reported (chapter 1, section 1.5.3).

It should be noted that since BQ-788 and NAC perfusions, at the concentrations tested, individually improved SP responses to approximately 80-90% of the control value, the combined treatment of these two drugs was not pursued.

In control rats, perfusion of SOD and NAC enhanced the vascular response to ES (126% & 111% of control) and the increase in this response after perfusing the combined BQ-123 + SOD (150% of control response) reached statistical significance. The vascular response to ES, in diabetic rats was improved after perfusing SOD (70% of control response) but normalized after using NAC, tirilazad mesylate, and the combined BQ-123 + SOD. These results suggest that ROS, especially hydroxyl radicals, are involved in microvascular changes in early diabetes. The similarity between ES responses after NAC perfusion and tirilazad mesylate treatment also suggests that part of the inhibitory action
of hydroxyl radicals could be related to oxidative damage. Since NAC was only perfused for a short period of time, the full potential of this drug in this experiment could not be extrapolated and therefore a true comparison of the effects of NAC and tirilazad mesylate could not be made.

The PE results showed the greatest improvement after perfusing combined BQ-123 + SOD, followed by treatment with tirilazad mesylate, and the least improvement was after SOD and NAC perfusions. These results suggest that free radicals are involved in altering endothelial permeability in 4 weeks diabetic rats.

Overall, results of investigations performed within chapter 7 revealed that free radicals play a role in modulating diabetic microvascular blood flow at this early stage (4 weeks post-diabetes). In addition, evidence was provided to suggest that the inhibitory effects of ETs and free radicals on microvascular blood flow were additive. Results in chapter 6 and 7, collectively, showed that ETs play a greater role than free radicals in altering endothelial cell permeability. Using different free radical scavengers, there were subtle differences in the relative contribution of different free radicals in altering microvascular blood flow. The results again showed that local pharmacological manipulation at the level of the peripheral skin microcirculation could normalize diabetic vascular responses.

The glycation process, a natural biochemical event, is enhanced in diabetes and can lead to excess formation of advanced glycated end-products (AGEs). It was postulated that AGEs are involved in the development of vascular damage and abnormalities in microvascular function observed in diabetes (chapter 1, section 1.5.2). The sixth empirical study, described in chapter 8, investigated the role of glycated products in modulating skin microvascular blood flow in early diabetes. The aims of this chapter were:

First, to examine the short- and long-term effects of amadori and AGEs (early and late products of glycation process) in modulating microvascular blood flow in 4 weeks diabetic rats. For this purpose, the diabetic microvasculature was examined after short-term exposure to amadori and AGEs and long-term treatment with aminoguanidine (AG).
Second, to examine the possible involvement of AGE receptors (RAGE) in altering microvascular blood flow in animals with early diabetes, using anti-RAGE antibody (IgG).

BBFs were not significantly changed in control or diabetic rats after amadori, AGEs, and anti-RAGE IgG perfusions, or after AG treatments. The results in diabetic rats suggest that in this disease condition, glycated products do not play a major role in modulating vascular tone.

In control rats, short-term exposure to amadori and AGEs increased vascular responses to SNP and SP. Amadori significantly enhanced the vasodilator responses to SNP and SP, reaching 150% and 173% of own control responses respectively. Although exposure to AGEs also increased these responses in control rats (143% & 162% of own control responses), only the response to SP reached statistical significance. Perfusion of anti-RAGE IgG did not have any effect on SNP or SP in control rats. It appears that under inflammatory conditions, control microvasculature enhances vascular responses in the presence of amadori and AGEs.

In diabetic rats, exposure to amadori enhanced the vasodilator responses to SNP and SP (55% & 74% of own control responses) but no changes in these responses were observed after perfusing AGEs. This increase in responses after amadori suggested that this product is capable of modifying vascular response in diabetic rats and this modification may be time-dependent. The lack of AGEs effect on SP and SNP responses, in diabetic rats, raised the possibility that endogenous AGEs are already involved in modulating these responses and, therefore, exogenous AGEs, used in this study, might not be capable of further modification. This result also suggests that modification of vascular responses by AGEs may depend on the duration of diabetes.

Anti-RAGE IgG did not have any effect on SP or SNP responses in control rats but improved SP responses to 72% of control value and normalized SNP responses in diabetic rats. The latter result not only provided further support for the proposition that AGEs are involved in the modulation of microvascular responses in diabetic rats but also suggest that RAGE may play a role in this modulation. Anti-RAGE IgG could improve vasodilatation by inhibiting AGE-RAGE interaction, which could prevent NO inhibition,
generation of free radicals, and depletion of the antioxidant defence system (chapter 1, sections 1.5.2 & 1.5.3).

It should be noted that since only a single dose of anti-RAGE IgG (100 μM/ml) was used, the results in this study could not reflect the true contribution of endogenous AGEs on the vascular response in 4 weeks diabetic rats. Endogenous AGEs true contribution can only be extrapolated from a dose-response effect of anti-RAGE IgG on the vascular response, an experiment that was not performed in the current study. The alternative approach was to examine the long-term role of endogenous AGEs using long-term treatment with AG (late and early onset treatments) to prevent AGEs.

Results showed that both 2 and 4 weeks (late and early onset) treatments with AG could significantly enhance the response to SP (both around 70% of control response), and CGRP (both around 90% of control response) in diabetic rats. These results could be partially due to enhancement in SNP responses (both about 80% of control response). By inhibiting AGEs formation, AG could improve these responses by protecting NO against deactivation. Early onset treatment with AG did not cause normalization of any of the post-terminal vascular responses in diabetic rats, suggesting that factors other than AGEs may be involved in reducing microvascular responses in early diabetes.

ES responses in diabetic rats were improved to 70% of control response after late onset and normalized after early onset treatment with AG. This may be related to preservation of sensory nerve function by AG and may depend on the duration of diabetes.

