The Effects of Diabetes on Sympathetic Neurovascular Transmission

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Abstract

Impaired neural control of arteries is implicated in the etiology of diabetic foot, a major complication of diabetes. The loss of sympathetic nerve-mediated control of blood flow to plantar skin may be an early change that contributes to the later development of microvascular disease in foot skin. The mechanisms that modify sympathetic regulation of arterial vessels are not understood, but are suggested to be due to diabetes-induced neuropathy. Therefore the primary aim of this thesis was to investigate the effects of diabetes on the sympathetic innervation and activation of plantar metatarsal arteries (PMAs) that supply blood to plantar skin of the hind paw digits in rats. The streptozotocin (STZ) rat model of type I diabetes was chosen as it has been widely used to investigate mechanisms that lead to diabetic complications.

Eight-week-old male Wistar rats were treated with STZ (60 mg/kg i.p.) or vehicle (citrate-buffer i.p.; controls). STZ-treated rats received no insulin (STZ-NI) or were treated with a low (~1 unit/day; STZ-LI) or a high (~4 units/day; STZ-HI) dose of insulin. The STZ-NI and STZ-LI rats were hyperglycemic (blood glucose >20 mM), whereas STZ-HI rats were normoglycemic (blood glucose <15 mM). Rats were maintained for 12 weeks when arteries were isolated for in vitro studies.

In the first study, wire myography was used to assess vascular function. In comparison with PMAs from control rats, those from STZ-NI rats had reduced nerve-evoked contractions. PMAs from STZ-NI rats also had a decreased density of perivascular nerve fibers revealed by immunolabeling for the pan-neuronal marker β-tubulin III. No changes in vascular function and innervation density were observed in PMAs from STZ-LI and STZ-HI rats. However, in PMAs from both STZ-NI and STZ-LI rats, the β-tubulin III immunoreactive (IR) nerve fibers were thickened. The majority of perivascular nerve fibers were tyrosine hydroxylase (TH)-IR (i.e. originated from sympathetic neurons) and the labeling intensity for this protein increased in PMAs from both STZ-NI and STZ-LI rats.

The effects of diabetes on mesenteric arteries (MAs) from STZ-NI rats were also determined. Compared to control MAs, nerve-evoked contractions were not changed in MAs from STZ-NI rats. The density of nerve fibers in the perivascular nerve plexus of MAs was reduced but this change could be explained by an increase in vascular dimensions.
There was no change in the width or TH immunolabeling of the nerve fibers. These findings suggest PMAs are particularly sensitive to the effects of diabetes.

Thickening of the sympathetic nerve fibers in the perivascular nerve plexus of PMAs suggests diabetes may induce axon remodeling. Peripherin and β-tubulin III are structural proteins that are reported to increase in regenerating axons. The second study investigated whether diabetes changed expression of these neuron-specific proteins in PMAs. Western blotting revealed an increase in peripherin protein content of PMAs from STZ-LI rats compared to those from STZ-HI and control rats. The number of fibers in the perivascular nerve plexus that were peripherin-IR also increased in PMAs from STZ-LI rats. Co-labeling with antibodies to peripherin and neuropeptide Y (a marker for sympathetic axons) revealed that peripherin expression increased in sympathetic axons. No changes in β-tubulin III protein content were detected. These findings are consistent with diabetes stimulating remodeling of the sympathetic nerve terminals. No changes in peripherin protein expression were detected in the tail artery, again suggesting that PMAs are selectively affected by diabetes.

The third study investigated whether changes in the structure of the perivascular nerve plexus were accompanied by changes in mRNA expression levels (assessed by quantitative RT-PCR) of genes involved in neurotransmission, axon structure, plasticity, neurotrophin signaling and stress. No diabetes-induced changes in mRNA expression were detected in neuronal cell bodies within the L1-L4 sympathetic chain ganglia.

In all experiments, changes observed in PMAs from STZ-NI and/or STZ-LI rats were not observed in those from STZ-HI, suggesting they are due to hyperglycemia. The possibility the changes are explained by loss of a direct influence of insulin on the sympathetic neurons/PMAs, however, cannot be excluded. PMAs appear to be particularly vulnerable to the effects of diabetes. This may be explained by these vessels, which are located close to the plantar surface of the hind paw, also being subjected to biomechanical stress from weight-bearing and locomotion. Together the findings indicate that PMAs provide a suitable model for the assessment of treatments for the prevention of diabetes-induced neurovascular dysfunction seen in diabetic humans.
Declaration

This is to certify that

• the thesis comprises only my original work towards the PhD except where indicated in the Preface,

• due acknowledgement has been made in the text to all other material used,

• the thesis is less than 100,000 words in length, exclusive of tables, figures and references.

______________________________
Niloufer J Johansen
Acknowledgments

“You don't write because you want to say something; you write because you've got something to say.” - F. Scott Fitzgerald

It has been a long journey to complete this thesis, however, I do hope this work does the quote justice (note to supervisors, reviewers and anyone else reading this document). There are some key individuals who have made this thesis possible who I would like to personally thank. To my main supervisor, Assoc. Prof. James Brock, thank you particularly for your critical editing of my work. I am grateful for the scientific knowledge that you have imparted to me. I am also in great debt to my co-supervisor, Prof. Heather Young, for steering me through, at times, the choppiest waters of science. I am also thankful to Prof. Elspeth McLachlan for showing me what ‘real’ science is about, especially during the first year of my graduate studies undertaken in Sydney. It was witnessing the science that resulted from labs of Prof. Elspeth McLachlan and Assoc. Prof. James Brock which cemented my vision for what I thought science was truly about – thank you. I am thankful to Assoc. Prof. Colin Anderson and Dr. Peter Kitchener for making sure my work was on (or close to being on) track. Finally, I would like to thank Prof. Tony Goodwin for aiding my work to proceed.

As much as I would love to say this thesis is only the product of my hard work, I am grateful to the research assistants who imparted their knowledge to me on the more technical aspects of experiments to save me some time from troubleshooting issues that I would otherwise had to have learned painfully myself. In particular, I would like to thank Diana T., Kirsty T., Rachael A., Yan Hong T., Billie H. and Nicole K.

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On a more personal note, I would like to dedicate this thesis to my parents, Dr. Chris Johansen and Mrs. Lutfi Johansen. A lot of blood and tears have gone into getting to this stage which I wouldn’t have gotten to without your unconditional love and support. Thank you for always being a phone call away, despite being at least 2,720 km (Melbourne-Perth) or 3,301 km (Sydney-Perth) away. To my closest relatives and friends around the world who were concerned about me and continually reminded me to get some form of a life, thank you.

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Now let’s get started shall we with what I have to say about autonomic diabetic neuropathy...
Conference presentations and publications resulting from the work presented in this thesis.

**PUBLICATION**


**ORAL COMMUNICATION**

Australian Physiological Society (AUPS)/ Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists (ASCEPT) 5 – 7 December 2011 at the Perth Convention Exhibition Centre, Perth, Western Australia, Australia.

**Perivascular sympathetic neuropathy in the streptozotocin type I diabetic rat model**

Johansen N.J., Abela R. and Brock J.A. - Dept of Anatomy and Neuroscience, University of Melbourne, VIC 3010, Australia.

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**Increased peripherin in sympathetic axons innervating plantar metatarsal arteries in diabetic rats – evidence for axonal remodeling?**

Johansen N.J., Frugier T., Hunne B.L. and Brock J.A. - Dept of Anatomy and Neuroscience, University of Melbourne, VIC 3010, Australia.

Australian Neuroscience Society (ANS) 29 January – 1 February 2012 at the Jupiters Hotel, Gold Coast, Queensland, Australia.

**Altered perivascular innervation of plantar metatarsal arteries in the streptozotocin-induced diabetic rat**

Johansen N.J., Frugier T., Hunne B.L. and Brock J.A. - Dept of Anatomy and Neuroscience, University of Melbourne, VIC 3010, Australia.


**Perivascular sympathetic neuropathy in the streptozotocin type I diabetic rat model**

Neurovascular transmission is impaired in arteries supplying skin of diabetic rats
Johansen N.J., Tripovic D., Abela R. and Brock J.A. - Prince of Wales Medical Research Institute, University of NSW, Sydney, NSW 2031, Australia;

Neurovascular transmission is impaired in arteries supplying skin of diabetic rats
Johansen N.J., Tripovic D. and Brock J.A. - Prince of Wales Medical Research Institute, University of NSW, Sydney, NSW 2031, Australia.
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<table>
<thead>
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<th>Full Form</th>
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<tbody>
<tr>
<td>AGEs</td>
<td>Advanced glycated end-products</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>ATF-2</td>
<td>Activating transcription factor-2</td>
</tr>
<tr>
<td>ATF-3</td>
<td>Activating transcription factor-3</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>AVAs</td>
<td>Arteriovenous anastomoses</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CMT2B</td>
<td>Charcot-Marie-Tooth type 2B</td>
</tr>
<tr>
<td>Ct-value</td>
<td>Cycle threshold value</td>
</tr>
<tr>
<td>DMI</td>
<td>Desmethylimipramine</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglia</td>
</tr>
<tr>
<td>EDHF</td>
<td>Endothelium-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>ETP</td>
<td>Effective transmural pressure</td>
</tr>
<tr>
<td>GAP-43</td>
<td>Growth associated protein 43</td>
</tr>
<tr>
<td>HSD</td>
<td>Honest significant difference</td>
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<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
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<td>Immunohistochemistry</td>
</tr>
<tr>
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<td>Interleukin-6</td>
</tr>
<tr>
<td>IR</td>
<td>Immunoreactive</td>
</tr>
<tr>
<td>MA</td>
<td>Mesenteric artery</td>
</tr>
<tr>
<td>miR</td>
<td>microRNAs</td>
</tr>
<tr>
<td>NET</td>
<td>Norepinephrine transporter</td>
</tr>
<tr>
<td>NF</td>
<td>Neurofilament</td>
</tr>
<tr>
<td>NF-H</td>
<td>Neurofilament-heavy</td>
</tr>
<tr>
<td>NF-L</td>
<td>Neurofilament-light</td>
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<tr>
<td>NF-M</td>
<td>Neurofilament-medium</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese type 1 diabetic</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>NRQ</td>
<td>Normalized relative expression value</td>
</tr>
<tr>
<td>NT-3</td>
<td>Neurotrophin-3</td>
</tr>
<tr>
<td>NT-4</td>
<td>Neurotrophin-4</td>
</tr>
<tr>
<td>NVT</td>
<td>Neurovascular transmission</td>
</tr>
<tr>
<td>p75&lt;sup&gt;NTR&lt;/sup&gt;</td>
<td>p75 neurotrophin receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phenylephrine</td>
</tr>
<tr>
<td>PMA</td>
<td>Plantar metatarsal artery</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>QSART</td>
<td>Quantitative sudomotor axon reflex test</td>
</tr>
<tr>
<td>RQ</td>
<td>Relative expression value</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SHR</td>
<td>Spontaneous hypertensive rats</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>STZ-HI</td>
<td>Streptozotocin-treated rats receiving a high dose of insulin</td>
</tr>
<tr>
<td>STZ-LI</td>
<td>Streptozotocin-treated rats receiving a low dose of insulin</td>
</tr>
<tr>
<td>STZ-NI</td>
<td>Streptozotocin-treated rats receiving no insulin</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TIDM</td>
<td>Type I diabetes mellitus</td>
</tr>
<tr>
<td>TIIDM</td>
<td>Type II diabetes mellitus</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>WB</td>
<td>Western blotting</td>
</tr>
<tr>
<td>WKY</td>
<td>Wistar Kyoto</td>
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CHAPTER 1 Evidence for Neurovascular Deficits Preceding the Development of Vascular-Related Complications of Diabetes
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1. Diabetes

Diabetes is a chronic disease characterized by aberrant carbohydrate metabolism whereby excess glucose is present within the blood circulation (hyperglycemia) which, if left untreated, results in systemic complications. In Australia, it is estimated that 1.7 million people are affected and globally the number is expected to reach 380 million by 2025, making diabetes the fastest growing chronic disease (Barr et al., 2006; Mbanya et al., 2008). Of all cases of diabetes, 10% are diagnosed as type I diabetes mellitus (TIDM) and 85-90% as type II diabetes mellitus (TIIDM). TIDM is triggered by autoimmune destruction of insulin producing pancreatic β-cells and is therefore treated by the administration of insulin. TIIDM is diagnosed by insulin resistance and/or abnormal insulin secretion. TIIDM is strongly influenced by unhealthy lifestyle choices (Barr et al., 2006). In both conditions, with or without treatment, numerous vascular-related complications develop. There is growing evidence that diabetes can alter blood flow in a variety of arterial beds and this may lead to the development of complications in many organs such as the skin, eye, skeletal muscle, heart and kidneys. Treating diabetes-related complications places a severe financial burden in Australia, costing A$10.3 billion annually (Barr et al., 2006). Further research is required to better understand the mechanisms underlying the development of vascular-related complications. This knowledge is necessary for the development of therapeutic strategies which prevent and/or reduce the detrimental effects of diabetes.

Approximately 50% of people with diabetes will develop neuropathies, although not all of these will have symptoms (Vinik & Mehrabyan, 2004). A distal symmetric neuropathy, which impairs sensation and produces muscle weakness in the arms and legs, is most commonly diagnosed. Diabetes also affects autonomic control of smooth muscle in many organs, and autonomic control of the heart and glands (Vinik et al.,
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2003; Vinik & Mehrabyan, 2004). Thus altered neural function is likely to contribute to many of the complications associated with diabetes.

Clinically, reductions in touch and vibration sensation in diabetic patients indicate damage to large myelinated sensory fibers (Vinik & Mehrabyan, 2004). Small fiber (C- and Aδ-fiber) sensory dysfunction can produce pain and sensory testing reveals decreased sensitivity to warm and cold stimuli (Vinik & Mehrabyan, 2004). Erectile dysfunction is an early sign of autonomic dysfunction and affects up to 75% of diabetic men (Vinik et al., 2003). The neural control of both heart and blood vessels is altered (Vinik et al., 2003). Loss of heart rate variability under resting conditions suggests decreased parasympathetic control. Reduced exercise tolerance implies diminished sympathetic control. Furthermore, diabetic patients often display orthostatic hypotension (reduced sympathetic vasomotor response to postural challenge). All these changes are attributed to degeneration of nerves (Vinik et al., 2003; Vinik & Mehrabyan, 2004).

As elaborated in section 3 below, diabetes can also impair neural control of arterial vessels in skin, and the resultant changes in skin blood flow have been suggested to contribute to the etiology of complications such as diabetic foot. For example, in patients mostly free of signs of diabetic neuropathy, impaired postural regulation of arterial vessels supplying plantar skin of the feet has been observed (Shore et al., 1994). This change has been suggested to contribute to the development of microangiopathy in plantar skin (Flynn & Tooke, 1995). Microangiopathy is a microvascular disease in which thickening of the capillary basement membrane leads to tissue ischemia and impaired nutrient transport across the capillary wall. Together with sensory nerve dysfunction, poor nutrition of skin results in an increased risk of skin injury, poor wound healing and ulceration (Flynn & Tooke, 1995). As a consequence, diabetes greatly increases the risk of gangrene and the need for lower
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limb amputation (Flynn & Tooke, 1995). Because the mechanisms by which nerves control blood vessels differ depending on the vascular bed studied, particular regions of the vasculature may be highly susceptible to the effects of diabetes. This literature review will outline the role of nerves in controlling arteries under normal conditions and the changes that have been described in diabetes in both human patients and animal models.

2. Normal control of blood flow

Blood flow into tissues is primarily regulated by resistance arteries and arterioles, which are precapillary arterial vessels with a sufficiently small lumen diameter (<400 µm) that they contribute both passively to the resting resistance to blood flow and actively to control blood flow to meet changing tissue demands (Christensen & Mulvany, 2001). Small resistance arteries (lumen diameter ~100-400 µm) are composed of a single-cell luminal lining of endothelial cells surrounded by multiple layers of smooth muscle cells enclosed within a connective tissue sheath (adventitia). The structure of arterioles <100 µm in luminal diameter is similar except they have only 1-2 layers of smooth muscle cells. Embedded within the adventitia and running parallel to the arterial wall lie sympathetic nerve axons which branch at multiple points to form a perivascular plexus (Figure 1.1). Axon terminals are in close proximity to the vascular smooth muscle and, when activated, release noradrenaline together with cotransmitters (e.g. adenosine 5'-triphosphate [ATP] and neuropeptide Y [NPY]), which trigger vasoconstriction (see section 2.2). Arterial vessels may also be innervated by the axon terminals of peptidergic primary afferent neurons that intermingle with the sympathetic axons (Figure 1.1) and, when activated, elicit vasodilation (see section 2.2). Blood flow through resistance arteries is also influenced by non-neural mechanisms that contribute to the regulation of vascular tone (i.e. the degree of vascular constriction). Non-neural mechanisms include intrinsic myogenic...
mechanisms that produce constriction or dilation of small arterial vessels in response to increases or decreases in blood pressure, respectively. In addition, vascular tone is regulated by chemical mediators released locally from the endothelium (e.g. nitric oxide [NO]) and surrounding tissues (e.g. tissue metabolites) and those delivered via the blood (e.g. hormones such as angiotensin II and vasopressin) (Noguera et al., 1997; Segarra et al., 1998; Macarthur et al., 2011).

**Figure 1.1.** Sensory and sympathetic axons forming the perivascular plexus innervating the plantar metatarsal artery from a normal rat as identified by antibodies to calcitonin gene-related peptide (CGRP; green) and tyrosine hydroxylase (TH; red), respectively (N. J. Johansen, unpublished).

2.1. **Endothelium-mediated vascular responses**

The endothelium can influence the level of vascular contraction via a variety of mediators, for review see Sandow et al. (2012). In general, endothelial cells mediate
vasodilation via the release of NO, prostacyclin and endothelium-derived hyperpolarizing factor (EDHF). Endothelial-derived constrictor agents include endothelin, thromboxane and superoxide. The mechanisms by which endothelial-mediated vasodilation occurs depends on the type (Clark & Fuchs, 1997) and location (Hwa et al., 1994) of the vascular bed. For example, in healthy rats, vasodilation in small mesenteric arteries is mediated by both NO and EDHF whereas in femoral arteries vasodilation is mediated almost entirely by NO (Zygmunt et al., 1995; Wigg et al., 2001). After 8 weeks of experimental diabetes, the EDHF-mediated vasodilation was markedly reduced in mesenteric arteries but the NO-mediated component was undisturbed in both mesenteric and femoral arteries (Wigg et al., 2001). Additional evidence that diabetes selectively reduces the vasodilator effect of EDHF in resistance arteries is reviewed by Fitzgerald et al. (2005). Details of diabetes-induced alterations in endothelial function will not be further discussed as it is outside the focus of this literature review.

2.2. Nerve-mediated control of arterial vessels

The regulation of vascular smooth muscle by nerve-released neurotransmitters is termed neurovascular transmission (NVT). Neurally evoked constriction of resistance arterial vessels is mediated by sympathetic vasoconstrictor neurons that contribute to the regulation of both baseline levels of vascular tone as well as peripheral reflex (e.g. baroreflex) mediated changes in vascular tone (Jänig, 2006). In addition, some arterial vessels (including those supplying skin and the intestine) are supplied by peptidergic primary afferent neurons that cause vasodilation when activated (Holzer, 1997; Dunn et al., 2003). As described above, the axon terminals of both the sympathetic and sensory neurons form a perivascular nerve plexus within the adventitia (Figure 1.1), and neurotransmitters are released from terminals at the adventitial-medial border.
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The primary neurotransmitter contained in postganglionic sympathetic neurons is noradrenaline, and in arteries this induces vasoconstriction through activation of α-adrenoceptors on the vascular smooth muscle. In addition, sympathetic neurons release cotransmitters NPY and ATP, which mediate vasoconstriction by acting on postjunctional NPY-Y1 receptors (Heath, 1998; Stephens et al., 2004; Kellogg, 2006; Hodges et al., 2009) and P2X-purinoceptors (Ziganshin et al., 2004; Pakdeechote et al., 2007), respectively. The relative roles of noradrenaline, NPY and ATP vary between arterial vessels (Burnstock, 1993).

The perivascular nerve terminals of peptidergic primary afferent neurons contain calcitonin gene-related peptide (CGRP) and substance P, which are released when these fibers are activated by sensory stimuli (Holzer, 1997). The vasodilator action of these nerves is mediated by CGRP which activates CGRP1-receptors on the vascular smooth muscle. Substance P does not act directly on the vascular smooth muscle, but neurokinin receptors are present on the endothelium and activation of these receptors can produce vasodilation through the release of NO from the endothelium (Ralevic et al., 1992; Biro et al., 1997; Gibbins, 1997). To date, most knowledge regarding mechanisms of neural control of vasculature comes from studies of arteries and arterioles in the cutaneous (skin) and mesenteric vascular beds.

2.2.1. Neural control of cutaneous arteries

At the dermal-subcutaneous junction, the arteries supplying skin divide to form a plexus of smaller arteries within the dermis. Capillary loops arising from these vessels supply the most superficial layer of the dermis, providing nutrients to the epidermis. In the dermis, opening of arterio-venous anastomoses (AVAs) shunts blood directly from the arterial to the venous side of the circulation, bypassing the capillary loops (Coffman, 1972). AVAs are concentrated in regions of skin that play important roles in thermoregulation, such as the palmar and plantar surfaces of the hands and feet,
Opening the AVAs can increase blood flow 100-fold in skin, allowing greater heat transfer between the blood and the external environment (Burton, 1961). Sympathetic neurons densely innervate the muscular component of the AVAs and their firing rates are sensitive to changes in body temperature, thereby regulating blood flow through the thermoregulatory circuit of the skin vascular bed (Gibbins, 1997). Colder temperatures increase the firing rate of these sympathetic neurons resulting in vasoconstriction and a reduction of blood flow through the AVAs (Morris, 1997). Conversely warmer temperatures decrease their firing rate, reducing the level of vasoconstriction and allowing greater blood flow via the AVAs. Sympathetic neurons regulating skin blood flow can also be strongly activated by alerting reactions (Yu & Blessing, 1997) and emotional stimuli (Jänig, 2006).