Amadori and late onset AG treatment improved only basal PE (R₁), whereas anti-RAGE IgG and early onset AG treatment normalized R₁ and the PE response to SP (SP₂, ₃ & ₄) in diabetic rats. The improvement in PE after anti-RAGE IgG and early onset AG treatment suggests the involvement of AGEs in altering endothelial cell permeability in early diabetes.

Overall, the findings presented in chapter 8 demonstrate that short-term exposure of control microvasculature to early (e.g. amadori) and late (e.g. AGEs) glycation products could modulate vascular function. Furthermore, amadori products exerted a positive enhancing effect on microvascular function in early diabetes. It was proposed that in
these rats, amadori products could provide a respite for the microvasculature and hence, improve the inflammatory response. When anti-RAGE IgG was used, the data showed that AGE receptors are involved in modulating the inflammatory responses in rats with early diabetes and the duration of treatment could play an important role with reference to preventing neurovascular damage.

Human and animal studies have demonstrated that strict glycaemic control, using both short- and long-term insulin treatments, can slow the progression of complications associated with diabetes (chapter 1, section 1.8.5.3). Previous studies also proposed a relationship between insulin and free radicals, ET-1, NO and AGEs (chapter 1, section 1.8.5.3).

The seventh empirical study, described in chapter 9, investigated the role of insulin in the modulation of skin blood flow in early diabetes. The aim was to examine the relationship between the time of commencement and duration of insulin treatment and the development of skin microvascular alterations. For this purpose, diabetic rats received insulin treatment either for 4 weeks, commencing shortly after diabetes onset (early onset insulin treatment) or for 2 weeks, commencing on day 14 after diabetes onset (late onset insulin treatment).

Early onset insulin treatment significantly improved BBF in diabetic rats (from 66% to 87% of control BBF), suggesting that this treatment may protect microvascular damage in diabetes.

Late onset insulin treatment significantly improved SNP responses (58% of control) but did not improve SP or CGRP responses in diabetic rats. Early onset insulin treatment, on the other hand, normalized all these responses in diabetic animals. The general improvement in smooth muscle reactivity in diabetic rats after both early and late onset insulin treatments might be partially due to an increase in cGMP via NO-mediated stimulation of guanylate cyclase (chapter 1, section 1.8.5.3). The partial increase in SNP response after late insulin intervention may be related to the effect of insulin on ET concentration and / or activity. Insulin may inhibit excess ET-1 production or down-regulate ET-A receptors on smooth muscle cells (chapter 1, section 1.8.5.3), preventing...
NO deactivation by ET-1 (see results in chapter 6) or improve the efficiency of the cGMP pathway (chapter 1, section 1.8.4).

The lack of improvement in SP response, in late onset insulin-treated diabetic rats, suggests the involvement of other factors that are affecting microvascular responses independent of the hyperglycaemic effect. Results in chapters 6, 7, and 8 demonstrated that ET receptor antagonists, antioxidants, their combination, and preventing AGEs formation could improve or normalize microvascular responses in 4 weeks diabetic rats. These results raised the possibility that production of substances such as free radicals, ETs, and AGEs might be enhanced during the first 2 weeks of diabetes and alter the microvascular function in these animals. It is possible that early onset insulin treatment could protect diabetic microvessels against the effects of these substances. Normalization of SP responses in diabetic rats using late onset insulin treatment was supportive of this notion. Early insulin intervention might protect NO against inactivation by inhibiting ROS formation and retarding the formation of AGEs and hence causing normalization of SP responses. Normalization of CGRP responses in diabetic rats after early onset insulin treatment could be partially due to an improvement in smooth muscle reactivity and partially due to an increase in cAMP levels in smooth muscles (chapter 1, section 1.8.5.3).

Late onset insulin treatment improved the ES response in diabetic rats to 81% of control response but early onset treatment normalized it. Part of the improvement in ES response could be related to an improvement in the smooth muscle reactivity. Early onset insulin treatment might also prevent nerve ischaemia, partially due to effective glycaemic control over time.

PE was normalized in diabetic rats only after early onset insulin treatment, demonstrating that effective treatment could restore normal endothelial function in diabetic rats (see chapter 1, section 1.8.5.3 & introduction in chapter 9).

Overall, the data presented in this study demonstrated that early onset insulin treatment could have an important role in preventing early skin microvascular alterations caused by diabetes. The possibility was raised that adequate control of hyperglycaemia at the early
stages of diabetes may reduce the long-term impact on the microvascular and tissue repair processes.

11.4. Factors involved in tissue repair process

Sensory nerves, especially unmyelinated afferent nerves, play an important role in initiating the wound repair process by mediating both early and late components of the neurogenic inflammatory responses (VD, PE & modulation of immune responses). It is generally known that in the diabetic condition, sensory nerve function is altered, inflammatory responses are reduced, and wound healing is impaired.

In previous empirical chapters, it was established that certain factors such as ETs, free radicals, AGEs, and hyperglycaemia are involved in modulating skin microvascular blood flow in early diabetes. It was also demonstrated that preventing the deleterious effects of these factors, using specific inhibitors / antagonists, could normalize diabetic vascular responses.

The final empirical study (chapter 10) was designed to examine the effects of certain treatment protocols (local and systemic), used in previous chapters, in modulating the repair of full-thickness skin wounds in 4 weeks diabetic rats. The aim was to establish if these treatment protocols could also improve tissue repair processes involved in full-thickness skin wounds. For this purpose, a thermal wound was induced in a control group and a number of test groups of diabetic rats. These animals then received several different treatments and changes in their wound sizes were monitored over time until full wound closure was achieved (see method in chapter 10).

The results showed that different stages of tissue repair processes were affected in early diabetes. A lack of a significant wound expansion in diabetic rats suggested a delay in response to injury, reflecting an impairment of the first stage of the tissue repair process (i.e. the inflammatory phase). This could be due to an alteration in post-terminal mechanisms, leading to inadequate inflammatory responses in 4 weeks diabetic rats (see results in chapter 3).

Delayed wound contraction, between days 2-7, suggested that the proliferative phase (re-epithelialization, neovascularization, and wound contraction) of the repair process might
also be impaired. Changes in post-terminal effects mediated by sensory neuropeptides such as SP could play an important role in this stage of the repair process. Retarded wound contraction after day 7 in diabetic rats suggested that the final stage of the repair process (i.e. tissue remodelling) could also be compromised. Changes in the action of sensory peptides and NO synthesis could affect extracellular matrix formation and collagen deposition (chapter 1, section 1.9.3).

The total number of days required for full wound contraction in diabetic rats was reduced in insulin-treated (16 ± 0.3 days) compared to non-treated animals (18 ± 0.2 days). Within the first 2 days after induction, wounds in insulin-treated diabetic rats expanded more (by 0.4 cm²) than non-treated diabetic rats (by 0.15 cm²). This suggests that hyperglycaemia is involved in impairing the inflammatory phase of the tissue repair process in diabetic rats. It was hypothesized that insulin could contribute to the improvement of this stage of the process by:

- It’s direct effect as an endothelium-dependent vasodilator (chapter 1, section 1.8.5.3)
- Inhibiting alterations in vascular permeability and response to vasoactive agents (see results in chapter 9)

Between days 2-7, reduction in wound size in insulin-treated diabetic rats (by 2.1 cm²) was significantly greater than non-treated diabetic animals (by 1.3 cm²). Similarly, between days 9-11, wound contracted more in insulin-treated animals (by 1 cm²) compared to non-treated diabetic rats (by 0.8 cm²). These results suggest that hyperglycaemia is also involved in impairing proliferative and tissue remodelling stages of the repair process in diabetic rats. It was hypothesized that insulin may contribute to the improvement of these stages of the repair process by:

- Adequate control of hyperglycaemia (i.e. blood glucose level close to normal range during the experimental period) (see chapter 9 results)
- Preventing NO inactivation (see chapter 9 results)

These results suggest that insulin is involved in improving both proliferative and remodelling stages of the tissue repair process in diabetic rats, but its effect is greater on the former stage.
The total number of days required for full wound contraction in diabetic rats was reduced in AG-treated (16 ± 0.2 days) compared to non-treated rats (18 ± 0.2 days). Within the first 2 days after induction, wound expansion in AG-treated diabetic rats (by 0.1 cm²) was similar to non-treated diabetic animals (by 0.15 cm²). This suggests that factors other than AGEs may be involved and they may exert a greater effect on the inflammatory phase of the tissue repair process in diabetic rats. Between days 2-7, wounds in AG-treated diabetic rats were significantly contracted (by 1.9 cm²) when compared to those in non-treated diabetic animals (by 1.3 cm²). This suggests that AGEs are involved in the proliferative stage of the tissue repair process. It was hypothesized that AG treatment could contribute to the improvement of this stage of the repair process by preventing AGEs-induced changes in post-terminal events (see results in chapter 8).

Between days 9-11, the reduction in wound size in AG-treated diabetic rats (by 0.75 cm²) was not different from non-treated diabetic rats (by 0.8 cm²). Again, this suggests that factors other than AGEs may be involved, exerting a greater effect on the tissue remodelling stage of the repair process in diabetic rats.

These results suggest that AG is involved mainly in improving the proliferative stage of the tissue repair process in diabetic rats.

The time required for full wound closure in tirilazad mesylate-treated diabetic rats (13 ± 0.2 days) was shorter than control animals (14 ± 0.2 days) but only reached statistical significance when compared to the period of time required by non-treated diabetic rats (18 ± 0.2 days). Within the first 2 days after induction, wound expansion in tirilazad mesylate-treated diabetic rats (by 0.4 cm²) was greater than AG-treated diabetic animals (by 0.1 cm²) but similar to those in the control group (by 0.55 cm²). Between days 2-7, contraction in wound size in tirilazad mesylate-treated diabetic rats (by 2.25 cm²) showed a similarity to those in the control group (by 2.5 cm²) but was greater than those observed in AG-treated animals (by 1.9 cm²). These results suggest that free radicals play a role in impairing both inflammatory and proliferative stages of the tissue repair process in diabetic rats. In addition, free radicals are more involved than AGEs in impairing the proliferative stage of this process. It was hypothesized that tirilazad mesylate may
contribute to the improvement of these stages of the tissue repair process by preventing free radical-induced changes in post-terminal events (see results in chapter 7). Between days 7-9, contraction in wound size in tirilazad mesylate-treated diabetic rats (by 0.9 cm²) was similar to those in the control group (by 1 cm²). This suggests that free radicals are also involved in impairing tissue remodelling stage of the repair process in diabetic rats. Tirilazad mesylate may contribute to the improvement of this stage of the process by scavenging free radicals and so protecting the microvasculature against damage by these agents.

These results suggest that tirilazad mesylate is involved in improving both proliferative and remodelling stages of the tissue repair process in diabetic rats, but its effect is greater on the latter stage.

The total number of days required for full wound contraction in diabetic rats treated with combined BQ-123 + SOD (13 ± 0.3 days) was similar to tirilazad mesylate-treated diabetic rats (13 ± 0.3 days). This raised the possibility that free radicals play a more important role than ETs in tissue repair processes in diabetes. Within the first 2 days after induction, the greatest wound expansion was observed in combined BQ-123 + SOD-treated diabetic rats (by 0.75 cm²). Between days 2-7 and among other treated diabetic rats, wounds contracted the most in rats treated with combined BQ-123 + SOD (by 2.35 cm²), which was similar to those observed in control animals (by 2.5 cm²). These results suggest that ETs and free radicals together could cause a greater impairment in the inflammatory and proliferative stages of the tissue repair process than individually in diabetes (additive effect). It was hypothesized that BQ-123 and SOD may improve these stages of the repair process by preventing changes in post-terminal events induced by ET-1 (involving both ET-A and ET-B receptors) and free radicals (see results in chapters 6 & 7).