In addition to the “steal” effect of shunting blood directly from the arterial to the venous circulation via the AVAs, blood flow through the nutritive capillary bed is regulated by nerve-mediated constriction of the small arteries and arterioles in the dermis. Nerve-evoked constriction of the larger feed arteries that supply blood to skin of the hands and feet can have an important influence on cutaneous blood flow, as demonstrated by Raynaud’s syndrome where neurally induced vasospasm of digital arteries can completely occlude blood flow to the fingers or toes (Garcia-Carrasco et al., 2008).

In arteries supplying the skin of both humans and rodents, noradrenaline is the primary neurotransmitter used by sympathetic neurons and it acts upon $\alpha_1$- and $\alpha_2$-adrenoceptors on vascular smooth muscle cells to produce vasoconstriction (Coffman & Cohen, 1988; Yeoh et al., 2004a, b). Present evidence suggests that $\alpha_1$-adrenoceptor-mediated constriction is more prominent in the larger feed arteries supplying blood to skin, with the relative role of $\alpha_2$-adrenoceptors increasing more distally in the arterial vascular bed (Morris, 1997). In feed arteries supplying the skin of
rodents, there is evidence that nerve-released ATP contributes to vasoconstriction (Morris, 1994; Johnson et al., 2001), but a study of small subcutaneous arteries isolated from human skin biopsies failed to identify a role for this cotransmitter (Stephens et al., 1992). In the guinea-pig ear, while ATP contributes to the neural activation of the main feed artery, it does not contribute to nerve-evoked constrictions of more distal arterial vessels (Morris, 1994, 1999). Nerve-evoked constrictions of the small cutaneous arteries in the guinea pig ear in vivo were, however, reduced by blockade of $\alpha_1$-adrenoceptors with prazosin and in the presence of this agent the remaining contraction was reduced by the NPY-receptor antagonist 1229U91, indicating a role for NPY in NVT (Morris, 1999). Studies in humans have demonstrated that blockade of $\alpha_2$-adrenoceptors (with yohimbine) and/or NPY-Y1 receptors (with BIBP-3226) attenuated the reduction in skin blood flow induced by body cooling, further demonstrating a role for both noradrenaline and NPY in neural control of cutaneous arterial vessels (Stephens et al., 2004). In rodent cutaneous arteries, the effects of NPY can be mediated either through a direct vasoconstrictor action (Morris, 1999) or indirectly by potentiating contractions produced by nerve-released noradrenaline (Heath, 1998; Stephens et al., 2004; Kellogg, 2006; Hodges et al., 2009).

In skin, activation of peptidergic primary afferent axon terminals in the epidermis by tissue damage elicits local vasodilation ("flare") due to the release of CGRP and the activation of CGRP1-receptors on small arterial vessels in the dermis (Holzer, 1997). This efferent function of the sensory fibers is mediated by axon collaterals that innervate the dermal blood vessels. Action potentials elicited by activation of the sensory nerve terminals in the epidermis travel antidromically within these collateral axons to elicit the release of CGRP from the perivascular nerve terminals (the axon reflex). Primary afferent axons also release substance P, which does not produce vasodilation in skin vessels but it does increase in venular endothelial permeability resulting in edema ("wheal") (Holzer, 1997).
2.2.2. Neural control of mesenteric arteries

Constriction of mesenteric arterial vessels contributes to the regulation of blood flow in the intestine (Furness & Marshall, 1974). To date there have been no studies that have investigated the relative contributions of noradrenaline, and the cotransmitters ATP and NPY, to nerve-mediated constrictions of human mesenteric arteries. In rat mesenteric arteries, vasoconstriction induced by short trains of stimuli is mediated by both noradrenaline and ATP (Brock et al., 2006; Rummery et al., 2007). In this case the effects of noradrenaline are mediated solely through \( \alpha_1 \)-adrenoceptors whereas the effects of ATP are mediated through P2X1-purinoceptors. The relative contributions of noradrenaline and ATP to NVT in mesenteric vessels appear to vary between experimental conditions. For example, in isometrically mounted rat mesenteric arteries, noradrenaline acting at \( \alpha_1 \)-adrenoceptors is primarily responsible for nerve-evoked vasoconstrictions (Angus et al., 1988; Sjoblom-Widfeldt et al., 1990). In pressurized arteries, however, the relative contribution of noradrenaline to nerve-evoked vasoconstriction is less and the contribution of ATP increases when the intraluminal pressure is raised from 30 to 90 mmHg (Rummery et al., 2007). As the pressurized condition is more physiological, this finding may indicate that ATP plays an important role in regulating these vessels in vivo but to date this has not been directly investigated. There is also evidence that the contribution of ATP to NVT increases in the more distal vessels in the mesenteric arterial vascular bed (Gitterman & Evans, 2000; Luo et al., 2003). NPY has also been demonstrated to contribute to neural activation of rat mesenteric arteries and this effect is mediated indirectly through a facilitatory effect of activating smooth muscle NPY-Y1 receptors on contractions mediated by noradrenaline (Donoso et al., 1997; Han et al., 1998).

Mesenteric arteries are relatively well supplied by peptidergic primary afferent neurons but the physiological role of this innervation remains obscure (Holzer et al.,...
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1982). Activating afferent fibers with capsaicin or electrical stimuli produces vasodilation of mesenteric arteries, which is mediated by CGRP acting at CGPR1-receptors (Han et al., 1990).

3. Diabetes alters neural control of arterial vessels in diabetic humans

There is evidence to suggest that changes in the neural control of resistance arteries contribute to the impairment of microvascular blood flow which precedes the onset of many diabetic complications (Gilmore et al., 1993; Cacciatori et al., 1997). The exact cause of the change in the neural control of arteries is not known, but it is widely believed that autonomic and sensory neuropathy leads to deficits in sympathetic nerve-mediated vasoconstriction and sensory nerve-mediated vasodilation, respectively (Levy et al., 1989; Cacciatori et al., 1997; Hamdy et al., 2001; Caselli et al., 2003; Lefrandt et al., 2003; Quattrini et al., 2007). Additionally, because studies in animals suggest that the mechanisms by which nerves control blood vessels differ between vascular beds (see section 2.2), it is possible that particular regions of the vasculature are more susceptible to the effects of diabetes. However, due to the ease of study, much of the available evidence for altered neural control of the vasculature in diabetes comes from studies of skin blood flow.

3.1. Neural regulation of skin blood flow can be impaired in diabetes

Changes in the blood flow in diabetic skin have largely been studied using plethysmography and laser Doppler flowmetry. Plethysmography has been used to measure changes in the finger or toe volume, which provides an overall measure of skin blood flow into the digits. In contrast, laser Doppler flowmetry provides a relative measure of changes in blood flow in the smaller blood vessels (including the capillaries) close to the epidermal-dermal junction. Using plethysmography, Archer et al. (1984), provided evidence that both TIDM and TIIDM patients with severe non-painful sensory neuropathy had impaired neural control of skin blood flow. Under
resting conditions, a 5-fold increase in big toe blood flow was observed in diabetic patients with severe non-painful sensory neuropathy compared to that of control subjects. A marked reduction in the decrease in blood flow (vasoconstriction) produced by sympathetic arousal induced by coughing, gasping, responding to loud noises and performing mental arithmetic was also demonstrated in this group of diabetic patients. This reduction in neurally evoked vasoconstriction was not observed in diabetic patients with severe painful neuropathy, indicating that their ability to induce sympathetic nerve-mediated vasoconstriction in the big toe was intact.

A similar reduction in vasoconstriction in the big toe to sympathetic arousal (induced by a deep inspiratory breath) was demonstrated using laser Doppler flowmetry in T2DM patients (Aso et al., 1997; Valensi et al., 1997). Valensi et al. (1997) found that the reduction in nerve-evoked vasoconstriction was not correlated with changes in cardiac autonomic function (a commonly used clinical measure of autonomic neuropathy), suggesting that the laser Doppler flowmetry method provides a sensitive and selective test for detecting sympathetic nerve dysfunction. Takahashi et al. (1998) demonstrated that T2DM patients with autonomic neuropathy had reduced sympathetically-mediated vasoconstriction of pedal, metatarsal and digital arteries evoked by a deep breath (a sympathetic stimulus) when compared to T2DM patients without autonomic neuropathy and control subjects. Additionally, this study found a high degree of correlation between diabetic patients with early signs of autonomic dysfunction and reduced vasoconstriction of the digital arteries evoked by a deep breath, suggesting impaired sympathetic vasoconstrictor function may precede more overt signs of autonomic neuropathy.

3.1.1. Diabetes impairs posturally induced changes in skin blood flow

Vasoconstriction of arterial vessels supplying the plantar skin of the big toe can be elicited by moving the foot from the level of the heart to below the heart
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(posturally mediated vasoconstriction). Blockade of centrally derived sympathetic nerve activity produced by epidural delivery of the local anesthetic, lidocaine, to the lumbar region of the spinal cord slightly reduced, but did not abolish, this response (Hassan & Tooke, 1988). In contrast, local blockade of nerve activity in plantar skin with lidocaine abolished posturally evoked vasoconstriction (Hassan & Tooke, 1988). This effect of posture has been hypothesized to be explained by a local sympathetic reflex mechanism within the microcirculation whereby an increase in transmural pressure in small veins is sensed and elicits a neurally mediated vasoconstriction of arterial vessels (the veno-arterial reflex; Henriksen, 1991). In diabetic patients with peripheral sensory neuropathy, posturally mediated reductions in blood flow in plantar skin were reduced compared to those in diabetics without peripheral sensory neuropathy and controls (Rayman et al., 1986). This finding provides further evidence that diabetes can impair sympathetic control of blood flow to the skin of the feet.

A comparison of the posturally mediated vasoconstriction in plantar skin of the big toe between prepubertal children and young postpubertal children revealed that this response increases with age (Shore et al., 1994). In prepubertal TIDM diabetic children, posturally evoked vasoconstriction in plantar skin did not differ from non-diabetic age-matched controls, but that in postpubertal TIDM children was significantly impaired (Shore et al., 1994). Importantly, most of the postpubertal subjects in this study did not display signs of diabetic complications, suggesting that this test may detect early diabetes-induced deficits in sympathetic control of arterial vessels in plantar skin. TIIDM patients without signs of peripheral sensory neuropathy also showed a reduction in posturally evoked vasoconstriction in the plantar skin of the big toe (Cacciatori et al., 1997; Figure 1.2). This impairment of posturally evoked vasoconstriction was progressively more pronounced in TIIDM patients with peripheral sensory neuropathy and those with peripheral sensory neuropathy and foot ulceration (Cacciatori et al., 1997). Further evidence of sympathetic dysfunction in diabetics
without complications is provided by a study where long-term TIDM patients with and without complications had smaller reductions in blood flow in the palmar skin of the big finger in response to venous occlusion compared to healthy controls (Tooke et al., 1987). This reduction in blood flow evoked by venous occlusion is also attributed to a veno-arterial reflex (Henriksen, 1976).

**Figure 1.2.** Type II diabetic patients with varying degrees of neuropathy have impaired posturally evoked vasoconstriction in the plantar skin of the big toe. Relative to control subjects, diabetics without sensory neuropathy (DN-) had reduced posturally evoked vasoconstriction. The extent of vasoconstriction was progressively reduced in diabetics with neuropathy (DN+) and in diabetics with sensory neuropathy and foot ulceration (DNU). Figure reproduced from Cacciatori et al. (1997).
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3.1.2. Diabetes impairs thermal control of skin blood flow

A number of studies have reported that plantar skin is warmer in diabetics with signs of peripheral neuropathy than in controls, which is attributed to increased blood flow through AVAs (Archer et al., 1984; Chan et al., 1991). Diabetes has also been reported to impair changes in skin blood flow produced by locally changing skin temperature. For example, locally heating the plantar surface of the big toe to 44°C increased blood flow 2- to 3-fold in control subjects and diabetic patients without neuropathy, but in diabetic patients with evidence of autonomic and somatic neuropathy the same stimulus produced a decrease in blood flow (Stevens et al., 1991). The authors attributed this effect of diabetes, in part, to loss of sympathetic control of the skin vasculature. However, current evidence suggests that vasodilation evoked by local heating is mediated via a sensory nerve-mediated axon reflex, with a more slowly developing component that is mediated by NO released from the endothelium (Johnson & Kellogg, 2010). The role of sympathetic nerves in this response is less clear (Johnson & Kellogg, 2010). Therefore, the findings of Stevens et al. (1991) might also be explained by an impaired sensory nerve-mediated axon reflex together with endothelial dysfunction.

In the dorsum of the foot, where there are few AVAs, the change in blood flow induced by heating the skin was significantly smaller in T1DM patients with retinopathy compared to diabetics without retinopathy (or other complications) and normal subjects (Wilson et al., 1992). The rate of increase in blood flow was also slower in diabetics with retinopathy compared to the other two groups, and diabetics without retinopathy also had slower responses than the controls. The diabetes-induced changes in the time course of vasodilation may reflect a relatively selective impairment of sensory nerve-mediated vasodilation. In support of this idea, Krishnan and Rayman (2004) detected impaired vasodilation to heating in T1IDM patients with and without
sensory neuropathy, and attributed this to reduced sensory nerve-mediated vasodilation. The reduced maximum blood flow in the dorsum of the foot in the patients with retinopathy may be caused by diabetes-induced structural changes to the microvasculature (i.e. peripheral vascular disease).

The decrease in skin blood flow evoked by local cooling is also defective in diabetic patients with signs of neuropathy (Wilson et al., 1992). Current evidence suggests that this response is largely due to a postjunctional change in the vascular muscle, with cooling inducing the mobilization of $\alpha_2$-adrenceptors from the Golgi apparatus to the plasma membrane (Johnson & Kellogg, 2010). As a result, there is an increase in the reactivity of vascular muscle to noradrenaline released from the perivascular sympathetic axons. Local cooling also increases the reactivity of the vascular muscle to constrictor agents by inhibiting the vasodilator action of endothelium-derived NO (Johnson & Kellogg, 2010). It is also possible that dysfunction of sympathetic vasoconstrictor fibers contributes to the deficits in vasodilatory responses to local cooling in diabetic patients. However, the question of whether local cooling increases sympathetic nerve activity has not been resolved (Johnson & Kellogg, 2010).

To examine whether diabetes affects sympathetic control of skin blood flow, a number of studies have investigated skin vascular responses in the hand produced by cooling the contralateral arm (Donk et al., 1990; Gilmore et al., 1990; Bornmyr et al., 1999). In control subjects, this stimulus evokes a decrease in skin blood flow that has been measured either by monitoring reductions in skin temperature (Donk et al., 1990) or with a Doppler flowmeter (Bornmyr et al., 1999). Donk et al. (1990) studied 23 diabetic patients with signs of autonomic neuropathy and found reduced or absent responses to cooling. Bornmyr et al. (1999) studied patients who were recently diagnosed with TIIDM and those with long-term TIDM and found that vasoconstriction
in the fingertips was reduced in both groups, suggesting this may be a sensitive test for detecting subclinical changes in sympathetic function. Cutaneous vasoconstriction to whole body cooling has also been studied with plethysmography in hands and feet of diabetic patients (Gilmore et al., 1990). In this group of patients those with signs of neuropathy had reduced vasoconstriction in the feet but no significant changes in the hands. While it is generally assumed that altered sympathetic control of the skin vasculature revealed in these studies is produced by dysfunction of sympathetic vasoconstrictor neurons, the studies do not exclude the possibility that the afferent side of the reflex is also affected by diabetes.

3.1.3. Diabetes-induced changes in sensory nerve-mediated vasodilation (axon reflex) in skin

A reduction in number of cutaneous peptidergic sensory nerves has been suggested to contribute to the decreased sensory axon reflex-mediated vasodilation in skin of diabetic humans (Krämer et al., 2004). Sensory axon reflex-mediated vasodilation (i.e. ‘flare’) was quantified in response to electrophoretic administration of 10% acetylcholine into the plantar skin of the feet of long-term TIDM patients with and without foot complications and of control subjects (Parkhouse & Le Quesne, 1988). While the neurogenic vasodilatory responses were similar between control subjects and diabetics without foot complications, the responses from diabetics with foot complications were markedly reduced. Additionally, the level of deficit in sensory axon reflex-mediated responses was greatest in those subjects with loss of pain sensation in their feet, consistent with the loss of nociceptive sensory fibers (Parkhouse & Le Quesne, 1988).

3.1.4. Diabetes changes blood flow oscillations in skin

Laser Doppler flowmetry has been used to measure the frequency of spontaneous fluctuations of blood flow in the most superficial layers of the human skin.
(Bernardi et al., 1989). In the volar skin of the forearm of normal subjects, oscillations in blood flow have low and high frequency components (Bernardi et al., 1989). The highest frequency component is produced by pulsatile increases in blood flow produced by heart beats, whereas the lowest frequency component peaks at around 0.1 Hz (10 second period) and is primarily due to sympathetic nerve-mediated vasoconstriction. Evidence that sympathetic nerve activity underlies this component of blood flow variation is provided by the finding that it is greatly reduced by sympathetic blockade (produced by brachial plexus block with a local anesthetic; Bernardi et al., 1989). Moreover, the relative size of this low frequency component is increased by a postural challenge when sympathetic nerve activity is increased. Bernardi et al. (1997) found that the 0.1 Hz fluctuations were selectively reduced in TIDM patients without overt signs of autonomic dysfunction. They concluded that laser Doppler flowmetry detects early signs of autonomic dysfunction. Similarly, reductions in the 0.1 Hz component of capillary blood flow variation in dorsal skin of the ring finger have been detected in TIDM and TIIDM patients with and without signs of cardiac parasympathetic neuropathy and/or sensory neuropathy (Meyer et al., 2003). For this reason, Meyer et al. (2003) suggested that monitoring changes in sympathetic nerve-mediated vasomotion provide a sensitive technique for detecting sympathetic dysfunction.

3.2. Role of modified neural control of skin blood flow in the development of diabetic foot

Changes in neural control of skin blood flow have been suggested to contribute to the poor nutrition of skin that contributes to the development of diabetic foot (Chao & Cheing, 2009). The hemodynamic hypothesis proposes that reduced arterial vasoconstriction increases capillary blood flow resulting in increased hydrostatic pressures and shear stress and that these changes induce basement membrane
thickening (microangiopathy) as an injury response (Tooke, 1995). The alternative capillary steal hypothesis proposes that increased blood flow through the AVAs reduces blood flow through the capillaries, resulting in reduced supply of nutrients to skin. However, the issue as to whether diabetes increases or decreases capillary blood flow remains controversial (Chao & Cheing, 2009).

Several studies have demonstrated that diabetes increases capillary blood flow. For example, in a group of diabetic patients with peripheral and autonomic neuropathy (but no signs of peripheral vascular disease), Flynn et al. (1988) observed a marked increase in AVA blood flow in plantar skin but they also demonstrated an increase in capillary blood flow in nail fold skin. They therefore concluded that AVA shunting does not reduce capillary blood flow. Similarly, Netten et al. (1996) reported that TIDM patients, with sensory neuropathy and reduced sympathetic nerve-mediated vasoconstriction in their feet, had increases in both AVA and capillary blood flow. In accord with the hemodynamic hypothesis, direct measurements of capillary pressures in finger nail fold skin revealed higher pressures in TIDM patients than in healthy control subjects (Sandeman et al., 1992). This change could be detected in patients that had diabetes for less than 1 year, indicating that changes in capillary pressure could precede the development of microangiopathy.

As indicated earlier in this section, there is clear evidence that diabetes can increase AVA blood flow. Jorneskog and Flagrell (1996) reported that capillary blood flow in TIDM patients was reduced in the nail fold skin of the big toe compared to that in healthy controls. No differences were detected in capillary blood flow in the nail fold of the fourth finger. The difference between the studies of Jorneskog and Flagrell (1996) and Flynn et al. (1988) may be explained by skin temperature, which did not differ between the control and diabetic subjects in the former study, but was 5°C higher in the diabetics subjects in the latter study (Jorneskog & Fagrell, 1996). In TIDM
patients with peripheral vascular disease, there was marked reduction in capillary blood flow in nail fold skin of the big toe but total blood flow in the skin microcirculation was normal (Jorneskog et al., 1995). It was concluded that sufficient blood reaches the skin but, owing to AVA shunting, it does not pass through the capillaries. The oxygenation of venous blood in the feet has been reported to be higher in diabetic patients with neuropathy and foot ulceration than in both healthy controls and diabetic patients without complications (Boulton et al., 1982). This difference is also suggested to be due to AVA shunting, with less oxygen uptake in skin. In summary, while changes in skin blood flow almost certainly contribute to etiology of diabetic foot, the precise mechanisms remain uncertain. Furthermore, the findings presented above appear to suggest that changes in capillary blood flow may depend on the degree of skin function impairment.

3.2.1. Evidence for sympathetic denervation in feet of human diabetics

While the changes in sympathetic nerve-mediated vasoconstriction observed in diabetic patients have been attributed to the loss of perivascular nerve fibers, there are no published studies that have directly demonstrated that diabetes produces denervation of the vasculature. Indirect evidence for sympathetic denervation was reported in a neurochemical study which strongly suggests that this occurs in the feet of diabetics with neuropathy. After loading the sympathetic nerve terminals with tritiated noradrenaline, Tack et al. (2002) measured the rate of tritium entry into the local venous circulation (noradrenaline spillover) in the arms and feet of normal subjects and diabetic patients with painful peripheral neuropathy of the feet but without evidence of generalized autonomic neuropathy. Comparisons were also made between diabetic control patients without neuropathy and diabetic control patients with painless neuropathy. Compared to both the normal subjects and the diabetic control subjects, there were no differences in tritiated noradrenaline spillover in the
arms, but noradrenaline spillover in the feet was significantly reduced in the patients with painful neuropathy. Following ganglionic blockade to silence the postganglionic sympathetic neurons, tritiated noradrenaline spillover in the arms and feet of normal subjects was reduced. However, in diabetic subjects with painful neuropathy, ganglionic blockade only reduced spillover in the arms and did not change the already low levels in the feet.