Between days 9-11, a significant reduction in wound size was observed in combined BQ-123 + SOD-treated diabetic rats (by 0.9 cm²), which was greater than those in tirilazad mesylate-treated diabetic rats (by 0.8 cm²) and far greater than the control group (by 0.45 cm²). This suggests that ETs and free radicals also play an important role in impairing
the tissue remodelling stage of the repair process. The combined BQ-123 + SOD may contribute to the final stage of the repair process by:

- Inhibiting ET-1 activity
- Preventing NO deactivation

These results suggest that ETs play a greater role than free radicals in inhibiting the tissue remodelling stage of the repair process.

Overall, results of the investigation in this chapter demonstrated that all treatments used have contributed positively, to varying degrees, to different stages of wound repair and to the overall improvement in repair processes in diabetic rats. Furthermore, pharmacological manipulations at the level of the microvasculature (e.g. local injection of combined BQ-123 + SOD) and systemic treatments (e.g. insulin, AG, Tirilazad mesylate) were shown to improve repair processes in early diabetes.

11.5. Concluding remarks and future directions

The primary aim of this thesis was to investigate mechanisms underlying changes in microvascular blood flow with relevance to tissue repair in early diabetes. Based on the findings presented in this thesis, it is reasonable to conclude that a number of factors that are known to cause long-term vascular complications also contribute to early changes in skin microvascular blood flow in early diabetes.

This thesis demonstrated that by reducing the effects of ETs, free radicals, AGEs and hyperglycaemia in early diabetes, using specific treatment protocols, the skin microvascular blood flow was significantly improved (chapters 6, 7, 8, and 9). When the same treatments were applied to diabetic rats with skin wounds, overall tissue repair processes were also enhanced. This work raised the exciting possibility that early intervention can prevent microvascular alterations in diabetes, which could decelerate the onset of long-term diabetic complications.

The overall information in this thesis could have significant clinical implications for treating diabetic patients with long-term vascular complications. The principle behind the use of non-invasive and easy to apply treatment protocols, which achieve a significant
improvement in tissue repair processes, was refreshing and worthy of further investigation. Future research is needed to determine the impact of early intervention on decelerating late diabetic complications.

There are a number of issues relevant to the topic of this thesis that were not pursued due to limitations of time and resources. However, investigating some of these issues (mentioned below) could be of great benefit in further expanding our knowledge in the areas of diabetes, skin microvasculature, and wound repair, including:

1. Investigating mechanisms underlying changes in skin microvascular blood flow in
   - rats with longer-duration of diabetes
   - type II model of diabetic rats
   - genetic model of type I diabetes
   - short- and long-term treatment with natural antioxidants (e.g. Vitamin E)
2. Examining structural changes in the microvasculature of diabetic rats in relation to the duration of diabetes, levels of hyperglycaemia, AGEs, and presence of excess free radicals, with or without treatment.
3. Investigating the potential clinical application of the treatment protocols used in chapter 10 in improving skin repair processes in diabetic patients with compromised microvascular blood flow.
CHAPTER 12

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APPENDICES
Appendix A:

Responses in control rats over time (Chapter 3, part 1)

Control (week zero) = 4-6 weeks old rats
Control (week 1) = 5-7 weeks old rats
Control (week 5) = 9-11 weeks old rats
Control (week 10) = 14-16 weeks old rats

<table>
<thead>
<tr>
<th>Control rats</th>
<th>BBF 95% CI</th>
<th>SNP 95% CI</th>
<th>SP 95% CI</th>
<th>CGRP 95% CI</th>
<th>ES 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0</td>
<td>(25.1 ± 0.9 cm²) (23.3-26.9)</td>
<td>(26.1 ± 1.8 cm²) (22.6-29.6)</td>
<td>(51.4 ± 2.9 cm²) (45.7-57.1)</td>
<td>(24.8 ± 4.9 cm²) (15.2-34.4)</td>
<td>(17.6 ± 1.3 cm²) (15-20.1)</td>
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<tr>
<td>Week 1</td>
<td>(26.7 ± 2.2 cm²) (22.4-31)</td>
<td>(28.4 ± 4.2 cm²) (20.2-36.6)</td>
<td>(50.1 ± 3.7 cm²) (42.8-57.3)</td>
<td>(25.1 ± 4.8 cm²) (15.7-34.5)</td>
<td>(17.7 ± 1.6 cm²) (14.6-20.8)</td>
</tr>
<tr>
<td>Week 5</td>
<td>(23.2 ± 1.4 cm²) (20.4-25.9)</td>
<td>(29.2 ± 3.5 cm²) (22.3-36.1)</td>
<td>(50.9 ± 2.5 cm²) (46-55.8)</td>
<td>(24.4 ± 4.6 cm²) (15.4-33.4)</td>
<td>(17.5 ± 2.4 cm²) (12.8-22.2)</td>
</tr>
<tr>
<td>Week 10</td>
<td>(23.3 ± 1.8 cm²) (19.8-26.8)</td>
<td>(23.6 ± 4 cm²) (15.7-31.4)</td>
<td>(52.3 ± 2.4 cm²) (47.6-57)</td>
<td>(23.3 ± 2.1 cm²) (19.2-27.4)</td>
<td>(16.4 ± 0.4 cm²) (15.6-17.2)</td>
</tr>
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</table>

Table A-1: Basal blood flow (BBF) and vascular responses to SNP (100μM), SP, CGRP (both 1μM) and ES (20V, 15Hz, 2 mSec, for 1 min) in control rats over time (n = 6-9). Data are presented as mean ± SEM and their 95% confidence intervals (95% CI).