To further assess sympathetic innervation of the feet, Tack et al. (2002) used positron emission tomography scanning to image uptake of 6-[¹⁸F]fluorodopamine in the feet of their subjects (Figure 1.3). This agent, which was delivered intravenously, is a substrate for the neuronal norepinephrine transporter and is selectively accumulated in sympathetic nerve terminals. Uptake of 6-[¹⁸F]fluorodopamine in the feet of diabetic patients with painful neuropathy was greatly reduced compared to that in normal subjects (Figure 1.3). In addition, inhibition of the neuronal norepinephrine transporter with desipramine reduced uptake of 6-[¹⁸F]fluorodopamine in the feet of normal subjects but did not significantly change that in the feet of diabetic patients with painful neuropathy. Together, the findings of Tack et al. (2002) provide strong neurochemical evidence for sympathetic denervation in the feet of diabetic patients with painful diabetic neuropathy.
**Figure 1.3.** Neurochemical assessment of sympathetic innervation in the feet of control subjects and diabetics with painful neuropathy. Accumulation of 6-\[^{18}\text{F}]\text{Fluorodopamine}\) in sympathetic nerve terminals via the norepinephrine transporter was imaged using positron emission tomography in the feet of normal subjects (A) and diabetics with painful neuropathy (B). Compared to control subjects, a reduction in the accumulation of 6-\[^{18}\text{F}]\text{Fluorodopamine}\) was measured in the feet of diabetics with painful neuropathy suggesting a reduction in sympathetic innervation (i.e. denervation). Image reproduced from Tack et al. (2002).
4. Diabetes impairs sympathetic control of sweat glands in the skin

The amount of sweat that is produced by activation of cholinergic sympathetic axons supplying sweat glands (sudomotor function) is commonly used as an assessment of sympathetic nerve function in skin (Low, 2004). In clinical tests, sweat production can be evoked by: (1) electrical stimulation of nerves (commonly the tibial or median nerves) or by sympathetic arousal (sympathetic skin response); (2) ionophoresis of acetylcholine into skin, which stimulates sweating at an adjacent site by evoking an axon reflex in the terminals of cholinergic sympathetic neurons that supply sweat glands at both the application and assay sites (quantitative sudomotor axon reflex test [QSART]) (Low, 2004). Using these tests, a number of studies have reported that diabetes can impair sudomotor function in the skin of the lower extremities compared to the upper limbs, and that these deficits are detectable in patients with no overt signs of sensory nerve deficits or other complications (Low et al., 1983; Kennedy & Navarro, 1989; Maselli et al., 1989; Hoeldtke et al., 2001; Sun et al., 2008). Because QSART assesses a locally evoked axon reflex, it can be used to quantify the regional extent of autonomic dysfunction. For instance, diabetic patients with early signs of neuropathy had reduced sweat production in the feet compared to the lower part of the leg, suggesting that diabetes affects sympathetic postganglionic neurons with longer axons first (Shimada et al., 2001). It is possible that cholinergic sympathetic neurons are more vulnerable to the effects of diabetes than noradrenergic sympathetic neurons. However, in T1IDM patients with and without signs of neuropathy, there was a positive correlation between the reductions in both posturally evoked vasoconstriction and the sympathetic skin response, suggesting that adrenergic and cholinergic fibers are simultaneously affected (Cacciatori et al., 1997). A combination of tests assessing sudomotor function and sympathetic nerve-mediated vasoconstriction in the skin has been proposed for the early detection of sympathetic dysfunction in diabetic patients (Hilz et al., 2000; Asahina et al., 2008).
4.1. Effects of diabetes on neural control of other vascular beds in humans

Very few studies have investigated the effects of diabetes on neural control of blood flow in vascular beds other than those in the skin of the hand and foot. In TIDM patients with and without postural hypotension (a sign of autonomic neuropathy), the proportional reductions in blood flow in the superior mesenteric artery produced by tilting from supine to vertical were similar to those in healthy control subjects (Purewal et al., 1995). This finding suggests that dysfunction of vasoconstrictor neurons supplying the splanchnic vascular bed does not contribute to the development of postural hypotension in these diabetic patients.

Forearm blood flow (primarily reflecting skeletal muscle blood) was found to be increased in type I diabetics without complications compared to healthy controls (Vervoort et al., 1999). In this group of diabetic patients, arterial plasma noradrenaline levels were lower than those of the controls but no differences in endothelial function in the forearm were detected between these groups. The reduction in plasma noradrenaline concentration was interpreted to be due to a decrease in whole-body sympathetic nerve activity in the diabetic patients. Vervoort et al. (1999) also demonstrated that the reduction in forearm blood flow produced by intrarterially-infused noradrenaline was increased in diabetic patients, a change they attributed to denervation-induced supersensitivity of the vascular muscle. They therefore concluded that the increase in blood flow is due to a decrease in sympathetic nerve-mediated vasoconstriction, which was most likely due to sympathetic denervation.

Cerebrovascular resistance is under autonomic modulation and impaired neural control of cerebral blood flow may contribute to the orthostatic intolerance seen in diabetic patients. Using measurements of blood flow variation in the middle cerebral artery during a head up tilt, it was demonstrated that the increase in power of the low frequency of component of variation (i.e. at ~0.1 Hz) was smaller in long-term TIIDM
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patients with vascular and autonomic abnormalities than in healthy controls (Cencetti et al., 1999). This was suggested to be due to a reduction in baroreflex-mediated control of cerebrovascular blood flow, although the authors could not exclude the possibility that the changes were due, at least in part, to stiffening of the arterial vessels due to atherosclerosis.

On the basis of these combined observations it is difficult to establish a general picture of the effects of diabetes on sympathetic neural control of the vasculature. However, the findings summarized above appear to suggest that the neural control of the cutaneous vasculature, particularly in the feet, is especially vulnerable to the effects of diabetes. This may be due to diabetes having a greater effect on sympathetic neurons with long axons (Vinik et al., 2003).

5. Animal models of diabetes

A number of animal models have been developed to study T1DM and T2DM, which have been reviewed in detail by Rees and Alcolado (2005). The effects of diabetes on NVT and peripheral neurons have primarily been investigated in models of T1DM where the β-cells in the pancreas are selectively destroyed by alloxan in rabbits or streptozotocin (STZ) in rodents. There are also genetic models of type I (e.g. non-obese diabetic mice, bio breeding rats) and type II (e.g. Ob/Ob mice, Zucker rats, Goto Kakizaki rats) diabetes, but these have not yet been used to study changes in NVT or neuropathy in detail. While diabetes has been reported to modify endothelial and vascular smooth muscle function of resistance arteries (Wigg et al., 2001; Wigg et al., 2004), the following sections will primarily focus on the effects of diabetes on sympathetic control of the vasculature and on sympathetic nerve morphology in animal models of diabetes.
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6. Effects of experimental diabetes on sympathetic neurons supplying arteries

In the following section, the small number of animal studies that have investigated the effects of diabetes on perivascular sympathetic innervation and/or NVT are summarized.

6.1. Rat tail artery

The only artery from the cutaneous vascular bed where the effects of diabetes have been studied is the rat tail artery. This vessel supplies blood to the skin of the tail and plays an important role in thermoregulation (Rand et al., 1965). The reported effects of diabetes on neural activation of the rat tail artery differ between studies. Hart et al. (1988) reported that 8 weeks of diabetes produced a small reduction in sympathetic nerve-mediated vasoconstriction. In contrast, Weber and MacLeod (1994) and Speirs et al. (2006) reported that 12 weeks of diabetes produced no change in sympathetic nerve-mediated vasoconstriction. Speirs et al. (2006) also assessed the perivascular sympathetic innervation of the tail artery and demonstrated no differences between vessels from control and diabetic rats.

Morrison et al. (2004) used high performance liquid chromatography to measure the concentration of noradrenaline in the proximal, middle and distal portions of the rat tail artery between 7.5 and 42 weeks after the induction of diabetes with STZ. They found increased noradrenaline contents in all portions of the tail artery, but the most marked and persistent changes occurred in the distal portion. Because the distal part of the tail artery is innervated by sympathetic neurons with the longest axons (Sittiracha et al., 1987; Anderson & McLachlan, 1991), the more marked increase in noradrenaline content in this region of the tail artery might suggest that these neurons are most susceptible to the effects of diabetes.
6.2. Rat mesenteric arteries

In the perfused mesenteric arterial bed, 12 weeks of STZ-induced diabetes reduced sympathetic nerve-mediated pressor responses (i.e. vasoconstrictions; Ralevic et al., 1995) but this effect was not apparent after 8 weeks (Ralevic et al., 1993). In addition to their sympathetic innervation, mesenteric arteries also receive an extensive innervation by peptidergic sensory axons which, when activated, produce vasodilation (see section 2.2.2 above). After 8 weeks of diabetes, the vasodilator effect of the peptidergic sensory axons was reduced (Ralevic et al., 1993). While it was assumed that the observed effects of diabetes were due to neuropathy, the innervation of the small mesenteric arteries was not histologically assessed. Therefore it is not possible to conclude conclusively that the reduced effectiveness of the sympathetic and peptidergic sensory innervations is due to nerve loss. Indeed, Belai et al. (1996) reported 8 weeks of STZ-induced diabetes produced no changes in the sympathetic innervation of the superior mesenteric artery, and an increase in both the density and fluorescence intensity of the substance P- and CGRP-immunolabeled perivascular sensory axons. While the perfused mesenteric arterial bed preparation includes the superior mesenteric artery, its large lumen diameter means it is unlikely to contribute significantly to the nerve-evoked changes in perfusion pressure recorded.

6.3. Rabbit carotid artery

The only other artery where the effects of diabetes on NVT have been studied is the carotid artery from rabbits made diabetic with alloxan. Six weeks after the induction of diabetes, nerve-evoked constrictions of the carotid artery were not different from those of age-match controls (Cohen et al., 1990). However, Cohen et al. (1990) concluded that the arteries from diabetic rabbits were partially denervated because: (1) they accumulated less tritiated noradrenaline; (2) they had increased sensitivity to noradrenaline in the absence, but not in the presence, of the neuronal
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Norepinephrine transporter inhibitor, cocaine; (3) in the presence of cocaine, nerve-evoked contractions were smaller than control. These findings reveal a decrease in norepinephrine transporter activity and this was interpreted as indicating partial denervation. However, their conclusion was not backed up by histological data.

7. Evidence for neuropathy of sympathetic axons in animal models of diabetes

7.1. Skin

Karanth et al. (1990) did not find evidence for diabetes-induced changes in the perivascular sympathetic axons present within the footpad skin of STZ-treated rats. In the plantar skin of STZ-induced diabetic rats, however, there are reductions in the numbers of CGRP fibers in the epidermis and dermis (Karanth et al., 1990) and sensory nerve-mediated vasodilation is reduced in hind paw skin (Bennett et al., 1998).

7.2. Abdominal prevertebral ganglia

The most extensive morphological studies of the effects of diabetes on sympathetic neurons in STZ-treated rats have been conducted by Schmidt and co-workers (1981; 1982; 1984; 1986; 1988). These studies investigated the effects of diabetes on the postganglionic sympathetic neurons that supply the ileum and colon, which have their cell bodies within the abdominal prevertebral ganglia (the celiac and superior mesenteric ganglia). The axons of prevertebral neurons reach the intestine via the paravascular mesenteric nerves, which run parallel to the mesenteric arteries that supply blood to the gut. The paravascular mesenteric nerves also contain the sympathetic axons that supply the mesenteric arteries (Hill et al., 1985).

Long-term STZ-induced diabetes (4-6 months onwards) produced swellings (neuroaxonal dystrophy) of axons located within the paravascular mesenteric nerves supplying the distal ileum and the colon (Schmidt et al., 1981; Schmidt & Scharp, 1982; Clark & Schmidt, 1984; Schmidt et al., 1988) (Figure 1.4). Importantly, while
immunohistochemical studies showed that sympathetic axons containing tyrosine hydroxylase (TH; Figure 1.4) and dopamine β-hydroxylase (without NPY or somatostatin) developed neuroaxonal dystrophy, those that also contained somatostatin or NPY did not develop axon swellings (Schmidt et al., 1988). Somatostatin is present in the subpopulations of sympathetic neurons which innervate the intramural ganglia and mucosa of the intestine (Costa & Furness, 1984), and NPY is present in sympathetic neurons that supply the mesenteric and intestinal vasculature (Schmidt et al., 1988). The sympathetic axons within the perivascular nerve plexus around the mesenteric arteries did not show signs of neuroaxonal dystrophy up to 1 year following the induction of diabetes (Clark & Schmidt, 1984; Schmidt et al., 1988).

Electron microscopy revealed that chronic diabetes did not reduce the number of axons present in each nerve fascicle within the paravascular mesenteric nerves (Schmidt & Plurad, 1986). Chronic diabetes also did not change the number of sympathetic neurons present in the superior mesenteric ganglion (Schmidt, 2001). Therefore no evidence was found to suggest that diabetes causes loss of sympathetic neurons. However, electron microscopy has revealed that diabetes produced neuroaxonal dystrophy of axons within the superior mesenteric ganglion and that some postganglionic neurons within this ganglion possessed dendrites that were dilated by distinctive branched tubular aggregates (Schmidt & Plurad, 1986). Similar changes have been observed in superior mesenteric ganglia obtained post-mortem from diabetic humans (Schmidt et al., 1993). In STZ-treated rats, the number of axons with neuroaxonal dystrophy within the paravascular mesenteric nerves and the superior mesenteric ganglion increased with the duration of diabetes (Schmidt & Scharp, 1982; Clark & Schmidt, 1984; Schmidt & Plurad, 1986).
Figure 1.4. Sympathetic axons within perivascular and paravascular nerves supplying mesenteric arteries and intestine in control and STZ-induced diabetic rats. Immunolabeling for tyrosine hydroxylase (A-D) reveals sympathetic fibers in the perivascular (arrows in A, B) and paravascular (A-D; indicated by arrowheads in A, B) nerve bundles in control (A, B) and diabetic rats (C, D). The arrows in C and D demonstrate diabetes-induced swellings in sympathetic axons in the paravascular nerves. Magnification for images as follows: A at 100x; B at 415x; C and D 800x. Images reproduced from Schmidt et al. (1988).
Although STZ-induced diabetes produced a decrease in dopamine β-hydroxylase activity measured in the terminals of sympathetic axons within the colon, no changes were detected within the celiac and superior mesenteric ganglia, where their cell bodies are located (Schmidt et al., 1981). This suggested that the distal axons are selectively affected by diabetes (distal axonopathy; Schmidt et al., 1981). In further support of this conclusion, neuroaxonal dystrophy was observed in the long axons supplying the distal ileum (12-15 cm long) but not in the shorter axons supplying the jejunum (2-4 cm long) (Schmidt & Plurad, 1986). In addition, within the paravascular mesenteric nerves supplying the distal ileum, more abnormal axons were found close to the intestine compared to a more a proximal region close to the superior mesenteric ganglion (Schmidt & Scharp, 1982).

The development of neuroaxonal dystrophy within the paravascular mesenteric nerves and/or superior mesenteric ganglion has also been observed in BB/Wor (Bio-breeding/Worcester) T1DM rats (Yagihashi & Sima, 1986; Schmidt et al., 2004) and non-obese type I diabetic (NOD) mice (Schmidt et al., 2003a). In contrast, in two models of T1IDM, Zucker diabetic fatty rats and Bio-breeding Zucker diabetic/Worcester rats, neuroaxonal dystrophy was not detected in the superior mesenteric ganglion (Schmidt et al., 2003b; Schmidt et al., 2004). However, the number of dystrophic axons increased in the paravascular mesenteric nerves of Bio-breeding Zucker diabetic/Worcester rats but they were substantially less frequent than in age-matched STZ-treated rats. As the blood glucose levels in these models of T1IDM are similar to those in STZ-treated rats, these findings suggest that hyperglycemia may not be the only factor producing this neuropathy.

7.3. Penile tissues

Erectile dysfunction is an early sign of diabetic autonomic neuropathy. Using glyoxylic acid-induced catecholamine fluorescence, Felten et al. (1983) reported that 4
months of STZ-induced diabetes produced a reduction in the number and intensity of noradrenergic varicosities within the corpus cavernosum, corpus spongiosum and the perivascular nerve plexuses of blood vessels in the rat penis. This decrease was paralleled by a decrease in the noradrenaline content of the penis. In contrast, Morrison et al. (2007a; 2007b) observed an increased intensity of immunolabeling for TH in the noradrenergic fibers innervating the corpus cavernosum of STZ-treated rats (Figure 1.5 A, B) and this was accompanied by an increase in the noradrenaline content of this tissue between 10 and 42 weeks after STZ treatment (Morrison et al., 2007b). There was also an increase in the noradrenaline content in the glans penis (Morrison et al., 2007b). Additionally, they observed that the TH immunoreactive nerve fibers in the corpus cavernosum were dilated and had engorged endings (Figure 1.5 A, B). At least some of the striking differences between the studies of Felten et al. (1983) and Morrison et al. (2007a; 2007b) might be explained by the different rat strains used, as the former used Sprague-Dawley rats whereas the latter used Wistar rats. Increased noradrenaline content in the penis has also been described in the corpus cavernosum of 52-week-old Goto-Kakizaki rats (Morrison et al., 2009) and of 65- to 70-week-old monosodium-glutamate-treated rats (Morrison et al., 2008), both models of T1IDM.

7.4. Seminal vesicles

As with the corpus cavernosum, the intensity of immunolabeling for TH in the noradrenergic fibers innervating the seminal vesicles was increased in rats with STZ-induced diabetes (Morrison et al., 2006). There was also an increase in the noradrenaline content of the seminal vesicles between 7 and 34 weeks after STZ treatment (Morrison et al., 2006).

7.5. Vas deferens

Nerve-evoked contractions of vasa deferentia from rats with 6 months of STZ-induced diabetes are reduced in size (Tomlinson et al., 1982). Electron microscopy
revealed that approximately half the axons containing dense-cored vesicles (i.e. sympathetic axons) appeared normal whereas the remaining axons were swollen, had fewer dense-core vesicles and were almost devoid of other organelles (Tomlinson et al., 1982).

7.6. Pineal gland

The pineal gland is innervated by neurons in the superior cervical ganglion (Bowers et al., 1984; Reuss, 1986; Hesp et al., 2012). Normally, the release of noradrenaline from sympathetic terminals innervating the pineal gland stimulates the production of melatonin (for review see Simonneaux & Ribelayga, 2003). Melatonin is involved in many biological functions including the circadian rhythm and inhibition of insulin secretion (for review see Peschke & Muhlbauer, 2010). The production of melatonin by the pineal gland is increased in rats made diabetic with STZ, but there is no change in the circadian pattern of melatonin release (Peschke et al., 2008). In the pineal gland of rats with ≥4 weeks of STZ-induced diabetes, both light and electron microscopy revealed swollen sympathetic (TH) axons that lacked clearly-defined varicosities (Figure 1.5 C, D; Tsai et al., 2008). The protein expression levels of TH, however, were not changed by diabetes (Tsai et al., 2008).
Figure 1.5. Sympathetic nerves, revealed by immunolabeling for tyrosine hydroxylase, in the corpus cavernosa and pineal gland from control and STZ-induced diabetic rats. In the corpus cavernosa (A, B) and the pineal gland (C, D), immunolabeling for tyrosine hydroxylase demonstrated the presence of swollen sympathetic axon terminals innervating tissues from diabetic rats (B, D) compared to that of controls (A, C). Images reproduced from Morrison et al. (2007b) (A, B) and Tsai et al. (2008) (C, D).
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8. Possible mechanisms underlying diabetes-induced changes in sympathetic axons

The proposed mechanisms by which diabetes produces neuropathy of sympathetic axons include altered trophic factor signaling, impaired axon transport, changes in gene expression and/or oxidative stress.

8.1. Impaired trophic factor signaling

8.1.1. Nerve growth factor

Nerve growth factor (NGF) is synthesized by effector tissues, and after binding to TrkA and p75 neurotrophin (p75\textsuperscript{NTR}) receptors on sympathetic nerve terminals is internalized and retrogradely transported to the cell body (Luther & Birren, 2009). Chronic STZ-induced diabetes reduces NGF protein expression levels in a wide range of sympathetically innervated tissues (Hellweg & Hartung, 1990; Fernyhough et al., 1994). There is also a reduction in the retrograde transport of NGF within the sciatic nerve (Hellweg et al., 1994; Delcroix et al., 1997). As approximately 25% of the unmyelinated axons in the sciatic nerve are of sympathetic origin (Chad et al., 1983), it is likely that retrograde transport of NGF in these axons is affected by diabetes. However, the majority of evidence for altered NGF signaling in sympathetic neurons is derived from studies investigating the effects of diabetes on the splanchnic innervation.

In the ileum, NGF content is increased in STZ-rats made diabetic for 1 month (Schmidt et al., 2000). In addition, diabetes produced a substantial increase in the length (Schmidt et al., 1985) and weight (Schmidt et al., 1981) of ileum so that the total amount of NGF available within the gut wall for uptake into sympathetic neurons increased. Assessed both \textit{in vivo} and \textit{in vitro} the retrograde transport of \textsuperscript{125}I-NGF is reduced in paravascular mesenteric nerves of STZ-induced diabetic rats (Schmidt et al., 1985; Schmidt et al., 1986). In addition, there was a reduction in the accumulation of intravenously administered \textsuperscript{125}I-NGF in the superior mesenteric ganglia of diabetic rats.
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(Schmidt et al., 1985). In contrast, the total content of NGF in the superior mesenteric and celiac ganglia increased in rats with up to 7 months of STZ-induced diabetes (Schmidt et al., 2000). This change was suggested to be due to the increased availability of NGF in the intestine. No changes were detected in TrkA and p75NTR receptor protein expression levels in superior mesenteric and celiac ganglia from rats with STZ-induced diabetes (Schmidt et al., 2000). Treatment of rats with NGF resulted in a further increase in the NGF content of the superior mesenteric and celiac ganglia, but did not prevent the development of dystrophic sympathetic axons within the paravascular mesenteric nerves (Schmidt et al., 2001a). Furthermore, control rats treated with anti-NGF antibodies (i.e. deprived of NGF) failed to develop dystrophic axons in the paravascular mesenteric nerves (Schroer et al., 1995). Together these findings indicate that reduced NGF signaling is not the cause of the dystrophic sympathetic axons. Indeed, it has been suggested that the increased level of NGF in the prevertebral ganglia may be the trigger for diabetes-induced changes in the postganglionic sympathetic nerve axons (Schmidt et al., 2000).