Appendix B:

Plasma extravasation results

B1: Chapter 3, part 1

<table>
<thead>
<tr>
<th>Rat</th>
<th>R1 (μg/ml)</th>
<th>SP2 (μg/ml)</th>
<th>SP3 (μg/ml)</th>
<th>SP4 (μg/ml)</th>
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<tbody>
<tr>
<td>Control</td>
<td>(65 ± 12.9) (95% CI 40-90)</td>
<td>(86.7 ± 13.1) (95% CI 61-112)</td>
<td>(111.7 ± 12) (95% CI 100-135)</td>
<td>(123 ± 9.2) (95% CI 105-134)</td>
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<tr>
<td>Diabetic (2 weeks)</td>
<td>(106 ± 12.5) (95% CI 81.5-130.5)</td>
<td>(133 ± 13.6) (95% CI 106-160)</td>
<td>(169.7 ± 21.3) (95% CI 128-211)</td>
<td>(158.4 ± 21.4) (95% CI 116.5-200)</td>
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<tr>
<td>Diabetic (4 weeks)</td>
<td>(153 ± 17.7) (95% CI 118-188)</td>
<td>(166.6 ± 36) (95% CI 96-237)</td>
<td>(177.4 ± 49.7) (95% CI 80-275)</td>
<td>(152.4 ± 35.6) (95% CI 83-222)</td>
</tr>
<tr>
<td>Diabetic (6 weeks)</td>
<td>(150 ± 23.6) (95% CI 104-196)</td>
<td>(186.3 ± 9.6) (95% CI 167.5-205)</td>
<td>(192 ± 13.4) (95% CI 166-218)</td>
<td>(173.1 ± 21.2) (95% CI 131.5-215)</td>
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<tr>
<td>Diabetic (9 weeks)</td>
<td>(159.6 ± 5) (95% CI 150-169)</td>
<td>(170.5 ± 6.2) (95% CI 158-183)</td>
<td>(186 ± 15) (95% CI 157-215)</td>
<td>(184.3 ± 6.2) (95% CI 172-196)</td>
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Table B-1: PE in diabetic rats over time (n = 6-9). Data are presented as mean ± SEM and their 95% confidence intervals (95% CI).
B2: Chapter 4

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<th>Animals</th>
<th>R1 (μg/ml)</th>
<th>SP2 (μg/ml)</th>
<th>SP3 (μg/ml)</th>
<th>SP4 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP (C)</td>
<td>(65 ± 12.9)</td>
<td>(86.7 ± 13.1)</td>
<td>(111.7 ± 12)</td>
<td>(123 ± 9.2)</td>
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<td></td>
<td>(95%CI 40-90)</td>
<td>(95%CI 61-112)</td>
<td>(95%CI 100-135)</td>
<td>(95%CI 105-134)</td>
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<td>SP (D)</td>
<td>(153 ± 17.7)</td>
<td>(166.6 ± 36.1)</td>
<td>(177.4 ± 49.7)</td>
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<td></td>
<td>(95%CI 118-188)</td>
<td>(95%CI 96-237)</td>
<td>(95%CI 80-275)</td>
<td>(95%CI 83-222)</td>
</tr>
<tr>
<td>SP (C) + L-NAME</td>
<td>(63 ± 8.2)</td>
<td>(69.5 ± 8.1)</td>
<td>(66.7 ± 7.2)</td>
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<td></td>
<td>(95%CI 57-88)</td>
<td>(95%CI 60-90)</td>
<td>(95%CI 65-75)</td>
<td>(95%CI 60-75)</td>
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<tr>
<td>SP (D) + L-NAME</td>
<td>(141 ± 14.2)</td>
<td>(156 ± 14.1)</td>
<td>(167 ± 13.6)</td>
<td>(143 ± 14.4)</td>
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<td>(95%CI 150-170)</td>
<td>(95%CI 130-150)</td>
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<td>BK (C)</td>
<td>(58.3 ± 6.2)</td>
<td>(79 ± 4.6)</td>
<td>(109 ± 7.5)</td>
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<tr>
<td>BK (C) + L-NAME</td>
<td>(53 ± 9.2)</td>
<td>(60 ± 6.9)</td>
<td>(64 ± 10.2)</td>
<td>(62 ± 10)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 45-59)</td>
<td>(95%CI 50-70)</td>
<td>(95%CI 40-50)</td>
<td>(95%CI 30-40)</td>
</tr>
<tr>
<td>BK (D) + L-NAME</td>
<td>(159 ± 16.1)</td>
<td>(169 ± 11.3)</td>
<td>(170.4 ± 9.7)</td>
<td>(161 ± 12.4)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 136-182)</td>
<td>(95%CI 153-170)</td>
<td>(95%CI 145-160)</td>
<td>(95%CI 130-140)</td>
</tr>
<tr>
<td>Muscarine chloride (C)</td>
<td>(73 ± 15)</td>
<td>(90.3 ± 11.4)</td>
<td>(121.8 ± 11.1)</td>
<td>(135.1 ± 16.9)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 54-94)</td>
<td>(95%CI 70-130)</td>
<td>(95%CI 100-120)</td>
<td>(95%CI 105-125)</td>
</tr>
<tr>
<td>Muscarine chloride (D)</td>
<td>(158.3 ± 17.2)</td>
<td>(170 ± 14.7)</td>
<td>(182.7 ± 20)</td>
<td>(154.2 ± 16.2)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 132-184)</td>
<td>(95%CI 140-160)</td>
<td>(95%CI 140-160)</td>
<td>(95%CI 120-140)</td>
</tr>
<tr>
<td>Nicotine (C)</td>
<td>(73.9 ± 2.7)</td>
<td>(89.2 ± 6.9)</td>
<td>(112.4 ± 12.7)</td>
<td>(121.5 ± 14.7)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 65-95)</td>
<td>(95%CI 70-100)</td>
<td>(95%CI 85-105)</td>
<td>(95%CI 80-100)</td>
</tr>
<tr>
<td>Nicotine (D)</td>
<td>(166 ± 16.9)</td>
<td>(173.3 ± 15.5)</td>
<td>(182.6 ± 12.8)</td>
<td>(150.4 ± 16.3)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 133-200)</td>
<td>(95%CI 140-170)</td>
<td>(95%CI 150-170)</td>
<td>(95%CI 115-185)</td>
</tr>
</tbody>
</table>