8.1.2. Neurotrophin-3

Adult postganglionic sympathetic neurons have a requirement for neurotrophin-3 (NT-3) for normal function (Walker et al., 2009). While NT-3 usually signals via TrkC receptors, in sympathetic neurons NT-3 appears to mediate its effects via both TrkA and TrkC receptors (Belliveau et al., 1997). Synthesis of NT-3 has been demonstrated in both vascular smooth muscle (Donovan et al., 1995) and the rat colon (Liu et al., 2010). In the rat colon, synthesis of NT-3 is decreased by STZ-induced diabetes (Liu et al., 2010). Both anterograde and retrograde transport of NT-3 in the sciatic nerve is also reduced by STZ-induced diabetes (Fernyhough et al., 1998). However, treatment with NT-3 did not decrease the formation of dystrophic sympathetic axons in paravascular mesenteric nerves of rats with STZ-induced diabetes (Schmidt et al., 2001a).
8.1.3. *Insulin-like growth factor 1*

Insulin-like growth factor 1 (IGF-1) has a molecular structure similar to insulin and plays an important role in sympathetic neuron development (DiCicco-Bloom & Black, 1988; Zackenfels *et al.*, 1995). IGF-1 has also been shown to promote regeneration of both somatic motor and sensory axons (Ishii, 1995). The circulating and tissue levels of IGF-1 are reduced in rats with STZ-induced diabetes (Bornfeldt *et al.*, 1989). Reductions in the plasma level of IGF-1 have also been demonstrated in humans with TIDM and TIIDM (Tan & Baxter, 1986). On the basis of these findings, a role for IGF-1 in the pathogenesis of diabetic neuropathy has been suggested (Ishii, 1995).

A 2-month treatment of chronically diabetic rats (i.e. starting 6 months post-STZ) with IGF-1 greatly reduced the frequency of dystrophic axons in the paravascular mesenteric nerves (Schmidt *et al.*, 1999). Interestingly, Zucker Diabetic Fatty rats, which while severely hyperglycemic do not develop dystrophic axons along the paravascular mesenteric nerves, do not exhibit changes in circulating levels of IGF-1 (Schmidt *et al.*, 2003b). These findings suggest that loss of tropic support from IGF-1, or perhaps insulin, may be a primary cause of neuroaxonal dystrophy of sympathetic neurons supplying the intestine (Schmidt *et al.*, 2003b).

8.2. *Biochemical mechanisms of hyperglycemia-induced neuropathy*

Several studies have suggested an important role for oxidative stress in the pathogenesis of diabetic neuropathy (for review see Figueroa-Romero *et al.*, 2008). Four biochemical mechanisms have been proposed to explain how hyperglycemia leads to production of reactive oxygen species (e.g. superoxide) (Brownlee, 2001): 1) the formation of advanced glycation end-products; 2) increased polyol pathway flux; 3) increased hexosamine pathway flux; 4) increased synthesis of diacyl glycerol leading to hyperstimulation of protein kinase C. The roles of the hexosamine pathway and
protein kinase C in the effects of diabetes on sympathetic neurons have not been investigated. However, the possibility that reactive oxygen species, advanced glycation end-products, and increased polyol pathway flux contribute to the effects of diabetes on sympathetic neurons have been investigated and this evidence is briefly summarized below.

8.2.1. Reactive oxygen species

In rats, STZ-induced diabetes increased both TH activity and TH immunolabeling in the superior mesenteric and celiac ganglia (Schmidt & Cogswell, 1989; Semra et al., 2006). In superior mesenteric and celiac ganglia isolated from normal rats, the induction of oxidative stress with menadione increased the number of neurons that had strong TH immunoreactivity. However, this stimulus also caused a loss of neuronal viability and increased TUNEL staining (a marker for apoptosis). This effect of oxidative stress on neuronal cell viability is not paralleled in STZ-treated rats where there is no evidence of sympathetic neuron loss within the superior mesenteric and celiac ganglia even during prolonged periods of diabetes (Schmidt & Plurad, 1986). In contrast, in the myocardium of the heart where STZ-induced diabetes produced a reduction in the number of catecholamine containing nerve fibers, treatment with the antioxidant vitamin E from the time of STZ treatment completely prevented this loss of nerve fibers (Rosen et al., 1995). In summary, these findings suggest the contribution of reactive oxygen species in the pathogenesis of diabetic neuropathy varies between tissues.

8.2.2. Advanced glycation end-products

Advanced glycation end-products (AGEs) form within the extracellular matrix of the sciatic nerve endoneurium as early as 3 weeks post-STZ treatment and this is suggested to contribute to poor collateral sprouting and regeneration of sensory neurons within the sciatic nerve of diabetic rats (Duran-Jimenez et al., 2009).
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Administration of aminoguanidine, which inhibits the formation of AGEs, to cultured sensory neurons grown on glycated extra-cellular matrix proteins improved neurotrophin-stimulated neurite outgrowth (Duran-Jimenez et al., 2009). This finding provides evidence for a potential role of AGEs in the development of sensory nerve dysfunction. However, present evidence indicates that AGEs are not involved in the development of sympathetic neuroaxonal dystrophy within the superior mesenteric ganglia as this was not reduced in STZ-induced diabetic rats treated with aminoguanidine (Schmidt et al., 1996).

8.2.3. Polyol pathway

In both TIDM and TIIDM, hyperglycemia increases metabolism of glucose by aldose reductase, which is the first enzyme in the polyol pathway. Aldose reductase converts glucose to sorbitol, which is then converted to fructose by sorbitol dehydrogenase. The precise mechanism(s) whereby increased flux of glucose through the polyol pathway results in neuropathy remains open to debate. However, a popular concept is that the polyol pathway is linked through its co-enzymes (NADP and NADPH) to other intracellular pathways, changing many metabolic processes (Oates, 2002). Importantly, aldose reductase is expressed in postganglionic sympathetic neurons where it plays a role in the metabolism of noradrenaline (Kawamura et al., 1999). Notably, other peripheral neurons do not express aldose reductase although it is present in satellite glial cells in dorsal root ganglia and myelinating Schwann cells in nerve trunks (Jiang et al., 2006). Therefore sympathetic neurons may be particularly vulnerable to the neurotoxic effects of stimulating metabolism of glucose by aldose reductase.

Increased flux of the polyol pathway has been investigated as a possible mechanism by which hyperglycemia mediates the development of dystrophy in sympathetic nerves innervating the ileum. The concentration of sorbitol and fructose is
increased in both the celiac ganglia and superior cervical ganglion from rats made diabetic with STZ and Zucker diabetic fatty rats (Schmidt et al., 2001b). Rats with STZ-induced diabetes receiving an aldose reductase inhibitor (zopolrestat) had fewer dystrophic sympathetic axons in the paravascular mesenteric nerves (Schmidt et al., 1998), supporting a role for the polyol pathway in mediating the neurotoxic effects of hyperglycemia on sympathetic neurons. In contrast, treatment with a sorbitol dehydrogenase inhibitor (SDI-158) increased the frequency of dystrophic axons within the paravascular mesenteric nerves (Schmidt et al., 1998). No dystrophic axons were detected in control rats treated with SDI-158 (Schmidt et al., 1998). Lastly, the co-administration of aldose reductase inhibitor (sorbinil) and SDI-158 produced a similar reduction in the frequency of dystrophic axons in the mesenteric paravascular nerves to that produced by sorbinil alone (Schmidt et al., 2001b). Together, these findings suggest a key role for sorbitol in mediating the neurotoxic effects of hyperglycemia on sympathetic neurons. The polyol pathway also appears to be involved in the reduction of neuronal noradrenaline uptake (a putative indicator of sympathetic denervation) in the heart of STZ-induced diabetic rats, as this was prevented by treatment with the aldose reductase inhibitor, epalrestat (Kurata et al., 1997).

8.3. Changes in Schwann cell support of sympathetic neurons

Schwann cells wrap around sympathetic fibers to maintain axonal function. No morphological changes were detected in Schwann cells wrapping both normal and dilated axons within the paravascular mesenteric nerves (Schmidt & Scharp, 1982). However, the number of Schwann cells, but not the number of axons, present per fascicle within the paravascular mesenteric nerves increases after 13-15 months of diabetes (Schmidt & Plurad, 1986). This increase in Schwann cell number may be triggered by hyperglycemia (Almhanna et al., 2002) or be part of a regenerative
response like that seen after a crush injury (Romine et al., 1976) or nerve transection (Ribeiro-Resende et al., 2009).

It is possible that diabetes impairs neurotrophin production from Schwann cells, and that this in turn impairs axon function of sympathetic neurons. Twelve weeks post-STZ produced a reduction in NT-3 and neurotrophin-4 (NT-4) mRNA expression in the sciatic nerve of diabetic rats suggesting that the trophic production of both neurotrophins from support cells (most likely Schwann cells) was reduced (Rodriguez-Pena et al., 1995).

9. Human clinical trials

Despite the wide range of organs affected by diabetic autonomic neuropathy, current treatment methods are primarily based on better glycemic control and the management of complications (Kamenov & Trajkov, 2012). Diabetes-induced alterations in biochemical pathways determined from in vitro and in vivo studies have led to clinical trials assessing the efficacy of inhibitors for aldose reductase in the polyol pathway (Schemmel et al., 2010), protein kinase C isoforms (Vinik et al., 2005; Casellini et al., 2007; Brooks et al., 2008) and oxidative stress (Tutuncu et al., 1998; Valensi et al., 2005; Laczy et al., 2009; Ziegler et al., 2009; Ziegler et al., 2011; Bertolotto & Massone, 2012; Hernandez-Ojeda et al., 2012). The results of such trials have either resulted in no, or limited, beneficial effects in diabetic patients. Human clinical trials investigating the effects of treatments directly involved in the hexosamine pathway and the formation of AGEs have not yet been performed.

Several experimental studies have provided evidence for impaired neurotrophin signaling in both autonomic and sensory diabetic neuropathy (see section 8.1 above). In particular, tissue production of NGF is thought to be impaired in diabetes and therefore administration of NGF was reasoned as a method to treat diabetic neuropathy (Apfel et al., 1998). A preliminary study reported diabetic patients
with peripheral neuropathy had improved sensory nerve function after treatment with recombinant human NGF for 6 months (Apfel et al., 1998). However, a large-scale randomized control trial assessing the efficacy of recombinant human NGF showed that even though administration of the neurotrophin was safe, it failed to provide a beneficial effect on diabetic neuropathy (Apfel et al., 2000). Additionally, a preclinical trial assessing the treatment of human diabetics with recombinant human BDNF concluded that supplementation of this neurotrophin did not result in improved sensory and autonomic nerve function (Wellmer et al., 2001). Together, these results show that although defects in neurotrophin signaling are present in diabetes, supplementation of individual growth factors does not improve diabetic neuropathy.

10. Effects of diabetes on neurovascular transmission – questions left unanswered

Apart from tight glycemic control, there are currently no effective treatments that significantly reverse or slow the progression of established diabetic neuropathy (including diabetic autonomic neuropathy). To date diabetic autonomic neuropathy has not been a specific target for drug trials targeting neuropathy and this is likely to reflect the complexities in the clinical diagnosis of autonomic neuropathy (Kamenov & Traykov, 2012). For this reason, most clinical trials and experimental studies have targeted changes in sensory and motor nerve function. There is, however, growing evidence that early impairment of sympathetic nerve-mediated control of cutaneous arteries contributes to the development of peripheral vascular disease in skin of diabetic humans. While there are clear demonstrations that diabetes reduces sympathetic nerve-mediated vasoconstriction in arterial vessels supplying the skin of the feet, the mechanisms that produce these changes have not been identified. It has been hypothesized that the primary cause is loss of perivascular sympathetic axons (Stevens & Watkins, 1991) but there is no direct evidence to support this hypothesis. For this reason it is important to establish an experimental model of diabetes that
CHAPTER 1 EVIDENCE FOR NVT DEFICITS IN VASCULAR COMPLICATIONS OF DIABETES

demonstrates impaired sympathetic NVT in arteries supplying skin. Secondly, it is important to establish the mechanisms that impair sympathetic NVT.

The primary aim of the work presented in this thesis was to investigate the effects of diabetes on sympathetic NVT in arteries supplying blood to the plantar skin of the hind paw digits (i.e. plantar metatarsal arteries) in rats with STZ-induced diabetes. The STZ rat model of TIDM was used in this project because the majority of previous studies investigating diabetic neuropathy have used this experimental model. The rats were made diabetic for 12 weeks because sensory neuropathy is established in the hind paw by this time point (Sugimoto et al., 2008). The following hypotheses were tested:


2. Diabetes alters the sympathetic innervation of plantar metatarsal arteries and that this change is associated with altered expression of nerve-specific proteins, and in particular proteins that are putatively involved in axon remodeling.

3. Diabetes changes gene expression in the sympathetic chain ganglia containing neurons that project to the plantar metatarsal arteries.

The results presented in this thesis provide knowledge of a new experimental model of diabetic autonomic neuropathy where the functional deficits of sympathetic axons innervating plantar metatarsal arteries are correlated with markers that give an insight into possible mechanisms contributing to this deficit. A better understanding of the underlying pathology of diabetic autonomic neuropathy in experimental models will provide a basis for more successful treatments for preventing and/or reversing neuropathy in humans with diabetes.
CHAPTER 2 Streptozotocin-induced diabetes differentially affects sympathetic innervation and control of plantar metatarsal and mesenteric arteries in the rat

Preface

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Abstract

In humans neural control of arterial vessels supplying skin in the extremities is particularly vulnerable to the effects of diabetes. Here the streptozotocin (STZ) rat model of type I diabetes was used to compare effects on neurovascular function in plantar metatarsal arteries (PMAs), which supply blood to skin of hind paw digits, with those in mesenteric arteries (MAs). Twelve weeks after STZ (60 mg/kg, i.p.), wire myography was used to assess vascular function. In PMAs, lumen dimensions were unchanged but both nerve-evoked contractions and sensitivity to α₁ (phenylephrine, methoxamine)- and α₂ (clonidine)-adrenoceptor agonists were reduced. The density of perivascular nerve fibers was also reduced by ~25%. These changes were not observed in PMAs from STZ-treated rats receiving either a low dose of insulin that did not greatly reduce blood glucose levels or a high dose of insulin that markedly reduced blood glucose levels. In MAs from STZ-treated rats, nerve-evoked increases in force did not differ from control but, because lumen dimensions were ~20% larger, nerve-evoked increases in effective transmural pressure were smaller. Increases in effective transmural pressure produced by phenylephrine or α₁,β-methylene ATP in MAs from STZ-treated rats were not smaller than control, but the density of perivascular nerve fibers was reduced by ~10%. In MAs, the increase in vascular dimensions is primarily responsible for reducing effectiveness of nerve-evoked constrictions. By contrast, in PMAs decreases in both the density of perivascular nerve fibers and the reactivity of the vascular muscle appear to explain impairment of neurovascular transmission.
CHAPTER 2 | IMPAIRED NEUROVASCULAR TRANSMISSION IN DIABETES

Introduction

In humans abnormal control of the vasculature has been implicated in the etiology of many diabetes-related complications such as neuropathy and diabetic foot ulceration (Vinik et al., 2003). These changes involve endothelium dysfunction (Schalkwijk & Stehouwer, 2005) as well as deficits in both sympathetic nerve-mediated vasoconstriction (see below) and sensory nerve-mediated vasodilation (Anand et al., 1996). However, the mechanisms whereby diabetes affects sympathetic and sensory nerve regulation of the vasculature are not well understood, although it is believed that diabetes causes degeneration of these nerve supplies (Sullivan & Feldman, 2005).

Many clinical studies have evaluated the effects of diabetes on sympathetic nerve-mediated vasoconstriction of arterial vessels supplying plantar skin of the foot. These studies have demonstrated that both type I (insulin dependent) and type II (noninsulin dependent) diabetic patients with signs of sensory and/or autonomic neuropathy (e.g. orthostatic hypertension) have increased skin blood flow under basal conditions (Archer et al., 1984; Stevens et al., 1991) and attenuated reductions in skin blood flow (i.e. vasoconstriction) in response to sympathetic arousal (e.g. produced by mental arithmetic (Archer et al., 1984) or postural stimuli (Rayman et al., 1986)). Importantly, deficits in sympathetic vasoconstriction of arterial vessels supplying plantar skin can be demonstrated in both type I and type II diabetic patients at an early phase of diabetic neuropathy (Shore et al., 1994; Takahashi et al., 1998), perhaps suggesting that sympathetic neurons supplying these vessels are particularly vulnerable to the effects of diabetes. Furthermore, it has been suggested that reduced sympathetic nerve-mediated vasoconstriction of these vessels is an early change that contributes to the etiology of microangiopathy in skin of the feet (Flynn & Tooke, 1995). In contrast, although type I diabetic patients with orthostatic hypertension had increased basal blood flow in the superior mesenteric artery, posturally evoked
reductions in blood flow in this vessel were not impaired (Purewal et al., 1995), indicating that sympathetic control of arterial vessels in the splanchnic vascular bed was not detectably changed. These findings may indicate that diabetes differentially affects sympathetic neurons supplying arterial vessels in different vascular beds.

There are only a small number of studies that have investigated the effects of diabetes on sympathetic neural control of arterial vessels in animals. Most of these studies have used the streptozotocin (STZ)-treated rat model of type I diabetes and have investigated effects in the rat tail artery, a cutaneous thermoregulatory vessel with a function similar to that of digital arteries in man, and mesenteric arteries (MAs). In tail artery, Hart et al. (1988) reported that 8 weeks of diabetes produced a small reduction in sympathetic nerve-mediated vasoconstriction, whereas, Weber and MacLeod (1994) and Speirs et al. (2006) reported that 12 weeks of diabetes did not reduce sympathetic nerve-mediated vasoconstriction. In the perfused mesenteric arterial bed, diabetes reduced sympathetic nerve-mediated pressor responses after 12 weeks of diabetes (Ralevic et al., 1995) but this effect was not present after 8 weeks (Ralevic et al., 1993). In mesenteric arteries, it is not known if the decrease in the sympathetic nerve-mediated pressor response produced by diabetes is associated with degeneration of the sympathetic nerve supply but in the tail artery the perivascular sympathetic innervation is not affected by 12 weeks of diabetes (Speirs et al., 2006).

In the present study, the STZ-treated rat model of type I diabetes was used to test the hypothesis that sympathetic control of plantar metatarsal arteries (PMAs), which supply blood to skin of the hind paw digits, is particularly vulnerable to the effects of diabetes. In this vessel, I investigated whether diabetes modified sympathetic neurovascular transmission and whether the contributions of the cotransmitters, noradrenaline and ATP, to transmission were changed. I also assessed if any of the effects of diabetes could be attributed to changes in reactivity of PMAs to
α-adrenoceptor and P2X1-purinoceptor agonists or the effectiveness of the neuronal norepinephrine transporter (NET). In addition, the effects of diabetes on peptidergic sensory nerve-mediated vasodilation and the perivascular innervation of PMAs were determined. In these arteries, the effects of insulin treatment were investigated to confirm that any observed effects were due to diabetes and not to the direct toxic effects of STZ treatment. For comparison, the effects of diabetes on sympathetic and peptidergic sensory nerve-mediated control and innervation of small MAs supplying the ileal segment of the gastrointestinal tract were investigated.

Materials and Methods

All experiments were approved by the Animal Ethics Committees at the University of New South Wales (January 2010 - December 2010) and the University of Melbourne (January 2010 - March 2012) and conformed to the Australian code of practice for the care and use of animals for scientific purposes. Male Wistar rats, 8 to 10 weeks old, were purchased from Animal Resources Center (Canning Vale, WA, Australia). The rats were treated intraperitoneally with 60 mg/kg STZ dissolved in 50 mM citrate buffer (pH 4.5). Age-matched control animals were treated with an equivalent volume of citrate buffer. Blood glucose levels were measured 5-7 days post-injection using a glucose meter (Accu-Check Performa, Roche Diagnostics Australia Pty Ltd) to confirm that the rats were diabetic (>15 mmol/L glucose). Blood glucose levels and body weights were monitored weekly thereafter until they were terminated 12 to 13 weeks after the induction of diabetes. Diabetic animals were left untreated or were treated with a low (~1 unit/day) or high dose (~4 units/day) of insulin delivered by sustained-release implants containing bovine or porcine insulin (Linplant, Linshin Canada Inc.) inserted subcutaneously beneath the nuchal skin. At 1 and 7 weeks after the induction of diabetes, the low dose group received half a Linplant pellet and the
high dose received 2 Linplant pellets. Animals were maintained on a 12:12 hour light/dark cycle and were provided with food and water ad libitum.

At termination the animals were deeply anaesthetized with isoflurane and exsanguinated by cutting the carotid arteries. To assay long-term glycemic control, ~1 ml of blood was collected in EDTA tubes from the STZ-treated rats receiving insulin and their controls to determine the glycosylated hemoglobin levels by high-performance liquid chromatography (CLC330 GHb Analyzer, Primus, Kansas City, MO). The five PMAs branch from the plantar arch (at the distal end of the median plantar artery) and the first four PMAs divide in the interdigital spaces to form the plantar digital arteries supplying the adjacent sides of the digits (the 5th PMA supplies the plantar digital artery on the lateral side of the fifth digit) (see Figure 2.1). The first and second PMAs or second-order MAs were dissected and placed in physiological saline containing (in mM): 133 NaCl, 4.7 KCl, 2.0 CaCl₂, 1.2 MgCl₂, 1.3 NaH₂PO₄, 16.3 NaHCO₃ and 7.8 glucose. This solution was bubbled with carbogen (5% CO₂-95% O₂). On each experimental day tissue was collected from both a control and an STZ-treated rat and the experiments described below were performed in parallel.

**Figure 2.1.** Diagram of the plantar surface of the rat hind paw showing the location of metatarsal arteries that branch from the plantar arch at the distal end of the median plantar artery.
Wire Myography

Two four-channel myographs (Multi Myograph model 610M, Danish Myo Technology, Aarhus, Denmark) were used to record mechanical responses of the arteries. In each device, two artery segments (~1.5 mm in length) from a control and an STZ-treated rat were mounted isometrically between two stainless steel wires (40 µm diameter). Arterial segments were bathed in 6 ml of physiological saline that was continuously bubbled with carbogen and maintained at 36° to 37°C. The basal conditions were normalized by gradually stretching the vessel in small steps until the effective transmural pressure (ETP) calculated using Laplace’s equation (transmural pressure = wall tension/(internal circumference/2π), where wall tension = force/(2 x vessel segment length)) was 13.3 mN/mm² (100 mmHg) (Mulvany & Halpern, 1977). For MAs, the circumference was then adjusted to 90% of that determined at 13.3 mN/mm² (Mulvany & Halpern, 1977). For PMAs, the ETP was set initially at 13.3 mN/mm². The vessels were then left to equilibrate for at least 30 minutes. After this period, the basal ETP in MAs stabilized at ~6.5 mN/mm² (~50 mmHg) and in PMAs at ~10.0 mN/mm² (~75 mmHg). Under these conditions, MAs (Mulvany & Halpern, 1977) and PMAs (Figure 2.2) are at the peak of their length-tension relationship. Because diabetes has been reported to change the diameter of MAs (Wigg et al., 2004), the responses to nerve stimulation and to agonists were also converted to ETP to account for any changes in vascular dimensions.

After equilibration, all tissues were exposed to three (PMAs) or four (MAs) applications of phenylephrine (PE; 3 µM, Sigma-Aldrich, Castle Hill, Australia). The first two applications of PE were for ~4 minutes and confirmed the viability of the vessels. Following the third addition of PE, when the contraction had plateaued, relaxation to carbachol (0.1 and 1 µM added cumulatively; Sigma-Aldrich) was determined to assess the function of the endothelium. In MAs, when the contraction to the fourth
application of PE had plateaued, the relaxation to capsaicin (0.01 and 0.1 \( \mu \text{M} \) added cumulatively; Sigma-Aldrich) was determined to assess the vasodilator effect of activating the perivascular peptidergic primary afferent axons. In MAs, after washout of the fourth application of PE, capsaicin (1 \( \mu \text{M} \)) was applied for 10 minutes to prevent the inhibitory effects of activating the peptidergic primary afferent axons (Holzer, 1991). After wash out of capsaicin, the MAs were left for a further 30 minutes before starting the experiments described below.
Figure 2.2. The relationship between the internal circumference and the increase in wall tension produced by raising the K⁺ concentration to 60 mM in plantar metatarsal arteries (PMAs). To construct this relationship, PMAs from 4 animals were set up as described in the Materials and Methods, after equilibration for 30 minutes, the level of basal effective transmural pressure was sequentially adjusted to 2.7, 5.3, 8.0, 10.6 and 13.3 mN/mm² (i.e. 20, 40, 60, 80 and 100 mmHg), with responses to 60 mM K⁺ determined at each pressure. On the x-axis the internal circumference at each pressure is expressed as a proportion of that at 13.3 mN/mm² (IC/IC_{13.3}). Each point represents the mean of both internal circumference and wall tension measures with X- and Y-SEM bars, respectively. In these experiments, the α-adrenoceptor antagonists, prazosin (10 nM) and idaxozan (0.1 µM), were applied to the tissues to prevent the contractile effects of noradrenaline released from the nerve terminals by K⁺-induced depolarization.
**CHAPTER 2 | IMPAIRED NEUROVASCULAR TRANSMISSION IN DIABETES**

**Contractions to neural stimuli**

Tissues mounted in one four-channel myograph were used to assess contractions to electrical stimulation of the perivascular axons. Electrical stimuli (15 V, 0.2 ms pulse width) were generated by a four-channel stimulator (EXP-ST-CH4; Experimetria Ltd, Balatonfüred, Hungary) and delivered via platinum plate electrodes mounted either side of the tissue. In preliminary experiments, it was established that contractions to these stimuli were blocked by tetrodotoxin (0.5 μM), confirming that they are due to action potential-evoked release of neurotransmitter from the perivascular axons. To construct frequency response curves, the PMAs were stimulated with 25 pulses at 0.1, 0.3, 0.5 and 1.0 Hz whereas the MAs were stimulated with 100 pulses at 1, 2, 3, 5 and 10 Hz. These different stimulus parameters have been selected because PMAs are more responsive to electrical stimulation of their perivascular nerves than MAs, which are only weakly activated by trains of stimuli at 1 Hz (cf. Figures 2.4 A and 2.9 A). In PMAs from the STZ rats that received no insulin support and their controls, the effects of a 10-minute application of capsaicin (1 μM) on contractions to 10 pulses at 1 Hz were then assessed. Following washout of capsaicin tissues were left for 30 minutes before any further assessments.

To assess the effects of neurotransmitter antagonists, the responses of PMAs to 100 stimuli at 1 Hz, and of MAs to 20 stimuli at 10 Hz, were compared before and during the application of the antagonists; the antagonists were in contact with the tissue for at least 20 minutes before changes to the contractions were determined. For PMAs, the effects of the α1-adrenoceptor antagonist prazosin (10 nM; Sigma-Aldrich) were assessed on one vessel segment and the effects of the α2-adrenoceptor antagonist idazoxan (0.1 μM; Sigma-Aldrich) were assessed on the other. The effects of these antagonists were tested because in other arteries supplying blood to skin both α1- and α2-adrenoceptors contribute to neurovascular transmission (Morris, 1997) and
the concentrations used are 10-50 times higher than those corresponding to the pA₂ values for prazosin at α₁-adrenoceptors (Medgett & Langer, 1984) and idazoxan at α₂-adrenoceptors (Doeby et al., 1983). Subsequently, both α-adrenoceptor antagonists were combined to assess the effects of blocking of all α-adrenoceptors. In addition, in a separate group of PMAs from the STZ rats that received no insulin support, the effects of the P2X₁-purinoceptor antagonist NF449 (10 µM, Tocris Bioscience, Bristol, UK) were assessed. For MAs, where neurotransmission is mediated via α₁-adrenoceptors and P2X-purinoceptors (Lamont et al., 2003), the effects of prazosin (10 nM) were assessed in one vessel segment and those of the P2-purinoceptor antagonist suramin (0.1 mM; Sigma-Aldrich) were assessed in the other. Subsequently, both antagonists were combined to assess the effects of blocking of both α₁-adrenoceptors and P2-purinoceptors.

Contractions to chemical stimuli

Tissues mounted in the other four-channel myograph were used to assess reactivity to exogenously applied agents. For both PMAs and MAs, cumulative concentration-response curves were constructed for the α₁-adrenoceptor agonist, PE (0.01-100 µM). To determine the effects of blocking NETs, concentration-response curves for PE were obtained in the absence and in the presence of desmethylimipramine (DMI; 30 nM; Sigma-Aldrich). In PMAs, cumulative concentration-response curves were also acquired for the α₁-adrenoceptor agonist methoxamine (0.01-100 µM; Sigma-Aldrich), which is not a substrate for NET (Trendelenburg et al., 1970), and the relatively selective α₂-adrenoceptor agonist, clonidine (0.001-3.0 µM; Sigma-Aldrich). In PMAs from the STZ rats that received no insulin support and MAs, reactivity to α,β-methylene ATP (0.5-1 µM; Sigma-Aldrich) was assessed. At the end of these experiments, contractions to depolarization of the smooth muscle with physiological saline containing 60 mM K⁺ (equimolar substitution
of KCl for NaCl) were assessed. Prior to raising the K⁺ concentration, prazosin (10 nM) and idaxozan (0.1 µM) were applied to the tissues to prevent the contractile effects of noradrenaline released from the nerve terminals by K⁺-induced depolarization.

**Immunohistochemistry**

PMAs and MAs were pinned at approximately the same length as they were *in situ* in the hind paw and fixed in Zamboni's Fixative overnight at 4°C. The next day tissues were washed in dimethyl sulfoxide (3 x 10 minutes) to allow for better antibody penetration and then in phosphate buffered saline (PBS, pH 7.1-7.2; 3 x 10 minutes). Tissues were stored in PBS containing 0.1% (w/v) sodium azide at 4°C. Arteries were blocked for 1 hour at room temperature in 10% normal horse serum in PBS containing 1% (v/v) Triton™ X-100 (Sigma-Aldrich), and then incubated overnight at 4°C in antibody diluent containing mouse anti-tyrosine hydroxylase (TH) antibody (1:1000, Cat. No. 22941, ImmunoStar Inc, Hudson, WI, USA) and goat anti-calcitonin gene-related peptide (CGRP) antibody (1:1000, Cat. No. 1720-9007, Biogenesis Ltd, England, UK). In addition, separate tissues were treated with the pan-neuronal labels rabbit anti-protein gene product 9.5 (PGP9.5; 1:1000, Cat No. RA95101, Ultraclone Limited, England, UK) or mouse anti-β-tubulin III (1:750, Cat. No. MMS-435P, Covance, Princeton, NJ, USA). Tissues were washed with PBS (3 x 10 minutes) and incubated at room temperature for 1 hour in antibody diluent containing fluorescent secondary antibodies raised in donkey (Molecular Probes, Inc., OR, USA); Alexa Fluor 564 anti-mouse (1:500), Alexa Fluor 647 anti-sheep (1:500) and Alexa Fluor 488 anti-rabbit (1:1000). Tissues were again washed with PBS (3 x 10 minutes) and coverslipped in fluorescence mounting medium (Dako Australia, Campbellfield, VIC, Australia). Z-stacks of the fluorescent images through the entire adventitial thickness were collected using a Zeiss Pascal confocal microscope system. For these images the laser power was set between 10 and 15% and the excitation/emission wavelengths were 488 nm/band.
pass 505-530 nm for Alexa Fluor 488, 561 nm/band pass 575-615 nm for Alexa Fluor 594 and 633 nm/long pass 650 nm for Alexa Fluor 647. Collection of Z-stacks through the full thickness of the adventitia (15-25 µm) took 8-12 minutes and each region of the artery was only imaged once to minimize the effects of bleaching. The specificity of secondary antibodies was tested with omission of the primary antibodies, which always resulted in no immunostaining.

**Data Analysis**

The output from the myographs was recorded and analyzed using a PowerLab data acquisition system and the program Chart (ADInstruments, Bella Vista, NSW, Australia). The amplitudes of contractions to trains of electrical stimuli and to contractile agents were measured and converted to ETP. The EC$_{50}$ values for the α-adrenoceptor agonists were determined by fitting the concentration response data to the Hill equation using the curve fitting functions in Igor Pro (Wavemetrics Inc., Lake Oswego, OR, USA) and are presented as their negative logarithm (pEC$_{50}$). All statistics were performed using SPSS 19 (IBM Corp, Armonk, NY, USA). The stimulus frequency response curves were compared by repeated measures ANOVA with a single independent variable for between group comparisons. Other comparisons were made with unpaired t-tests or Mann Whitney U-tests if the variances were not homogeneous (indicated by Levene’s tests). When multiple pairwise comparisons were made in individual tissues, the P-values were adjusted using the false discovery rate procedure (Curran-Everett, 2000). Data are presented as means and SEMs or medians and interquartile ranges (IQR) if comparisons were made with Mann Whitney U-tests. P-values <0.05 were considered to indicate significant differences. Unless otherwise indicated in the text, P-values were obtained using unpaired t-tests. In all cases, n indicates the number of animals studied.
The density of the immunolabeled axon plexus was quantified using maximum intensity Z-projection images collected with x20 (MAs; pixel size 1.01 µm²) or x40/x63 objectives (PMAs; pixel size 0.38 µm²/0.24 µm² respectively). This was done by collecting two line profiles at the same points on all images (one placed in the upper half and the other in the lower half of the image) using the program ImageJ (National Institute of Health, Bethesda, MD, USA). After subtracting the mean maximum background value (determined from 10 points on the image where there was no detectable fluorescence), the number (intercept number) and widths (intercept widths) of the regions along the line profiles with pixel values >0 (see Figures 2.6 A and B) were determined using an in-house Igor Pro routine. The percentage area of the vessel surface covered by the immunolabeled nerve plexus in each image was also measured using ImageJ. For images of TH immunoreactivity (TH-IR), the mean value of the peaks along the line profiles was measured to ascertain if there was a difference in the intensity of fluorescence of the intercepts (nerve bundles). The integrated TH-IR fluorescence for the entire vessel surface in each image was also measured after subtraction of the mean background level (determined at 10 points on the image where there was no detectable fluorescence) and this was normalized to 100 µm² of vessel surface. For CGRP immunoreactive (CGRP-IR) axons in both PMAs and MAs, the numbers of fluorescent intercepts were determined along three lines placed at the same points (one placed in the upper half, one in the middle and the other in the lower half of the image) on images collected with a x20 objective. Measures of intercept number along the line profiles were calculated per 100 µm. For each measure, one-sample t-tests were used to determine if the measures in arteries from STZ-treated animals expressed as a percentage of those in the control tissues collected on the same day (and processed in parallel) differed significantly from 100%. For each animal and vessel type, the measures were the average obtained from at least three image Z-stacks.
CHAPTER 2 IMPAIRED NEUROVASCULAR TRANSMISSION IN DIABETES

Results

Animals

When assessed 5-7 days post injection the STZ rats had blood glucose levels in excess of 20 mmol/L, and the rats receiving no insulin (STZ-NI) and those receiving a low dose of insulin (STZ-LI) maintained levels >20 mmol/L. In contrast, the STZ rats receiving a high dose of insulin (STZ-HI) maintained blood glucose levels <15 mmol/L. At termination, the body weights of all three STZ-treated groups were less than their controls (Table 2.1). Additionally, STZ-NI rats had little net body weight gain whereas STZ-LI and STZ-HI rats had greater body weight gain, but this was still smaller than that of their controls (Table 2.1). However, body weight gain in STZ-HI rats was greater than in STZ-LI rats (Table 2.1). At termination the blood glucose levels in STZ-NI and STZ-LI rats were greater than those of their controls, but those of STZ-HI rats were more variable and did not differ significantly from their controls (Table 2.1). However, the % glycosylated hemoglobin levels were significantly higher in both STZ-LI and STZ-HI rats when compared with their controls, but those of STZ-HI rats were less markedly increased (Table 2.1).

Plantar metatarsal arteries (PMAs)

*The dimensions and endothelium-dependent relaxations of PMAs were not changed by diabetes, but K⁺-evoked contractions were reduced in vessels from STZ-NI rats*

The lumen diameter (estimated from the measured lumen circumference of the isometrically mounted vessels) and the basal ETP after equilibration for PMAs from STZ-NI, STZ-LI and STZ-HI rats did not differ from those of their controls (Table 2.2). Similarly, the % relaxation of vessels constricted with PE (3 μM) when their endothelium was stimulated with carbachol (0.1 and 1 μM) did not differ between PMAs from each of the groups of STZ rats and their controls (Figure 2.3 A-C). The peak increases in ETP produced by depolarization of the vascular muscle with 60 mM K⁺
were smaller in the PMAs from STZ-NI rats than in their controls (Figure 2.3 D). In contrast, the peak increases in ETP produced by 60 mM K⁺ in PMAs from STZ-LI or STZ-HI rats did not differ from those of their controls (Figure 2.3 E, F).

**Table 2.1.** Measures of terminal body weight, net body weight gain, blood glucose and % glycosylated hemoglobin at termination in control and STZ-treated rats.

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<tr>
<th></th>
<th>n</th>
<th>Terminal body weight (g)</th>
<th>Body weight gain (g)</th>
<th>Blood glucose concentration (mmol/L)</th>
<th>% Glycosylated hemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18</td>
<td>538 ± 11</td>
<td>156 ± 8</td>
<td>6.5 ± 0.3</td>
<td>-</td>
</tr>
<tr>
<td>STZ-NI</td>
<td>18</td>
<td>388 ± 12</td>
<td>12 ± 10</td>
<td>29.4 ± 0.8</td>
<td>-</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>560 ± 24</td>
<td>240 ± 9</td>
<td>6.3 (5.3 - 7.3)</td>
<td>5.3 (4.7 - 5.8)</td>
</tr>
<tr>
<td>STZ-LI</td>
<td>10</td>
<td>442 ± 10</td>
<td>126 ± 12</td>
<td>23.9 (23.1 - 25.2)</td>
<td>14.9 (13.6 - 15.9)</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>600 ± 17</td>
<td>242 ± 9</td>
<td>5.7 (5.2 - 7.8)</td>
<td>4.1 (3.7 - 4.6)</td>
</tr>
<tr>
<td>STZ-HI</td>
<td>10</td>
<td>544 ± 14</td>
<td>201 ± 9</td>
<td>9.1 (4.8 - 12.0)</td>
<td>8.3 (7.2 - 8.7)</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>&lt;0.05*</td>
<td>&lt;0.01*</td>
<td>0.22</td>
<td>&lt;0.01*</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEMs or medians and interquartile ranges (in parentheses). Statistical comparisons between control and STZ groups were made with Student’s unpaired t-tests or Mann Whitney U-tests as appropriate. *Significant differences. STZ-NI, STZ rats that received no insulin; STZ-LI, STZ rats that received a low dose of insulin; STZ-HI, STZ rats that received a high dose of insulin.
**Table 2.2.** The estimated lumen diameter and effective transmural pressure of plantar metatarsal arteries under basal conditions.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Diameter (µm)</th>
<th>Basal transmural pressure (mN/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>405 ± 19</td>
<td>10.1 ± 0.4</td>
</tr>
<tr>
<td>STZ-NI</td>
<td>10</td>
<td>392 ± 10</td>
<td>9.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>0.55</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>392 ± 4</td>
<td>10.2 ± 0.4</td>
</tr>
<tr>
<td>STZ-LI</td>
<td>6</td>
<td>394 ± 8</td>
<td>10.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>0.94</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>393 ± 6</td>
<td>10.1 ± 0.1</td>
</tr>
<tr>
<td>STZ-HI</td>
<td>6</td>
<td>394 ± 8</td>
<td>10.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEMs. Statistical comparisons between control and STZ groups of arteries were made with Student’s unpaired t-tests.
**Figure 2.3.** In PMAs, diabetes did not impair endothelium-dependent relaxation but it did reduce contractions evoked by 60 mM K⁺. (A-C) The % relaxations produced by carbachol (0.1 and 1 µM) in phenylephrine (3 µM)-constricted PMAs from STZ-treated rats receiving no insulin (A; STZ-NI, n = 10), a low dose of insulin (B; STZ-LI, n = 6) or a high dose of insulin (C; STZ-HI, n = 6) and their controls (n = 10 for STZ-NI and 6 for STZ-LI and STZ-HI). (D-F) Peak increases in effective transmural pressure evoked by application of physiological saline containing 60 mM K⁺ in PMAs from STZ-NI (D), STZ-LI (E) and STZ-HI (F) and their controls. The data are presented as means and SEMs. In D, * indicates a significant difference between responses of PMAs from STZ-NI and control rats (P < 0.01, unpaired t-test).
Nerve-evoked increases in ETP were reduced in PMAs from STZ-NI rats

Figure 2.4 shows stimulus frequency contraction data for PMAs from STZ-NI rats (Figure 2.4 A, B), STZ-LI rats (Figure 2.4 C) and STZ-HI rats (Figure 2.4 D) together with those for their controls. At all frequencies of stimulation, the peak increases in ETP for PMAs from STZ-NI rats were smaller than control (Figure 2.4 A, B). In contrast, stimulation frequency-contraction data for PMAs from STZ-LI and STZ-HI rats did not differ significantly from control (ANOVA between groups P = 0.23 and 0.36 respectively; Figure 2.4 C, D).

The effects of capsaicin (1 µM) on responses to 10 pulses at 1 Hz were only determined in PMAs from STZ-NI rats (n = 6) and their controls (n = 6). In both these groups of vessels, the peak amplitudes of the stimulus-evoked contractions measured 3 minutes following the addition of capsaicin were reduced by about 60% (Control 56 ± 11%; STZ-NI 64 ± 16%). During the 15-minute application of capsaicin the contraction amplitudes returned close to control levels, consistent with this agent activating and desensitizing the peptidergic primary afferent axons. The contractions measured 20 minutes after washout of capsaicin did not differ significantly from those measured before its addition (% of pretreatment contractions: control 98 ± 12%; STZ-NI 100 ± 9%; paired t-test P > 0.80 for both comparisons), indicating that capsaicin desensitization of the peptidergic primary afferent axons did not change the nerve-evoked contractions.

In all groups of PMAs (n = 6 for all STZ and control groups), the contractions to 100 pulses at 1 Hz were reduced by the α₁-adrenoceptor antagonist prazosin (10 nM: ~75-85% blockade) and by the α₂-adrenoceptor antagonist idazoxan (0.1 µM; ~20-30% blockade) and together these agents reduced the contractions by about 80-90% (see Table 2.3 for the individual group data). The magnitude of the blockades produced by the α-adrenoceptor antagonists did not differ between the arteries from any of the
STZ-treated groups and their control groups (P > 0.10 for all comparisons). The P2X1-purinoceptor antagonist NF449 (10 µM) blocked contractions to 100 pulses at 1 Hz by ~20% in arteries from both STZ-NI (19 ± 8%, n = 5) and control rats (18 ± 6%, n = 5; P = 0.92). The effects of NF449 were not tested in arteries from STZ-LI and STZ-HI rats.

Table 2.3. % Blockade of contractions to 100 pulses at 1 Hz produced by prazosin, idazoxan or the combination of both agents.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Prazosin</th>
<th>Idazoxan</th>
<th>Prazosin + Idazoxan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>75 ± 2</td>
<td>30 ± 9</td>
<td>83 ± 2</td>
</tr>
<tr>
<td>STZ-NI</td>
<td>6</td>
<td>81 ± 6</td>
<td>34 ± 6</td>
<td>78 ± 4</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.79</td>
<td>0.70</td>
<td>0.26</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>78 ± 4</td>
<td>26 ± 5</td>
<td>83 ± 3</td>
</tr>
<tr>
<td>STZ-LI</td>
<td>6</td>
<td>83 ± 7</td>
<td>19 ± 7</td>
<td>85 ± 4</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.93</td>
<td>0.40</td>
<td>0.84</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>84 ± 2</td>
<td>29 ± 7</td>
<td>88 ± 2</td>
</tr>
<tr>
<td>STZ-HI</td>
<td>6</td>
<td>89 ± 3</td>
<td>19 ± 6</td>
<td>91 ± 2</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.22</td>
<td>0.31</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Mean ± SEM. P values are for comparisons between control and STZ treated groups using unpaired t-tests.
PMAs from STZ-NI rats had reduced sensitivity to α-adrenoceptor agonists

Both in the absence and in the presence of the NET inhibitor, DMI (30 nM), there was a small but significant decrease in the pEC$_{50}$ for PE in PMAs from STZ-NI rats compared to their controls (Figure 2.5 A, B). This decrease in pEC$_{50}$ to PE was not observed in PMAs from STZ-LI (Figure 2.5 C, D) or STZ-HI rats (-DMI: control 6.03 ± 0.08, STZ-HI 5.98 ± 0.13, P = 0.72; +DMI: control 6.58 ± 0.13, STZ-HI 6.52 ± 0.10, P = 0.74).