Table B-2: PE in control and diabetic rats after /SP (1μM), SP (1μM) + L-NAME (100μM), control and diabetic rats after /BK (10μM), BK (10μM) + L-NAME (100μM), control rats after Nicotine (100μM) or Muscarine chloride (100μM), and diabetic rats after Nicotine (100μM) or Muscarine chloride (100μM) (n = 6-9). Data are presented as mean ± SEM and their 95% confidence intervals (95% CI). C=Control D=Diabetic

B3: Chapter 5

<table>
<thead>
<tr>
<th>Animal</th>
<th>R1 (μg/ml)</th>
<th>SP2 (μg/ml)</th>
<th>SP3 (μg/ml)</th>
<th>SP4 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (N)</td>
<td>(65 ± 12.9)</td>
<td>(86.7 ± 13.1)</td>
<td>(111.7 ± 12)</td>
<td>(123 ± 9.2)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 40-90)</td>
<td>(95%CI 61-112)</td>
<td>(95%CI 100-135)</td>
<td>(95%CI 105-134)</td>
</tr>
<tr>
<td>Control (T)</td>
<td>(77 ± 3.8)</td>
<td>(99.5 ± 8)</td>
<td>(111.2 ± 9.7)</td>
<td>(131 ± 20.2)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 70-84)</td>
<td>(95%CI 84-115)</td>
<td>(95%CI 92-130)</td>
<td>(95%CI 91-171)</td>
</tr>
<tr>
<td>Diabetic (N)</td>
<td>(153 ± 17.9)</td>
<td>(166.6 ± 36.1)</td>
<td>(177.4 ± 49.7)</td>
<td>(152.4 ± 35.6)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 118-188)</td>
<td>(95%CI 96-237)</td>
<td>(95%CI 80-275)</td>
<td>(95%CI 83-222)</td>
</tr>
<tr>
<td>Diabetic (T)</td>
<td>(154.7 ± 33.9)</td>
<td>(197.4 ± 29.9)</td>
<td>(215.7 ± 25.9)</td>
<td>(190.9 ± 10.1)</td>
</tr>
<tr>
<td></td>
<td>(95% CI 88-221)</td>
<td>(95%CI 139-256)</td>
<td>(95%CI 165-266.5)</td>
<td>(95%CI 171-211)</td>
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</tbody>
</table>

Table B-3: PE in control and diabetic rats before and after guanethidine treatment (50mg/kg, i.p) (n = 4-10). Data are presented as mean ± SEM and their 95% confidence intervals (95% CI). N = Guanethidine non-treated T = Guanethidine-treated
### Table B-4: PE in control, diabetic (Diab), control and diabetic rats after perfusing BQ-123, BQ-788 (both at 10μM), and diabetic rats after perfusing BIM (1μM) (n = 4-10). Data are presented as mean ± SEM and their 95% confidence intervals (95% CI).

<table>
<thead>
<tr>
<th>Animal</th>
<th>R1 (μg/ml)</th>
<th>SP2 (μg/ml)</th>
<th>SP3 (μg/ml)</th>
<th>SP4 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(65 ± 12.9)</td>
<td>(86.7 ± 13.1)</td>
<td>(111.7 ± 12)</td>
<td>(123 ± 9.2)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 40-90)</td>
<td>(95%CI 61-112)</td>
<td>(95%CI 100-135)</td>
<td>(95%CI 105-134)</td>
</tr>
<tr>
<td>Control + BQ-123</td>
<td>(70 ± 9.7)</td>
<td>(84.4 ± 8.9)</td>
<td>(106.3 ± 18.9)</td>
<td>(128.5 ± 24.5)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 51-89)</td>
<td>(95%CI 66.9-102)</td>
<td>(95%CI 69.2-143)</td>
<td>(95%CI 80-176.5)</td>
</tr>
<tr>
<td>Control + BQ-788</td>
<td>(66 ± 17.4)</td>
<td>(98 ± 11.4)</td>
<td>(102.6 ± 11.6)</td>
<td>(131.7 ± 14.2)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 31-100)</td>
<td>(95%CI 75.6-120)</td>
<td>(95%CI 80-125)</td>
<td>(95%CI 104-159)</td>
</tr>
<tr>
<td>Diabetic</td>
<td>(153 ± 17.7)</td>
<td>(166.6 ± 36.1)</td>
<td>(177.4 ± 49.7)</td>
<td>(152.4 ± 35.6)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 118-188)</td>
<td>(95%CI 96-237)</td>
<td>(95%CI 80-275)</td>
<td>(95%CI 83-222)</td>
</tr>
<tr>
<td>Diab+BQ-123</td>
<td>(50.5 ± 7.4)</td>
<td>(72.2 ± 7.4)</td>
<td>(88.4 ± 6.8)</td>
<td>(83.7 ± 5.7)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 36-65)</td>
<td>(95%CI 58-87)</td>
<td>(95%CI 75-102)</td>
<td>(95%CI 72.5-95)</td>
</tr>
<tr>
<td>Diab+BQ-788</td>
<td>(110 ± 24)</td>
<td>(140.7 ± 13.7)</td>
<td>(191.7 ± 27.2)</td>
<td>(212.2 ± 30)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 63-157)</td>
<td>(95%CI 114-168)</td>
<td>(95%CI 138-245)</td>
<td>(95%CI 153-271)</td>
</tr>
<tr>
<td>Diab+BIM</td>
<td>(153.9 ± 22)</td>
<td>(161.5 ± 21)</td>
<td>(196 ± 26)</td>
<td>(207 ± 21.9)</td>
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<tr>
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<td>(95%CI 111-197)</td>
<td>(95%CI 120-203)</td>
<td>(95%CI 145-247)</td>
<td>(95%CI 164-249)</td>
</tr>
</tbody>
</table>

### Table B-5: PE in control, diabetic (Diab), diabetic rats after perfusing SOD (100U/ml), NAC (100μM), BQ-123 (10μM), combined [BQ-123 (10μM) + SOD (100U/ml)], and diabetic rats treated with Freedox (Tirilazad mesylate) (5mg/kg/i.p) (n = 4-10). Data are presented as mean ± SEM and their 95% confidence intervals (95% CI).