The leftward shift in the EC$_{50}$ for PE produced by blockade of NET with DMI (measured by the EC$_{50}$ ratio) did not differ between PMAs from any of the STZ-treated groups and their controls (P > 0.2 for all comparisons). While there was a tendency for the maximum increase in ETP produced by PE to be smaller than control in PMAs from STZ-NI rats (Figure 2.5 A, B) and larger than control in PMAs from both STZ-LI (Figure 2.5 C, D) and STZ-HI rats (-DMI: control 30.1 ± 1.8 mN/mm$^2$, STZ-HI, 33.9 ± 3.1 mN/mm$^2$, P = 0.31; +DMI: control 33.9 ± 3.1 mN/mm$^2$, STZ-HI 36.0 ± 3.2 mN/mm$^2$, P = 0.27), these differences did not reach the level of statistical significance.

In comparison with their controls, PMAs from STZ-NI rats had a reduction in their pEC$_{50}$ for both methoxamine and clonidine but, in addition, these vessels had a reduction in their maximum contraction to both these agents (Table 2.4). These reductions in reactivity to methoxamine and to clonidine were not observed in PMAs from STZ-LI rats (Table 2.4). However, PMAs from STZ-HI rats had an increase in their pEC50 for clonidine and these vessels also had a trend towards an increased sensitivity to methoxamine (Table 2.4).

In arteries from STZ-NI rats, the contractions to α,β-methylene ATP (1 μM) did not differ significantly from those of their controls (control 11.2 ± 1.5 mN/mm$^2$, n = 5;
CHAPTER 2 | IMPAIRED NEUROVASCULAR TRANSMISSION IN DIABETES

STZ-NI 9.9 ± 0.8 mN/mm², n = 5; P = 0.49). Reactivity to α,β-methylene ATP was not tested in arteries from STZ-LI or STZ-HI rats.

PMAs from diabetic rats had an increased level of TH-IR labeling and those from STZ-NI rats had a reduced density of perivascular axon bundles

There was no difference between the widths of the wholemount vessel segments from each of the groups of STZ rats and their controls (Table 2.5). The perivascular plexus of PMAs revealed with pan-neuronal markers (anti-PGP9.5 or β-tubulin III) was comprised of a dense network of axon bundles (Figure 2.6 A-F). The great majority of fibers within the perivascular plexus were TH-IR (Figure 2.6 G, H). Both the mean peak intensity of the TH-IR fluorescent intercepts along the line profiles and the integrated TH-IR fluorescence were higher than control in vessels from STZ-NI and STZ-LI rats, but not STZ-HI rats (Figure 2.6 L, M). However, because TH-IR did not resolve the finest fibers well (Figure 2.6 G, H), I chose to use the pan-neuronal markers to assess changes in the density of fibers in the perivascular plexus (Figure 2.6 A-F). When compared with that of their controls, the % area of the PMA surface covered by the axon plexus was reduced in STZ-NI, increased in STZ-LI and unchanged in STZ-HI rats (Figure 2.6 I). There was also a ~25% reduction in the frequency of fluorescent intercepts along the line profiles in PMAs from STZ-NI rats, but this change was not seen in PMAs from STZ-LI and STZ-HI rats (Figure 2.6 J). In PMAs from STZ-NI and STZ-LI rats, there was an increase in the widths of the fluorescent intercepts along the line profiles (Figure 2.6 K). In addition to TH-IR fibers, a small number of CGRP-IR fibers were also observed within the perivascular plexus (Figure 2.7 A, B) but the frequency of CGRP-IR intercepts along the line profiles in vessels from each of the STZ-treated groups did not differ significantly from those in their control vessels (Figure 2.7 C).
Figure 2.4. In plantar metatarsal arteries (PMAs), diabetes reduced nerve-evoked contractions. (A) Representative traces showing contractions evoked by trains of 25 pulses at 0.1-1 Hz in segments of PMA from a control rat (upper) and a STZ-treated rat receiving no insulin (lower; STZ-NI). (B-D) The peak increases in effective transmural pressure produced by these stimuli in PMAs from STZ-NI rats (B; n = 10), and STZ-treated rats receiving a low dose of insulin (C; STZ-LI, n = 6) or a high dose of insulin (D; STZ-HI, n = 6) and their controls (n = 10 for STZ-NI and 6 for STZ-LI and STZ-HI). The data are presented as means and SEMs. In B, * indicates significant differences between responses of PMAs from STZ-NI and control rats (P < 0.05; unpaired t-test with P-values adjusted for multiple comparisons using the false discovery procedure).
Figure 2.5. In plantar metatarsal arteries (PMAs), diabetes reduced sensitivity to phenylephrine (PE). (A-D) Concentration response curves and pEC$_{50}$s for PE in PMAs from STZ-treated rats receiving no insulin (A, B; STZ-NI, n = 10) or a low dose of insulin (C, D; STZ-LI, n = 6) and their controls (n = 10 for STZ-NI and 6 for STZ-LI) in the absence (A, C) or in the presence of the norepinephrine transporter (NET) inhibitor desmethylimipramine (DMI) (B, D). The data are presented as means and SEMs. In A and B, * indicate that the pEC$_{50}$ values were significantly smaller than control in the PMAs from STZ-NI rats both with and without NET blockade ($P < 0.05$; unpaired t-tests).
CHAPTER 2 | IMPAIRED NEUROVASCULAR TRANSMISSION IN DIABETES

Table 2.4. Effects of diabetes on concentration-response curves for methoxamine and clonidine in plantar metatarsal arteries.

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<th></th>
<th>Methoxamine</th>
<th>Clonidine</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>pEC$_{50}$</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>6.35 ± 0.08</td>
</tr>
<tr>
<td>STZ-NI</td>
<td>10</td>
<td>6.11 ± 0.06</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>6.11 ± 0.07</td>
</tr>
<tr>
<td>STZ-LI</td>
<td>6</td>
<td>6.16 ± 0.03</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>6.14 ± 0.10</td>
</tr>
<tr>
<td>STZ-HI</td>
<td>6</td>
<td>6.36 ± 0.05</td>
</tr>
<tr>
<td>P</td>
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<td>0.08</td>
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Data are presented as means ± SEMs. Statistical comparisons between control and STZ groups of arteries were made with Student’s unpaired t-tests and significant differences are indicated by *.

Table 2.5. Widths of wholemount plantar metatarsal artery segments.

<table>
<thead>
<tr>
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<th>Vessel width (µm)</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>n</td>
</tr>
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<tr>
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<td>7</td>
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<tr>
<td>Control</td>
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<tr>
<td>STZ-LI</td>
<td>10</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
</tr>
<tr>
<td>STZ-HI</td>
<td>10</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEMs.
CHAPTER 2 IMPAIRED NEUROVASCULAR TRANSMISSION IN DIABETES
Chapter 2 Impaired Neurovascular Transmission in Diabetes

Figure 2.6. In PMAs, diabetes reduced the density of the perivascular nerve plexus and increased the intensity of tyrosine hydroxylase immunoreactivity (TH-IR). (A-F) The perivascular plexus revealed with a pan-neuronal marker (A, B anti-PGP9.5, C-F anti-β-tubulin III) imaged with a x63 objective in a PMA segment from STZ-treated rats receiving no insulin (B; STZ-NI), a low dose of insulin (D; STZ-LI) or a high dose of insulin (F; STZ-HI) and their controls (A, C and E respectively). Above A and B are shown line profiles measured along the blue lines on the images. (G, H) The TH-IR perivascular plexus imaged with a x40 objective in a PMA segment from a control and an STZ-NI rat. (I-K) The % area of the vessel surface covered by the immunolabeled nerve plexus (I) and the fluorescent intercept frequency (J) and width (K) along line profiles for artery segments labeled with a pan-neuronal marker from STZ-NI (n = 7), STZ-LI (n = 10) and STZ-HI (n = 10) rats expressed as a % of these measures in paired control tissues. (L, M) The mean peak value for the TH-IR fluorescent intercepts (L; Peak TH) along the line profiles, and the integrated TH-IR fluorescence/100 µm² of vessel surface (M; Integrated TH) for artery segments from STZ-NI (n = 7), STZ-LI (n = 10) and STZ-HI (n = 10) expressed as a % of these measures in paired control tissues. The data are presented as means and SEs. Statistical assessments were made with one-sample t-tests (* P < 0.05, ** P < 0.01). The scale bars in A and G indicate 50 µm and also apply, respectively, in B-F and H.
Figure 2.7. In plantar metatarsal arteries (PMAs), diabetes did not detectably change their innervation by calcitonin gene-related peptide immunoreactive (CGRP-IR) fibers. (A, B) CGRP-IR perivascular fibers imaged with a x20 objective in a PMA segment from an STZ-treated rat receiving no insulin (B; STZ-NI) and a control rat (A). (C) Measures of CGRP-IR intercept frequency along line profiles in PMAs from STZ-NI rats (n = 7) and STZ-treated rats receiving a low dose of insulin (STZ-LI, n = 10) or a high dose of insulin (STZ-HI, n = 10) expressed as a % of measures in paired control tissues. The data are presented as means and SEMs. The scale bar in A indicates 100 µm and also applies in B.
Mesenteric arteries (MAs)

MAs from diabetic rats had increased lumen diameters and impaired endothelial vasodilator function but no change in their responses to capsaicin or $K^+$.

Only MAs from STZ-NI rats were studied. After the normalization procedure the lumen diameter was about 20% larger for MAs isolated from the STZ-NI rats compared to their controls (control $382 \pm 17 \mu m$, $n = 8$; STZ-NI $459 \pm 19 \mu m$, $n = 8$; $P < 0.01$). However, after equilibration, the basal ETP did not differ significantly between these two groups (control $7.1 \pm 0.3 \text{ mN/mm}^2$; STZ $6.4 \pm 0.4 \text{ mN/mm}^2$; $P = 0.15$). In MAs from STZ-NI rats, the % relaxation of vessels constricted with PE (3 $\mu M$) when the endothelium was stimulated with carbachol (0.1 and 1 $\mu M$) was significantly smaller than in control MAs (Figure 2.8 A). In contrast, the % relaxation produced when the peptidergic sensory axons were activated with capsaicin (0.01 and 0.1 $\mu M$) did not differ between STZ-NI and control MAs (Figure 2.8 B). The peak increase in ETP produced by application of physiological saline containing 60 mM $K^+$ also did not differ between these groups of vessels (Figure 2.8 C).
Figure 2.8. In mesenteric arteries (MAs), diabetes impaired endothelium-dependent relaxation to carbachol but did not change relaxation produced by activation of the peptidergic sensory nerves with capsaicin or contractions evoked by depolarizing the vascular muscle with 60 mM K⁺. (A) The % relaxation produced by carbachol (0.1 and 1 μM) in phenylephrine (3 μM) constricted MAs from control (n = 8) and STZ-treated rats (n = 8). (B) The % relaxation produced by capsaicin (0.01 and 0.1 μM) in phenylephrine (3 μM) constricted MAs from control and STZ-treated rats. (C) The peak increases in effective transmural pressure produced by application of physiological saline containing 60 mM K⁺ in MAs from control and STZ-treated rats. The data are presented as means and SEMs. In A, * indicates significant differences between responses of MAs from control and STZ-treated rats (P < 0.05; unpaired t-tests).
Nerve-evoked increases in ETP were reduced in MAs from diabetic rats

Over the range of stimulation frequencies studied, the nerve-evoked increases in ETP in MAs from STZ-NI rats were smaller than those in MAs from control rats (Figure 2.9 A, B; ANOVA between groups P < 0.05). However, post-hoc comparisons between the responses of these groups of vessels at each stimulation frequency did not reach the level of statistical significance. As the increased dimensions of the MAs from STZ-NI rats might cause the reductions in nerve-evoked increases in ETP, Figure 2.9 C displays the same data plotted as increases in wall tension (force/(2 x vessel segment length)) (Mulvany & Halpern, 1977). When expressed in this manner the nerve-evoked responses did not differ between MAs from STZ-NI and control rats (ANOVA between groups P = 0.65), indicating that the absolute increase in force generated by nerve stimulation did not differ between these groups of vessels.

In MAs from STZ-NI rats, there was a small but significant decrease in the % blockade of contractions to 20 pulses at 10 Hz produced by prazosin (10 nM: control 89 ± 2%, n = 8; STZ-NI 76 ± 5%, n = 8; P < 0.05). However, there was no difference in the % blockade produced by the P2-purinoceptor antagonist suramin (0.1 mM: control 36%, IQR 31-38%, n = 8; STZ-NI 43%, IQR 28-66%, n = 8; Mann Whitney U-test P = 0.63) or by the combination of prazosin and suramin (control 99 ± 1%, n = 8; STZ 97 ± 2%, n = 8, P = 0.28) between MAs from STZ-NI and control rats.

MAs from diabetic rats had no change in their sensitivity to phenylephrine

Both in the absence and in the presence of DMI (30 nM), the pEC50 and maximum increase in ETP to PE did not differ between MAs from STZ-NI and control rats (Figure 2.10 A, B). The magnitude of the change in the pEC50 for PE produced by DMI (measured by the EC50 ratio) also did not differ between these groups of vessels (P = 0.73).
MA from diabetic rats had no change in their responses to α,β-methylene ATP

The peak increase in ETP to α,β-methylene ATP (0.5 μM) did not differ between MA from STZ-NI and control rats (control 12.4 ± 1.7 mN/mm², n = 8; STZ-NI 11.3 ± 1.1 mN/mm², n = 8; P = 0.59).

MA from diabetic rats had a reduced density of TH-IR perivascular axon bundles but this change was associated with an increase in vascular dimensions

The widths of the wholemount MA from STZ-NI rats were about ~25% larger than those of their controls (control 322 ± 1 μm, n = 7, STZ-NI 407 ± 10 μm, n = 7, P < 0.01). In MA from both STZ-NI and control rats, the perivascular plexus was composed of TH-IR axon bundles of fairly uniform thickness that formed a network pattern across the medial surface (Figure 2.11 A, C). In addition, the perivascular plexus of MA contained a higher density of CGRP-IR fibers than that of PMAs (Figure 2.11 B, D; cf. Figure 2.7 A, B). Both the % area of the vessel surface covered by the axon plexus and the frequency of TH-IR intercepts along the line profiles in MA from STZ-NI rats were ~12% lower than in controls (Figure 2.11 E), but the widths of the intercepts were similar in both groups of vessels (Figure 2.11 E). There was also a reduction in the integrated TH-IR fluorescence and tendency for the mean peak intensity of the TH-IR fluorescent intercepts along the line profiles to be less in MA from STZ-NI rats (Figure 2.11 E). Relative to control tissues, the frequency of CGPR-IR intercepts along the line profiles in MA from STZ rats was not significantly different (91 ± 12% of control; one-sample t-test P = 0.48).
Figure 2.9. In mesenteric arteries (MAs), diabetes reduced nerve-evoked increases in effective transmural pressure but did not significantly change these responses when expressed as increases in wall tension. (A) Representative traces showing contractions evoked by trains of 100 pulses at 1-10 Hz in a segment of MA from a control rat (upper) and an STZ-treated rat (lower). (B, C) The peak increases in effective transmural pressure (B) or wall tension (C) produced by these stimuli in MAs from control (n = 8) and STZ-treated rats (n = 8). The data are presented as means and SEMs. In B and C, the ANOVA P-values are for between group comparisons. Although the ANOVA indicated that nerve-evoked increases in effective transmural pressure in MAs from STZ-treated rats were smaller than those of their controls, comparisons between the responses of these groups of vessels to each frequency of stimulation did not reach the level of statistical significance.
Figure 2.10. In mesenteric arteries (MAs), diabetes did not change sensitivity to phenylephrine (PE). Concentration response curves and pEC\textsubscript{50}s for phenylephrine in MAs from control (n = 8) and STZ-treated rats (n = 8) in the absence (A) or in the presence of the neuronal norepinephrine transporter inhibitor desmethylimipramine (B). The data are presented as means and SEMs.
Figure 2.11. In mesenteric arteries (MAs), diabetes reduced the density of tyrosine hydroxylase immunoreactive (TH-IR) perivascular fibers but this change was associated with an increase in vascular dimensions. (A-D) The TH-IR (A, C) and calcitonin gene-related peptide immunoreactive (B, D; CGRP-IR) nerve plexus imaged with a x20 objective in a MA segment from a control (A, B) and an STZ-treated (C, D) rat. (E) The % area of the vessel surface covered by the TH-IR nerve plexus, the frequency, width and mean peak value of the TH-IR fluorescent intercepts (Peak TH) along line profiles and the integrated TH-IR fluorescence/100 µm² of vessel surface (Integrated TH) for MA segments from STZ-treated rats (n = 7) expressed as a % of these measures in paired control tissues. The data are presented as means and SEMs. Statistical assessments were made with one-sample t-tests and * indicates a significant difference (P < 0.05). The scale bar in A indicates 100 µm and also applies in C to D.
Discussion

This study provides the first evidence that neurovascular function is differentially affected in arteries supplying blood to skin of the hind paw digits (PMAs) and to the intestine (MAs). In STZ-NI rats, nerve-evoked increases in ETP were reduced in both PMAs and MAs. The change in neurovascular transmission in PMAs was associated with reduced reactivity to \( \alpha \)-adrenoceptor agonists and to high [\( K^+ \)], whereas no change in responsiveness to exogenously applied agents was detected in MAs. PMAs from STZ-LI and STZ-HI rats had no change in their nerve-evoked contractions and their reactivity to exogenously applied agents was not reduced, confirming that the changes observed in PMAs from STZ-NI rats were not the result of a direct toxic action of STZ. The frequency of nerve bundles was reduced in the perivascular nerve plexus of PMAs and MAs from STZ-NI rats, and in PMAs both the intensity of TH-IR and thickness of the nerve bundles (intercept widths) were increased. While the density of perivascular nerve bundles was not significantly changed in PMAs from STZ-LI rats, the intensity of TH-IR and the thickness of bundles were increased. These changes were not detected in PMAs from STZ-HI rats.

Previous studies investigating changes in sympathetic neurovascular function in rats with STZ-induced diabetes have not provided any insulin support. As reported by others (Fox \textit{et al.}, 1999), treatment of the STZ-treated rats with a low dose of insulin that leaves them markedly hyperglycemic, improved the overall health of the animals in comparison with those receiving no insulin support as indicated by the increase in body weight gain. In comparison with STZ-LI rats, the terminal glycosylated hemoglobin levels in STZ-HI rats were reduced by about 50% indicating effective long-term glycemic control. However, in comparison with control rats, the STZ-HI rats at termination still had elevated levels of glycated hemoglobin and reduced body weight gain.
CHAPTER 2 IMPAIRED NEUROVASCULAR TRANSMISSION IN DIABETES

The effects of STZ-induced diabetes on PMAs have not previously been reported, but they have been described for small MAs. As demonstrated in this study, there are several reports that diabetes impairs endothelium-mediated vasodilation of MAs (e.g. (Taylor et al., 1992; Heygate et al., 1996; Wigg et al., 2001). In rats with STZ-induced diabetes, Kiff et al. (1991) demonstrated increased blood flow in the mesenteric vascular bed under basal conditions. This finding questions the functional significance of the impairment of endothelium-mediated vasodilation in MAs. Perhaps the increase in blood flow reflects a decrease in sympathetic nerve-mediated activation of arterial vessels in the mesenteric vascular bed. An increase in splanchnic blood flow (which includes the mesenteric vascular bed) has been reported in human type I diabetics with postural hypotension (Purewal et al., 1995). As observed in the present study, Wigg et al. (2004) also found in rats that STZ-induced diabetes increased the lumen diameters of MAs and these investigators demonstrated that this change occurred without alterations in the thickness or the mechanical properties of the vascular wall. A chronic increase in blood flow can increase the lumen diameter of MAs (Gao et al., 2008), so a possible explanation for change in vessel dimensions observed in STZ-treated rats is an increase in intestinal perfusion to meet the demands produced by diabetes-induced hyperphagia (Wigg et al., 2004). The reported effects of STZ-induced diabetes on the reactivity of MAs to α-adrenoceptor agonists are variable, with sensitivity to these agents being either increased (Taylor et al., 1992) or unchanged (Heygate et al., 1996; Wigg et al., 2001).

With passive (i.e. unconstricted) diameters of ~400 µm, the size of second order MAs in control rats is at the upper end of the arterial vessels that produce resistance to blood flow (Christensen & Mulvany, 2001). However, as sympathetic nerve activation of MAs in vivo can reduce their internal diameter by 50-70% (Furness & Marshall, 1974) it is suggested that arterial vessels of this size play a more important role in neural regulation of peripheral resistance. Nerve-evoked pressor responses of
the perfused isolated mesenteric arterial bed were reduced by 12 weeks of diabetes (Ralevic et al., 1995). As the basal perfusion pressure was also decreased by diabetes (Ralevic et al., 1995), the reduced nerve-evoked pressor responses are possibly explained by the increased lumen dimensions of the resistance arteries in this vascular bed rather than a change in neurovascular transmission. This appears to be the case in the present study because, while the nerve-evoked increases in ETP were reduced in the MAs from STZ rats, the absolute increases in force produced by nerve stimulation in these vessels did not differ from those in control MAs. These findings contrast with those for the increases in ETP produced by 60 mM K⁺, PE and α,β-methylene ATP, which did not differ between MAs from STZ and control rats and therefore appear to scale with the change in vascular dimensions.

Like second order MAs, the PMAs had passive diameters of ~400 µm and it is therefore likely that constriction of these vessels contributes to the neural regulation of blood flow in the hind paw digits. The dimensions of PMAs from STZ-NI rats were similar to those of their controls and in these vessels the increases in absolute force produced by nerve stimulation (not shown) were reduced to a similar extent as the nerve-evoked increases in ETP. Therefore neurovascular transmission was reduced in PMAs from STZ-NI rats. The reduction in neurovascular transmission appears to be accounted for, at least in part, by postjunctional changes in the vascular muscle because these vessels also had reduced sensitivity to α-adrenoceptor agonists with decreases in their pEC\textsubscript{50}s for PE, methoxamine and clonidine and smaller maximum contractions to methoxamine and clonidine. Because the contractions to 60 mM K⁺ were also reduced in PMAs from STZ-NI rats, there appears to be a generalized decrease in the reactivity of these vessels to contractile agents. However, I cannot exclude the possibility that the expression of α\textsubscript{1}- and α\textsubscript{2}-adrenoceptors in the vascular muscle of PMAs is reduced by diabetes. Contractions to 1 µM α,β-methylene ATP were not reduced in PMAs from STZ-NI rats, but these responses were considerably smaller
than those produced by highest concentrations of α-adrenoceptor agonists tested or by 60 mM K⁺. Perhaps a reduced contraction would have been seen had a higher concentration of α,β-methylene ATP been used.

In contrast to the finding in MAs, no changes in endothelial-mediated vasodilation were detected in PMAs from STZ-treated rats. Endothelial-mediated vasodilation is thought to occur via the release of nitric oxide (NO) and other endothelial-derived relaxing factors. The relative contribution of these different vasodilator factors varies depending on the vessel studied. For instance, in MAs both NO and an endothelium-derived hyperpolarizing factor (EDHF) contribute to the vasodilation elicited by stimulation of endothelial muscarinic receptors with acetylcholine (Randall & Hiley, 1988; Baisch et al., 1994), whereas in the femoral arteries vasodilation to this stimulus is predominantly mediated by NO (Zygmunt et al., 1995). In a comparative study, Wigg et al. (2001) found that STZ-induced diabetes selectively decreased EDHF-mediated vasodilation in MAs but did not change the NO-mediated vasodilation in either mesenteric or femoral arteries. In the present study, the endothelial-derived relaxing factors contributing to the vasodilation triggered by the muscarinic agonist carbachol were not investigated. Perhaps the vasodilation in the PMAs, like that in femoral arteries, is primarily mediated by NO and that this is not significantly affected by diabetes.

PMAs are likely to be under strong thermoregulatory control like the rat tail artery that supplies blood to the skin of the tail (Ootsuka et al., 2004). In rat tail artery, 8 weeks of STZ-induced diabetes has been reported to produce a small decrease in nerve-evoked contractions (Hart et al., 1988) whereas another study found no change in nerve-evoked contractions after 12 weeks of diabetes (Speirs et al., 2006). In both these studies, the sensitivity to noradrenaline was assessed with the former study reporting no changes (Hart et al., 1988) and the latter reporting an increased
sensitivity to this agent (Speirs et al., 2006). In the study of Speirs et al. (2006), the tail arteries from diabetic rats were studied in physiological saline containing a high concentration of glucose (25 mM) and in control vessels bathed in the same solution the sensitivity to noradrenaline was similarly increased. In preliminary studies, the effects of increasing the glucose to 25 mM were assessed on reactivity of PMAs to nerve stimulation and to PE, methoxamine and clonidine and no changes were observed. Speirs et al. (2006) also reported an increased contribution of purinoceptors to nerve-evoked constrictions of tail arteries from diabetic rats. The relative contribution of P2X1-purinoceptors to neurovascular transmission (assessed with NF449) in PMAs from STZ-NI rats was not changed. Furthermore, blockade of $\alpha_1$- and $\alpha_2$-adrenoceptors with prazosin and idazoxan, respectively, reduced nerve-evoked contractions to a similar extent in PMAs from all the STZ and control groups of rats. In MAs, the blockade of nerve-evoked contractions produced by the P2-purinoceptor antagonist suramin was not changed by diabetes.

PMAs from STZ-LI and STZ-HI rats did not have reduced nerve-evoked contractions and their sensitivity to $\alpha$-adrenoceptor agonists was also not reduced. The findings in PMAs from STZ-LI rats, which remained markedly hyperglycemic, may be explained by the improved health of these animals and it is possible that changes would have been observed had a longer period of diabetes been studied. However, it is also possible that some of the changes observed in the PMAs from STZ-NI rats are the result of the marked reduction in insulin levels rather than hyperglycemia (Muniyappa et al., 2007) and that these are prevented in the STZ-LI rats. In PMAs from STZ-HI rats, an increase in sensitivity to clonidine and a trend towards an increase in sensitivity to methoxamine was observed. These effects may also be explained by a direct effect of insulin treatment, which has been reported to increase vascular reactivity to $\alpha$-adrenoceptor agonists (Kobayashi & Kamata, 1999).
The effects of STZ-induced diabetes on the sympathetic innervation of arteries have rarely been investigated and where they have been examined no changes have been detected (tail artery (Speirs et al., 2006); cerebral arteries (Lagnado et al., 1987)). In MAs from STZ rats, there was a ~12% decrease in both the frequency of TH-IR intercepts along the line profiles of the perivascular plexus and the % area of the vessel surface covered by the immunolabeled nerve plexus. This reduction in nerve fiber density cannot be attributed to a difference in fixation-induced shrinkage because, as with the estimated lumen diameters of the myograph mounted vessels, the widths of the wholemount MAs from STZ rats were 20-25% larger than those of control MAs. However, as the surface area of the vessels was increased, the total number of nerve bundles supplying a segment of artery was not reduced. Instead, it appears that the number of axon bundles in the perivascular plexus did not increase proportionately with the increase in vascular dimensions. This possibility may explain why the absolute increase in force produced by nerve stimulation did not differ between MAs from STZ and control rats.

In PMAs from STZ-NI rats, where the vascular dimensions were not changed, the ~25% reduction in the frequency of fluorescent intercepts along the line profiles indicates that the total number of nerve bundles supplying a segment of artery was reduced. In rat tail arteries, a reduction in innervation density due to axon loss is associated with a decrease in the activity of neuronal NET (Tripovic et al., 2011). As the effects of blocking NET on sensitivity to PE did not differ between PMAs from STZ-NI and control rats, perhaps this indicates that the changes in the perivascular plexus are produced by rearrangement of the terminal axons rather than loss of axons. In PMAs from both STZ-NI and STZ-LI rats, the fluorescent intercept widths along the line profiles were larger indicating thicker axon bundles. As a result, the % area of the vessel surface covered by the immunolabeled nerve plexus was only reduced by ~12% in STZ-NI rats, whereas it was increased by ~21% in STZ-LI rats. The cause of thickening
of the axon bundles was not further investigated but it has been reported that STZ-induced diabetes causes swelling of sympathetic axons supplying the intestine (Clark & Schmidt, 1984), corpus cavernosum (Morrison et al., 2007a), seminal vesicle (Morrison et al., 2006) and pineal gland (Tsai et al., 2008). In addition to the changed morphology of the perivascular nerve plexus, both the mean peak intensity of the TH-IR fluorescent intercepts along the line profiles and the integrated TH-IR fluorescence were increased in PMAs from both STZ-NI and STZ-LI rats, suggesting an increased content of TH. An increase in the intensity of TH-IR fluorescence has been reported for sympathetic axons supplying the corpus cavernosum (Morrison et al., 2007a) and seminal vesicle (Morrison et al., 2006) in STZ rats. The changes in the structure of the axon plexus and in TH-IR suggest that plastic changes are taking place in the sympathetic innervation of PMAs. No changes were detected in the perivascular axon plexus of PMAs from STZ-HI rats, indicating that the effects seen in PMAs from both STZ-NI and STZ-LI rats are most likely explained by hyperglycemia.

Sensory nerve-mediated vasodilation of the perfused isolated mesenteric arterial bed has been reported to be reduced in rats 8 weeks following induction of diabetes with STZ (Ralevic et al., 1993). However, in the present study, no changes in the peptidergic innervation of either MAs or PMAs were observed. Furthermore, no changes were detected in the inhibitory effects of activating the perivascular peptidergic axons with capsaicin on PE constricted MAs, or on nerve-evoked contractions of PMAs, from STZ-NI rats.

Together these findings suggest that sympathetic neurons supplying the PMAs are more markedly affected by diabetes than those supplying the mesenteric arteries. For somatic sensory and motor neurons, it is known that neurons with long axons (i.e. supplying the limbs) are most susceptible to diabetes-induced damage (Anand et al., 1996). Little is known about the length dependence of the effects of diabetes on
autonomic neurons. However, as deficits in sympathetic nerve-mediated vasoconstriction of arteries supplying skin of the hands and feet can be an early sign of diabetic autonomic neuropathy (Takahashi et al., 1998; Hilz et al., 2000), sympathetic neurons with long axons may also be particularly susceptible to the effects of diabetes. In rat tail artery, STZ-induced diabetes is reported to produce a length dependent increase in the neuronal content of biogenic amines (noradrenaline, adrenaline, serotonin and dopamine), with the most marked changes occurring in the most distal region of this vessel (Morrison et al., 2004). This length dependence raises the possibility that axonal transport is affected. Previous studies indicated that diabetes affects both anterograde and retrograde axonal transport in the sciatic nerve (Tomlinson & Mayer, 1984), so in the nerve terminal axons there may be changes in the turnover of proteins required for the normal processing of these biogenic amines (e.g. enzymes and transporters). A change in axonal transport may also explain the increased level of TH-IR detected in PMAs from STZ-NI and STZ-LI rats.

In conclusion, this study demonstrates that the effects of diabetes differ between PMAs and MAs. While in both vessels there were reductions in nerve-evoked responses, only in PMAs did diabetes appear to affect the sympathetic nerve terminals. In MAs, the observed reduction in innervation density produced by diabetes can be explained by the increased size of these vessels. In PMAs, the reduction in nerve-evoked contractions may also be explained by a decrease in the reactivity of their vascular muscle to α-adrenoceptor agonists. Importantly, in PMAs all effects of STZ-induced diabetes observed in this study were prevented in the rats receiving a high dose of insulin, demonstrating their dependence on hyperglycemia and/or loss of insulin signaling. Therefore, PMAs provide a suitable model to investigate the effects of diabetes on sympathetic vasoconstrictor neurons and neurovascular transmission, and to assess the efficacy of neuroprotective treatments.
CHAPTER 3 Increased peripherin in sympathetic axons innervating plantar metatarsal arteries in diabetic rats - evidence for axonal remodeling?
CHAPTER 3 DIABETES INCREASES PERIPHERIN IN SYMPATHETIC AXONS INNERVATING PMAs

Abstract

It was previously demonstrated that streptozotocin (STZ)-induced diabetes produced a change in the morphology of perivascular sympathetic nerve plexus of rat plantar metatarsal arteries (PMAs), suggesting remodeling of the sympathetic nerve terminal axons. In addition, diabetes produced an increase in the immunolabeling for tyrosine hydroxylase in the perivascular nerve plexus of PMAs. In this study, Western blotting was used to assess if diabetes increases protein expression levels for tyrosine hydroxylase and the putative indicators of axon regeneration, peripherin and β-tubulin III, in PMAs. In addition, diabetes-induced changes in the peripherin and β-tubulin III immunoreactive nerve plexus of PMAs were assessed. PMAs from STZ-treated rats (60 mg/kg, i.p.) receiving a low dose of insulin (STZ-LI; n = 22) were compared with those from STZ-treated rats receiving high dose of insulin (STZ-HI, n = 19) and vehicle-treated controls (n = 30). In PMAs from STZ-LI rats there was an increase in the protein expression level for peripherin but no change in that for tyrosine hydroxylase or β-tubulin III. In addition there was an increase in the number of peripherin immunoreactive nerve fibers in the perivascular nerve plexus of PMAs from STZ-LI rats. As previously demonstrated, the β-tubulin III immunoreactive nerve fibers were thickened in these vessels. Co-immunolabeling for peripherin and neuropeptide Y (a marker for sympathetic axons) revealed that peripherin immunoreactivity increased in sympathetic axons. These findings indicate that increased peripherin in sympathetic axons is likely due to hyperglycemia and suggests that axons are regenerating within the perivascular nerve plexus. No changes in peripherin protein expression were detected in the tail artery suggesting that the sympathetic neurons supplying the PMAs may be particularly vulnerable to the effects of diabetes. Together these findings make PMAs a suitable model to assess the development and prevention of diabetes-induced sympathetic neuropathy.
CHAPTER 3 DIABETES INCREASES PERIPHERIN IN SYMPATHETIC AXONS INNERRVATING PMAs

Introduction

Diabetes increases the intensity of immunolabeling for tyrosine hydroxylase (TH) in the perivascular plexus of plantar metatarsal arteries (PMAs) (see Chapter 2). Other studies have reported diabetes-induced thickening of sympathetic axons in the intestine, the corpus cavernosum and pineal gland in streptozotocin (STZ)-induced diabetic rats (Clark & Schmidt, 1984; Morrison et al., 2007b; Tsai et al., 2008). In the pineal gland, the protein expression of TH was unchanged and thus did not explain sympathetic axonal thickening (Tsai et al., 2008). In the corpus cavernosum, immunofluorescence for TH was increased but TH protein was not quantified (Morrison et al., 2007b). It is possible that increases in TH immunofluorescence in diabetic PMAs are due to diabetes-induced axon swelling rather than increases in cytoplasmic protein concentration (see Chapter 2), and thus studies of proteins known to be involved in axon remodeling are warranted.

The mechanisms involved in the thickening of sympathetic axons are currently unknown. The circulating and tissue levels of insulin-like growth factor 1 (IGF-1) are reduced in rats with STZ-induced diabetes (Bornfeldt et al., 1989), and treatment of STZ-induced diabetic rats with IGF-1 reduces the frequency of swollen sympathetic axons within the paravascular mesenteric nerves (Schmidt et al., 1999). In contrast, although it has been suggested that deficiencies in nerve growth factor (NGF) support are a cause of sympathetic neuropathy (Anand et al., 1996), treatment of diabetic rats with NGF did not change the number of swollen sympathetic axons (Schmidt et al., 2001a). However, there may be slowing of retrograde axon transport in diabetes (Hellweg et al., 1994), which would impair the ability of the axons to respond to NGF.

In addition to swelling of the sympathetic axons within the paravascular nerves that supply the distal ileum and colon (see Chapter 1 section 7.2), Schmidt et al. (1981) also described growth cone-like structures within these nerves suggesting axons were
undergoing regeneration. In normal adult rats, growth associated protein 43 (GAP-43), a marker for synaptic plasticity (Skene et al., 1986), is expressed at relatively high levels in nerve terminals of many postganglionic sympathetic neurons, including those supplying the vasculature, which suggests that they undergo continuous cycles of degeneration and regeneration. Schmidt et al. (1991) investigated the possibility that STZ-induced diabetes increases the expression of GAP-43 in prevertebral (superior cervical ganglion) and paravertebral ganglia (superior mesenteric and celiac ganglia), but they detected no changes in either immunolabeling for GAP-43 in the cell bodies or overall protein expression for GAP-43 in the ganglia. However, it remains possible that diabetes selectively increases expression of GAP-43 in the sympathetic nerve terminals, which contributes to the observed morphological changes. This possibility is difficult to investigate immunohistochemically because of the already high levels of GAP-43 in normal sympathetic nerve terminals. Increases in immunolabeling for peripherin, another putative marker for axonal regeneration (Oblinger et al., 1989b), have also been described in the swollen “dystrophic” axons within the superior mesenteric ganglia of diabetic humans (Schmidt et al., 1997). The possibility that diabetes produces changes in peripherin expression in the nerve terminals of postganglionic sympathetic neurons has not yet been investigated.

Peripherin is a type III intermediate filament protein that is expressed principally in subpopulations of peripheral neurons (i.e. sensory, autonomic and somatic motor neurons) (Escurat et al., 1990; Gorham et al., 1990). It can assemble into filaments by itself or with the three neurofilament (NF) proteins NF-light (NF-L), NF-medium (NF-M) and NF-heavy (NF-H) (Parysek et al., 1991; Beaulieu et al., 1999b). However, the exact function of peripherin in neurons is unknown. In the rat, peripherin expression is highest at late stages of embryonic development where it is present in newly differentiated neurons (Escurat et al., 1990; Gorham et al., 1990). As peripherin expression is coincident with axonogenesis, a role in axon growth has been postulated.
In the normal adult rat, peripherin immunoreactivity is primarily localized to a subset of small-diameter sensory neurons in the dorsal root ganglia (DRG) and some pre- and postganglionic sympathetic neurons (Parysek & Goldman, 1988; Oblinger et al., 1989b; Fornaro et al., 2008). Immunolabeling for peripherin has been described in the nerve fibers surrounding blood vessels in rat tongue (Parysek & Goldman, 1988) and cerebellum (Errante et al., 1998), but it was not established if these fibers were sympathetic axons. In sensory neurons, there is evidence that peripherin plays an important role in axon regeneration after nerve injury. Axotomy of the sciatic nerve produced an increase in the peripherin protein expression in the ipsilateral L5 DRG and the protein was transported to the newly formed axon sprouts distal to the site of injury (Oblinger et al., 1989b). No changes in other structural proteins such as NF-H, NF-M and NF-L were detected (Oblinger et al., 1989a). In further support for a role for peripherin in regeneration, Fornaro et al. (2008) demonstrated that upregulation of peripherin expression in DRG following axotomy is temporally correlated with the beginning of axon regrowth. To date there are no reports of peripherin expression being upregulated during regeneration of sympathetic axons, but if the diabetes-induced morphological changes in sympathetic nerve terminal axons are due to degeneration followed by sprouting and regeneration, it is possible that they will be accompanied by increases in peripherin expression.

In DRG neurons, axotomy also increases expression and transport of the neuron-specific microtubule protein β-tubulin III (Moskowitz & Oblinger, 1995). These elevated levels of β-tubulin III are suggested to change the composition and organization of the axonal cytoskeleton to promote axon regeneration. Expression levels of β-tubulin III are also increased in DRGs from STZ-treated rats (Liuzzi et al., 1998). Changes in the levels of β-tubulin III during regeneration of sympathetic neurons have not yet been examined.
CHAPTER 3 DIABETES INCREASES PERIPHERIN IN SYMPATHETIC AXONS INNERVATING PMAs

The present study tested the hypothesis that diabetes increases the protein expression of TH, peripherin and β-tubulin III in the perivascular nerve plexus of PMAs. Protein levels were quantified by Western blotting, and for both peripherin and β-tubulin III, changes to both the structure and fluorescence intensity of the immunolabeled nerve plexus were quantified. To assess if diabetes produced similar effects in another artery supplying blood to skin, TH, peripherin and β-tubulin III protein expression levels were also assessed in the proximal and distal parts of the tail artery.

Materials and Methods

Animal Handling and Tissues

All experiments were approved by the University of Melbourne Animal Ethics Committee and conformed to the Australian code of practice for the care and use of animals for scientific purposes. Male Wistar rats, 8-10 weeks old, from the Animal Resources Centre (Canning Vale, WA, Australia), were treated intraperitoneally with 60 mg/kg STZ dissolved in 50 mM citrate buffer (pH 4.5). Age-matched vehicle control animals were treated with an equivalent volume of citrate buffer. Blood glucose levels were measured 5-7 days post-injection using a glucose meter (Accu-Check Performa, Roche Diagnostics Australia Pty Ltd) to confirm that the STZ-treated rats were diabetic (>15 mM glucose). The STZ-treated rats were then divided into two groups, one of which received a low dose of insulin and were hyperglycemic (STZ-LI; blood glucose >20 mM) and the other received a high dose of insulin and were normoglycemic (STZ-HI; blood glucose <15 mM). Insulin was delivered by sustained-release implants (Linplant, Linshin Canada Inc.) inserted subcutaneously beneath the nuchal skin. The STZ-LI rats received half a Linplant pellet (~1 insulin unit/day) at 1 week and again at 6-7 weeks post-STZ treatment. The first cohort of STZ-HI rats received 2 Linplant pellets (~4 insulin units/day) at 1 week and 6-7 weeks after STZ treatment. To better maintain
CHAPTER 3 DIABETES INCREASES PERIPHERIN IN SYMPATHETIC AXONS INNERVATING PMAS

normoglycemic levels and prevent the animals from becoming transiently hypoglycemic, the second cohort of STZ-HI rats initially received 1.5 pellets at 1 week post-STZ treatment and then received additional pellets (1-1.5 pellets) at ~30 day intervals when the blood glucose levels increased to between 10 and 15 mM. The third cohort consisted of STZ-LI rats and their age-matched vehicle controls. Blood glucose levels and body weights were monitored at weekly intervals until rats were terminated 12-13 weeks after the induction of diabetes or the injection of citrate buffer. Animals were maintained on a 12:12 hour light-dark cycle and were provided with food and water ad libitum.

At termination the animals were deeply anaesthetized with isoflurane and exsanguinated by cutting the carotid arteries. Approximately 1 ml of blood was collected in EDTA tubes from all rats to determine the glycosylated hemoglobin levels by high-performance liquid chromatography (CLC330 GHb Analyzer, Primus, Kansas City, MO). From each hind paw, the median plantar artery and the five plantar metatarsal arteries (PMAs) branching from the plantar arch were dissected in cold phosphate buffered saline (PBS) containing 1:1000 of 50 mM protease inhibitor phenylmethylsulfonyl fluoride (PMSF; 1.74 mg/ml in isopropanol stored at -20°C; P7626, Sigma). The first two PMAs from the medial side of each hind paw were used for immunohistochemistry. The remaining arteries from both hind paws were pooled for each animal and snap frozen in liquid nitrogen and stored at -80 °C. Additionally, segments of tail artery were dissected from the proximal (2-3 cm from base of tail) and the distal (12-15 cm from base of tail) part of the tail to assess whether diabetes equally affected protein expression in both the plantar and tail vascular beds.

In all immunohistochemical experiments, PMAs collected from STZ-treated and control rats terminated on the same day were performed in parallel on the same slide (i.e. for each set of immunolabels). Western blotting experiments on PMAs and tail
arteries were conducted on tissue samples from the first two cohorts that contained all three treatment groups.