<table>
<thead>
<tr>
<th>Animal</th>
<th>R1 (μg/ml)</th>
<th>SP2 (μg/ml)</th>
<th>SP3 (μg/ml)</th>
<th>SP4 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(65 ± 12.9)</td>
<td>(86.7 ± 13.1)</td>
<td>(111.7 ± 12)</td>
<td>(123 ± 9.2)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 40-90)</td>
<td>(95%CI 61-112)</td>
<td>(95%CI 100-135)</td>
<td>(95%CI 105-134)</td>
</tr>
<tr>
<td>Diabetic</td>
<td>(153 ± 17.7)</td>
<td>(166.6 ± 36.1)</td>
<td>(177.4 ± 49.7)</td>
<td>(152.4 ± 35.6)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 118-188)</td>
<td>(95%CI 96-237)</td>
<td>(95%CI 80-275)</td>
<td>(95%CI 83-222)</td>
</tr>
<tr>
<td>Diab+SOD</td>
<td>(85 ± 7.8)</td>
<td>(94.4 ± 5)</td>
<td>(115.3 ± 4)</td>
<td>(119.3 ± 2.7)</td>
</tr>
<tr>
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<td>(95%CI 70-100)</td>
<td>(95%CI 85-104)</td>
<td>(95%CI 107.5-123)</td>
<td>(95%CI 114-125)</td>
</tr>
<tr>
<td>Diab+NAC</td>
<td>(107.6 ± 17.2)</td>
<td>(118.2 ± 17.9)</td>
<td>(160.7 ± 12.1)</td>
<td>(165.8 ± 5.2)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 74-141)</td>
<td>(95%CI 83-153)</td>
<td>(95%CI 137-184)</td>
<td>(95%CI 156-176)</td>
</tr>
<tr>
<td>Diab+BQ-123</td>
<td>(50.5 ± 7.4)</td>
<td>(72.2 ± 7.4)</td>
<td>(88.4 ± 6.8)</td>
<td>(83.7 ± 5.7)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 36-65)</td>
<td>(95%CI 58-87)</td>
<td>(95%CI 75-102)</td>
<td>(95%CI 72.5-95)</td>
</tr>
<tr>
<td>Diab+BQ-123 +SOD</td>
<td>(74.8 ± 11.1)</td>
<td>(91.9 ± 22.7)</td>
<td>(130.4 ± 18.8)</td>
<td>(142 ± 24)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 53-97)</td>
<td>(95%CI 47-137)</td>
<td>(95%CI 94-167)</td>
<td>(95%CI 95-189)</td>
</tr>
<tr>
<td>Diab+Freedox</td>
<td>(108.6 ± 14.1)</td>
<td>(129.8 ± 14.2)</td>
<td>(162.8 ± 8.6)</td>
<td>(172.2 ± 7)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 81-136)</td>
<td>(95%CI 102-158)</td>
<td>(95%CI 146-180)</td>
<td>(95%CI 158.5-186)</td>
</tr>
</tbody>
</table>
### Table B-6: PE in control, diabetic (Diab), diabetic rats after perfusing amadori (1mg/ml), AGE (10mg/ml), anti-RAGE (100μg/ml), and diabetic rats treated with AG (25mg/kg, i.p) (2 or 4 weeks). Data are presented as mean ± SEM and their 95% confidence intervals (95% CI).

<table>
<thead>
<tr>
<th>Animals</th>
<th>R1 (μg/ml)</th>
<th>SP2 (μg/ml)</th>
<th>SP3 (μg/ml)</th>
<th>SP4 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(65 ± 12.9)</td>
<td>(86.7 ± 13.1)</td>
<td>(111.7 ± 12)</td>
<td>(123 ± 9.2)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 40-90)</td>
<td>(95%CI 61-112)</td>
<td>(95%CI 100-135)</td>
<td>(95%CI 105-134)</td>
</tr>
<tr>
<td>Diabetic</td>
<td>(153 ± 17.7)</td>
<td>(166.6 ± 36.1)</td>
<td>(177.4 ± 49.7)</td>
<td>(152.4 ± 35.6)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 118-188)</td>
<td>(95%CI 96-237)</td>
<td>(95%CI 80-275)</td>
<td>(95%CI 83-222)</td>
</tr>
<tr>
<td>Diab + amadori</td>
<td>(124 ± 2.3)</td>
<td>(162 ± 20.9)</td>
<td>(179.6 ± 18.6)</td>
<td>(184 ± 25)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 119-128)</td>
<td>(95%CI 121-203)</td>
<td>(95%CI 143-216)</td>
<td>(95%CI 135-233)</td>
</tr>
<tr>
<td>Diab + AGes</td>
<td>(161.8 ± 19.2)</td>
<td>(182.3 ± 15.7)</td>
<td>(194.6 ± 14.8)</td>
<td>(168.2 ± 23.8)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 124-199)</td>
<td>(95%CI 151-213)</td>
<td>(95%CI 166-224)</td>
<td>(95%CI 122-215)</td>
</tr>
<tr>
<td>Diab + anti-RAGE</td>
<td>(131.6 ± 22.2)</td>
<td>(216.7 ± 36.3)</td>
<td>(261 ± 36)</td>
<td>(245 ± 19.9)</td>
</tr>
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<td>(95%CI 190-332)</td>
<td>(95%CI 206-284)</td>
</tr>
<tr>
<td>Diab (2 W)</td>
<td>(110.2 ± 12.9)</td>
<td>(115.9 ± 17.3)</td>
<td>(145.8 ± 19.7)</td>
<td>(156 ± 23.9)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 85-135)</td>
<td>(95%CI 82-150)</td>
<td>(95%CI 107-184)</td>
<td>(95%CI 109-203)</td>
</tr>
<tr>
<td>Diab (4 W)</td>
<td>(92.5 ± 17.9)</td>
<td>(140.7 ± 20.6)</td>
<td>(172.3 ± 14.3)</td>
<td>(168.2 ± 15.6)</td>
</tr>
<tr>
<td></td>
<td>(95%CI 57-128)</td>
<td>(95%CI 100-181)</td>
<td>(95%CI 144-200)</td>
<td>(95%CI 138-199)</td>
</tr>
<tr>
<td>Diab (4 W) + late</td>
<td>(125 ± 5)</td>
<td>(154.2 ± 18.5)</td>
<td>(158.5 ± 18)</td>
<td>(176 ± 12.6)</td>
</tr>
<tr>
<td>insulin intervention</td>
<td>(95%CI 115-135)</td>
<td>(95%CI 118-190)</td>
<td>(95%CI 123-194)</td>
<td>(95%CI 151-201)</td>
</tr>
<tr>
<td>Diab (4 W) + early</td>
<td>(77.4 ± 10.4)</td>
<td>(119 ± 5)</td>
<td>(157.1 ± 12.2)</td>
<td>(168.3 ± 14.1)</td>
</tr>
<tr>
<td>insulin intervention</td>
<td>(95%CI 57-98)</td>
<td>(95%CI 109-129)</td>
<td>(95%CI 133-181)</td>
<td>(95%CI 141-196)</td>
</tr>
</tbody>
</table>

### Table B-7: PE in control, diabetic (Diab) (2 & 4 weeks), and diabetic rats (4 weeks) treated with insulin (2-6IU/day) (late and early interventions) (n = 4-10). Data are presented as mean ± SEM and their 95% confidence intervals (95% CI).
Appendix C:

Commercial Amadori and AGEs (Produced by Peptech, Australia)

C₁: The production of amadori and AGEs

For making AGEs, glucose-6-phosphate (G-6-P) and a five week incubation time were used, and for amadori, D-glucose and only one week incubation were used, otherwise they were made by the same protocol provided by Peptech as followed for both G-6-P and glucose respectively.

The method was carried out in a sterile environment. Glucose was filter-sterilized using a 0.2\(\mu\)m filter. Working in a tissue cultured hood, the weighed out bovine serum albumin (BSA) powder was dispensed gradually into a sterile beaker of 200ml sterile phosphate buffer saline (PBS) and stirred into solution. 200\(\mu\)l trasylol, 800\(\mu\)l phenylmethylsulfonyl fluoride (PMSF), 4ml ethylenediaminetetraacetic acid (EDTA), and 200mg ampicillin was added and sterile filtered with 0.2\(\mu\)m filter. 5ml of filtered 2M glucose was dispensed into three 50ml tubes (set up in triplicates) and subsequently added up to 50 ml with the rest of the filtered ingredients. A fourth 50ml tube was prepared without any glucose, i.e. BSA only. Samples were incubated in a 50°C waterbath for required time according to G-6-P or D-glucose respectively. 500\(\mu\)l samples were taken every few days to establish the time course of amadori and AGEs formation. Samples were frozen and stored at -20°C.

C₂: The purification and analysis

The samples were dialyzed against distilled water and subsequently dialyzed against 100mM glycine pH 9.2. Dialysis removes free G-6-P, glucose, and schiff bases. The 500\(\mu\)l samples were tested in a periodate assay [Ahmed, 1991 #627] and the glycine dialyzed samples were run on a phenyl boronate agarose (PBA) column in order to purify amadori. The periodate assay was used to identify whether there were any amadori products present.
The PBA affinity chromatography purification of amadori products is based on the amadori products’ co-planar cis-hydroxyl groups which enables them to bind strongly to the column. BSA and AGEs lack this structure, and therefore do not bind to the column. The samples were applied directly after dialysis into the PBA column equilibrated in glycine buffer. The samples were washed through with glycine buffer at a flow rate of 1 ml/min. Non-glycosylated BSA and AGEs were in the flow through fraction. The amadori were eluted by lowering the pH, making PBA unable to bind the cis-diol groups of the amadori. This was done by pumping through tri-sodium citrate or citrate buffer (100mM, pH 5.8-6). The BSA alone-fraction and the amadori were then dialyzed extensively in 10mM tri-sodium citrate. The amadori were then further analyzed with PBS to remove any tri-sodium citrate and then quantified for amadori and total protein concentration using a periodate and PBA protein assay respectively.

A one week incubation at 50°C of D-glucose and BSA gave amadori products which were purified by column chromatography. The G-6-P counterpart showed no evidence of the presence of amadori products as found in the periodate assay. The failure to detect amadori during time course experiment and after enrichment procedures on PBA columns, strongly suggests that amadori is not a long lived intermediate of non-enzymatic glycosylation when G-6-P is the reducing sugar.
Appendix D:

The average urine glucose level in insulin-treated (2 and 4 weeks) diabetic rats over time.

Urine glucose levels indicated by Diastix strips:

Negative

Trace (5mmol/lit)
+ 15 (15mmol/lit)
++ 30 (30 mmol/lit)
+++ 60 (60 mmol/lit)
++++ 110 (110 mmol/lit)
Author/s:
Bassirat, Maryam

Title:
Mechanisms underlying changes in microvascular blood flow in a diabetic rat model: relevance to tissue repair

Date:
2002-08

Citation:

Publication Status:
Published

Persistent Link:
http://hdl.handle.net/11343/38988

File Description:
Mechanisms underlying changes in microvascular blood flow in a diabetic rat model: relevance to tissue repair

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