**Tissue Homogenization**

The following components (purchased from Sigma-Aldrich Pty. Ltd., Sydney, NSW, Australia) were added to each arterial tissue sample (wet weight ≤ 30 mg): 300 µl Radio-Immunoprecipitation Assay buffer (R0278), 3 µl protease inhibitor cocktail (P8340), 0.5 µl of 50 mM PMSF. Samples were kept at 4°C during the procedures. Arteries were diced using scissors, sonicated for 3-4 seconds twice (speed 6; XL2000, Misonix, NY, USA), centrifuged at 17,970 x g for 5 minutes, and then the supernatant was collected.

**Assaying Protein Content**

The protein content of each sample was quantified using Bio-Rad’s Dc™ Protein Assay Kit (Cat. No. 500-0116, Bio-Rad Laboratories Pty., Ltd., Gladesville, NSW, Australia). To generate a standard curve, samples containing a known content of bovine serum albumin (BSA; 0, 0.2, 0.5, 0.75, 1.0, 1.5 and 2.0 µg/µl) were prepared by diluting a 2 mg/ml stock solution (Cat. No. 500-0007, Bio-Rad Laboratories Pty., Ltd.) with distilled water. The tissue supernatants were diluted 1:3 and 1:9 with distilled water and the concentrations of both these dilutions were quantified. Three 5 µl samples of each standard solution and tissue sample dilution were placed in separate wells of a flat-bottomed 96-well plate (Cat. No. 3599, Costar, Corning, NY, USA). As per manufacturer’s instructions, 25 µl of Solution A’ (2% v/v Solution S mixed with Solution A) followed by 200 µl of Solution B were added to each well. After plating out, the samples were incubated on a shaker (speed 3.5, Cat. No. EOM5, Ratek Instruments Pty Ltd, Boronia, VIC, Australia) for 15 minutes at room temperature. Samples were shaken at 420 rpm for 10 seconds and were read at an absorbance wavelength of 690 nanometers using the Multiskan Ascent plate reader (Thermofisher Scientific Australia)
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Pty Ltd, Scoresby, VIC, Australia) and the Ascent Software (v. 2.6). A standard absorbance curve was generated for the samples containing known BSA concentrations and this was fitted by linear regression. Using this standard curve, the protein concentrations (in µg/µl) of the 1:3 and 1:9 dilutions of each sample were estimated using the average absorbance values for the three replicates, and these were multiplied by 3 and 9, respectively, to calculate the protein concentration of the undiluted sample. All absorbance values for the diluted samples were within the range of the standard curve. Samples were then aliquoted in volumes required to load 20 µg (PMAs) or 40 µg (proximal and distal tail arteries) of protein per well on the gels and stored at -80°C. The volume required per well of the gel was calculated by dividing the protein concentration of the undiluted sample (µg/µl) by the total amount of protein required per well on the gel.

Western Blotting

SDS-PAGE

Proteins were separated using the Mini-PROTEAN 3 Electrophoresis System (Cat. No. 456-1094, Bio-Rad), using up to 4 pre-cast 4-20% Mini-PROTEAN® TGX™ precast gradient gels (Cat. No. 456-1094, Bio-Rad; 2 gels per tank). To ensure equal volumes of the samples were loaded in each well, distilled water was added to the aliquots of the more concentrated samples so that their final protein concentration was identical to that of the sample with the lowest protein concentration. To each sample an equal volume of loading dye (Laemmli sample buffer; Cat. No. 161-0737, Bio-Rad) was added. In addition, 2-mercaptoethanol was added so that its final concentration in the sample was 2% (v/v; Cat. No. M3148, Sigma-Aldrich). All samples were heated to 95-100°C for 5 minutes using a heat block (DBH10, Ratek Instruments Pty Ltd, Boronia, VIC, Australia). After gels were placed into the electrophoresis tank, each tank was filled with 500 ml of 1 x Tris/glycine buffer (Cat. No. 161-0732, Bio-Rad). In each gel, 6
µl of a molecular weight marker (Cat. No. 161-0374, Bio-Rad) was loaded into at least one well. In addition, to allow comparisons between the protein content of samples run on different gels, at least one of the wells per gel contained a protein sample common to all gels. After all samples were loaded into the gel, the tanks were connected to a PowerPac™ power supply (Cat. No. 164-5050, Bio-Rad) and the proteins separated electrophoretically by applying 100 V for 1 hour 40 minutes at room temperature.

*Protein transfer*

Proteins separated electrophoretically on the gels were transferred to nitrocellulose membrane (pore size 0.22 µm; Cat. No. 926-31092, Li-Cor, NE, USA) using Bio-Rad’s Mini Trans-Blot® Electrophoretic Transfer Cell (Cat. No. 170-3930). The membrane was superimposed on the gel and both were sandwiched between two filter papers and two fiber pads within a transfer cassette. The assembly was carried out with all the cassette components pre-wetted with Tris/glycine buffer (Cat. No. 161-0734, Bio-Rad) containing 20% (v/v) methanol. Two cassettes were mounted in each tank, which was filled with 700 ml of 1 x Tris/glycine buffer containing 20% (v/v) methanol and connected to a PowerPac™ power supply. Transfers were carried out overnight at 30 V and 4°C. After the membranes were removed from the cassettes they were stained for 5 minutes with 0.1% (w/v) ponceau red (Sigma) in 5% (v/v) acetic acid (Sigma) and washed 3 x with distilled water. This step reveals all the protein bands on the membranes and was performed to ensure protein transfer was successful. Membranes were washed 3 x with distilled water before they were air-dried for 1 hour at room temperature.
Antibody Labeling for Western Blotting

Membranes were blocked in casein blocking buffer (Cat. No. 927-40000, Li-Cor, NE, USA) for at least 1 hour at room temperature. Both, primary and secondary antibodies were diluted in casein buffer with 0.01% Tween-20 (P5927, Sigma-Aldrich). The membranes were incubated with primary antibodies directed against TH, β-tubulin III or peripherin at 4°C overnight (antibody dilution and source are summarized in Table 3.1). Unbound antibodies were removed by washing with PBS containing 0.001% Tween20 (3 x 10 minutes). Membranes were then incubated with secondary antibodies (Table 3.1) for 1 hour at room temperature before they were again washed with PBS-0.001% Tween-20 (3 x 10 minutes) and then with PBS alone (1 x 10 minutes). β-tubulin III labeling was visualized by applying a biotin-streptavidin step to enhance signal detection. After excess β-tubulin III antibodies were washed off the membrane with PBS-0.001% Tween-20 (3 x 10 minutes), biotinylated horse anti-mouse IgG antibody was applied for 1 hour at room temperature and then the membrane was washed with PBS-0.001% Tween-20 (3 x 10 minutes). Subsequently, membranes were fluorescently labeled with streptavidin 680LT dye for 1 hour at room temperature before being washed off with PBS-0.001% Tween-20 (3 x 10 minutes) and then with PBS alone (1 x 10 minutes). Membranes were imaged using Li-Cor’s Odyssey® near infrared imager and images were acquired using the program Odyssey v2.1. (Li-Cor, NE, USA). After the protein of interest was imaged, the membranes were co-labeled for the loading control protein β-actin using the same protocol as described above. If the primary antibodies for the protein of interest were raised in rabbit or mouse, the primary antibody used for β-actin was raised in either mouse or rabbit (Table 3.1), respectively. After applying the fluorescent secondary antibody to reveal β-actin labeling, both the protein of interest and housekeeper protein were imaged simultaneously.
**Table 3.1.** Summary of primary and secondary antibodies used in Western blotting (WB) and immunohistochemistry (IHC).

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Primary Antibody Concentration for IHC</th>
<th>Secondary Antibody for IHC</th>
<th>Predicted Molecular Weight</th>
<th>Primary Antibody Concentration for WB</th>
<th>Secondary Antibody for WB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-tyrosine hydroxylase</td>
<td>1:1000</td>
<td>1:500 donkey anti-mouse 594 IgG H+L (Cat. No. A21203, Molecular Probes, Inc., OR, USA)</td>
<td>60 kDa</td>
<td>1:1000</td>
<td>1:5,000 IRDye® donkey anti-mouse IgG H+L 680LT (Cat. No. 926-68022, Li-Cor, Nebraska USA)</td>
</tr>
<tr>
<td>Mouse anti-β-tubulin III</td>
<td>1:750</td>
<td>1:500 donkey anti-mouse 647 IgG H+L (Cat. No. A31571, Molecular Probes, Inc., OR, USA)</td>
<td>50 kDa</td>
<td>1:1000</td>
<td>1:5000 biotinylated horse anti-mouse IgG H+L (Cat. No. BA-2000, Vector Labs, Burlingame, CA, USA)</td>
</tr>
<tr>
<td>Rabbit anti-peripherin</td>
<td>1:500</td>
<td>1:1000 donkey anti-rabbit 488 IgG H+L (Cat. No. A21206, Molecular Probes, Inc., OR, USA)</td>
<td>57 kDa</td>
<td>1:5000</td>
<td>1:1000 IRDye® donkey anti-rabbit 800CW IgG H+L (Cat. No. 926-32213, Li-Cor, Nebraska USA)</td>
</tr>
</tbody>
</table>
### CHAPTER 3 DIABETES INCREASES PERRIPHERIN IN SYMPATHETIC AXONS INNERVATING PMAs

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Secondary Antibody</th>
<th>Dilution</th>
<th>Molecular Weight</th>
<th>rf</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep anti-neuropeptide Y (Clone E2210, Gift from J. Oliver)</td>
<td>1:2000</td>
<td>1:500 donkey anti-sheep 647 IgG H+L (Cat. No. A21448, Molecular Probes, Inc., OR, USA)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mouse anti-β-actin (Cat. No. 926-42212, Li-Cor, Nebraska USA)</td>
<td>-</td>
<td>-</td>
<td>45 kDa</td>
<td>1:10,000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rabbit anti-β-actin (Cat. No. 926-42210, Li-Cor, Nebraska USA)</td>
<td>-</td>
<td>-</td>
<td>45 kDa</td>
<td>1:1000</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1:5000 IRDye® donkey anti-mouse IgG H+L 680LT (Cat. No. 926-68022, Li-Cor, Nebraska USA)

1:1000 IRDye® donkey anti-rabbit 800CW IgG H+L (Cat. No. 926-32213, Li-Cor, Nebraska USA)
The fluorescence intensities of the bands were analyzed using ImageJ (NIH, Bethesda, USA) and the protocol described at the following website: http://rsb.info.nih.gov/ij/docs/menus/analyze.html#gels. The protein content for each of the bands was quantified by measuring its integrated pixel intensity. This was done by defining a rectangular area that completely covered the protein band in each of the lanes, and then placing this on the protein band in each lane and measuring the integrated pixel intensity for this fixed area. β-actin was confirmed as an appropriate housekeeper protein for Western blotting, as the protein content of the β-actin bands did not differ between the samples from the control, STZ-HI and STZ-LI groups of rats. The relative protein expression level for each protein of interest (i.e. TH, β-tubulin III or peripherin) was determined by dividing the integrated pixel intensity of the band of interest by that for the β-actin band for the same sample. To correct for inter-gel variability, the relative values for the test samples on each gel were normalized to that for the intra-gel control on the same gel (i.e. expressed relative to the intra-gel control value). This enables comparison of samples run on different gels.

**Tissue Fixation and Immunohistochemistry**

Tissues used for immunohistochemistry were fixed at their *in vivo* length in Zamboni’s fixative overnight at 4°C. Samples were washed 3 x 10 minutes with dimethyl sulfoxide followed by 3 x 10 minutes with PBS (pH 7.1-7.2), and then stored in PBS with 0.01% (v/v) sodium azide at 4°C. They were then blocked for at least 1 hour at room temperature in PBS-1%Triton with 10% normal horse serum. Tissues were labeled with antibodies raised against peripherin, β-tubulin III and neuropeptide Y (NPY) antibodies (Table 3.1). Fluorescent secondary antibodies were raised in donkey (Table 3.1). Primary antibodies were diluted in antibody diluent (290.9 mM NaCl, 7.5 mM Na₂HPO₄, 2.5 mM NaH₂PO₄ and 0.1% (w/v) sodium azide; pH 7.1) and incubated overnight at 4°C. Excess antibody was washed off 3 x 10 minutes with PBS and
secondary antibodies were applied for 1 hour at room temperature. Additionally, no primary antibody control experiments were performed for each sample to ensure specificity of secondary antibodies. Samples were washed for 3 x 10 minutes in PBS and mounted and coverslipped on Starfrost® slides (Waldemar Knittel Glasbearbeitungs GmbH, Braunschweig, Germany) with fluorescence mounting medium (Dako North America, Inc., CA, USA). Samples were viewed with a Zeiss Pascal confocal microscope system and imaged as Z-stacks through the entire adventitial thickness.

Data Analysis

All statistical analyses were performed using SPSS 19 (IBM Corp, Armonk, NY, USA) or GraphPad Prism (GraphPad Inc., La Jolla, CA, USA). For Western blotting experiments and the measurements of net body weight gain, % glycosylated hemoglobin and blood glucose levels at termination, comparisons were first made between control, STZ-HI and STZ-LI groups using one-way ANOVA, or a Kruskal-Wallis test if the data variance differed significantly between the experimental groups (assessed using Levene’s test). Post-hoc pairwise comparisons were made with Tukey’s honest significant difference (HSD) tests or Dunn’s tests. Pearson’s product-moment correlation coefficient was used to calculate the strength of the correlation between the peripherin protein expression levels in PMAs and the % of glycosylated hemoglobin in the blood of the animal from which they are derived. Prior to making this comparison, it was confirmed that both sets of data were normally distributed using Kolmogorov-Smirnov tests.

The density of the β-tubulin III and peripherin immunolabeled perivascular axon plexus innervating PMAs was quantified using maximum intensity Z-projection images collected with a x63 objective (pixel size 0.24 μm²). This was done by collecting two line profiles at the same points on all images (one placed in the upper half and the
other in the lower half of the image) using ImageJ (National Institute of Health, Bethesda, MD). After the mean maximum background value (determined from 10 points on the image where there was no detectable fluorescence) was subtracted, the number (intercept number), widths (intercept widths), and peak pixel values of the regions along the line profiles with pixel values >0 were determined using an in-house Igor Pro routine. In addition, ImageJ was used to measure the percentage area of the vessel surface covered by the β-tubulin III or peripherin immunoreactive (IR) nerve plexus after subtraction of the mean maximum background. The integrated peripherin and β-tubulin III-IR fluorescence for the entire vessel surface in each image (also measured with ImageJ) was calculated after subtraction of the mean maximum background level and normalized to 100 µm² of vessel surface. For each nerve plexus measure, one-sample t-tests were used to determine if the measures in arteries from STZ-treated animals expressed as a percentage of those in the control tissues collected on the same day (and processed in parallel) differed significantly. For each animal, the measures were the average obtained from at least three image Z-stacks.

For studies assessing co-localization of peripherin and NPY, line profiles for each label were obtained at the same two points on each RGB image (one in the upper half and the other in the lower half of the image). Prior to making comparisons, the mean maximum background value for each label (determined as above) was subtracted from the line profile for that label. For each set of line profiles, co-labeling was assessed by determining the percentage of fluorescence peaks along each line profile at which the pixel values at the same location on each line profile were >0. In addition, the number of regions along each line profile with pixel values >0 (i.e. intercept number) was determined for each immunolabel.
Results

Animals

All STZ-treated rats had elevated blood glucose levels (>19 mM) prior to insulin treatment (at 1 week after STZ treatment), and the blood glucose levels for the STZ-LI rats was >15 mM throughout the 12-week period (Figure 3.1). In comparison, the blood glucose levels of STZ-HI rats were greatly reduced by insulin treatment (between STZ-LI and STZ-HI group comparison P < 0.001). At termination, the blood glucose levels did not differ between control and STZ-HI rats, but these levels were significantly lower than for STZ-LI rats (Table 3.2). The net body weight gain for STZ-LI animals at termination was less than for both STZ-HI and control rats, and that for the STZ-HI rats was also less than that for the controls (Table 3.2). Terminal % glycosylated hemoglobin levels in blood from STZ-LI rats (~16%) were about twice those in STZ-HI rats (~8%), but levels in STZ-HI rats were slightly higher than those in control rats (~5%) (Table 3.2).

β-actin protein expression levels did not vary between treatment groups in PMAs and tail arteries

The β-actin protein expression in all samples was identified as a 45 kDa band in PMAs (Figure 3.2 B, D, F) and tail arteries. The intensity values relative to the inter-gel control for β-actin protein expression for arteries from each treatment group (i.e. controls, STZ-HI and STZ-LI) were compared to ensure diabetes did not selectively alter β-actin protein content. Between the three groups, no differences in β-actin protein expression (Table 3.3) were detected. β-actin was therefore an appropriate loading control for standardizing TH, β-tubulin III and peripherin protein expression for PMAs and tail arteries.
CHAPTER 3 DIABETES INCREASES PERIPHERIN IN SYMPATHETIC AXONS INNERVATING PMAS

Figure 3.1. Blood glucose levels (mM) measured at weekly intervals after i.p. injection of streptozotocin (STZ). Before the insertion of insulin pellets (at 1 week) all STZ-treated rats had elevated blood glucose levels. Throughout the 12-week period, STZ-treated rats given a low dose of insulin (STZ-LI, n = 22) maintained blood glucose levels >15 mM (i.e. hyperglycemic). Those given a high dose of insulin (STZ-HI, n = 19), at 3 weeks and thereafter had blood glucose measurements <15 mM. The graph also shows blood glucose measurements at 1 week and at 12 weeks in the citrate buffer-treated control rats (n = 30) and these data points are joined by the dashed line. The first arrow indicates the time at which the STZ-LI and STZ-HI rats received their first insulin pellet(s). The second arrow indicates the time the STZ-LI rats and the first cohort of STZ-HI rats received their second insulin pellet(s). The other cohorts of STZ-HI rats were given additional insulin pellets when their blood glucose increased to between 10-15 mM.
CHAPTER 3 DIABETES INCREASES PERIPHERIN IN SYMPATHETIC AXONS INNERVATING PMAS

Table 3.2. Net body weight gains, % glycosylated hemoglobin levels and blood glucose levels at termination in STZ-treated rats given a high (STZ-HI) or low (STZ-LI) dose of insulin and their controls.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Net body weight gain (g)</th>
<th>% Glycosylated hemoglobin</th>
<th>Terminal blood glucose level (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 30)</td>
<td>241 ± 9</td>
<td>5.3 ± 0.4</td>
<td>6 (5.4 - 6.7)</td>
</tr>
<tr>
<td>STZ-HI (n = 19)</td>
<td>196 ± 9</td>
<td>7.7 ± 0.7</td>
<td>5.8 (4.7 - 8.5)</td>
</tr>
<tr>
<td>STZ-LI (n = 22)</td>
<td>104 ± 8</td>
<td>16.4 ± 0.5</td>
<td>26.1 (23.8 - 27.8)</td>
</tr>
</tbody>
</table>

Comparison between groups

<table>
<thead>
<tr>
<th>Comparison between groups</th>
<th>Net body weight gain</th>
<th>% Glycosylated hemoglobin</th>
<th>Terminal blood glucose level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vs STZ-HI</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P = 0.61</td>
</tr>
<tr>
<td>Control vs STZ-LI</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>STZ-HI vs STZ-LI</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

Data are presented as means and SEMs or medians and interquartile ranges (in parentheses). Comparisons between the control, STZ-HI and STZ-LI groups were initially made using one-way ANOVA or the Kruskal-Wallis test and pairwise comparisons between the groups were made with Tukey’s HSD or Dunn’s tests.
Table 3.3. Comparison of β-actin protein expression levels (integrated fluorescent intensity of band/pixel) in arterial samples between control, STZ-HI and STZ-LI rats.

<table>
<thead>
<tr>
<th>Plantar Metatarsal Arteries</th>
<th>Distal Tail Artery</th>
<th>Proximal Tail Artery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>STZ-HI</td>
<td>STZ-HI</td>
<td>STZ-HI</td>
</tr>
<tr>
<td>STZ-LI</td>
<td>STZ-LI</td>
<td>STZ-LI</td>
</tr>
<tr>
<td>2.64 (1.51 - 3.70)</td>
<td>2.20 ± 0.29</td>
<td>3.49 ± 0.49</td>
</tr>
<tr>
<td>1.56 (1.19 - 2.83)</td>
<td>1.94 ± 0.23</td>
<td>4.35 ± 0.63</td>
</tr>
<tr>
<td>2.09 (1.77 - 5.23)</td>
<td>2.59 ± 0.36</td>
<td>3.82 ± 0.66</td>
</tr>
<tr>
<td>n = 13</td>
<td>n = 13</td>
<td>n = 13</td>
</tr>
<tr>
<td>n = 12</td>
<td>n = 12</td>
<td>n = 12</td>
</tr>
<tr>
<td>Kruskal-Wallis Test P = 0.22</td>
<td>ANOVA P = 0.32</td>
<td>ANOVA P = 0.59</td>
</tr>
</tbody>
</table>

Data are presented as means and SEMs or medians and interquartile ranges (in parentheses). Statistical comparisons between the control, STZ-HI and STZ-LI groups were made using one-way ANOVA or the Kruskal Wallis test.
CHAPTER 3 DIABETES INCREASES PERIPHERIN IN SYMPATHETIC AXONS INNERVATING PMAS

Tyrosine hydroxylase content in PMAs was not changed by diabetes

In Chapter 2 data are presented showing an increase in both the integrated TH-IR immunofluorescence per 100 µm² of vessel surface and the peak immunofluorescence of the intercepts along the line profiles in PMAs from STZ-LI rats. Western blots for TH had a single band at approximately 60 kDa, which is the predicted weight of TH (Figure 3.2 B). Although there were higher outliers for the TH contents measured for PMAs from STZ-LI rats, comparison using the Kruskal-Wallis test indicated no significant differences (P = 0.21) between the TH content of PMAs from control, STZ-LI and STZ-HI rats (Figure 3.2 A, B).

A subset of the perivascular axons innervating the PMAs was peripherin-IR

Most, if not all, of the axons within the nerve plexus of PMAs are β tubulin III-IR (see Chapter 2). Figure 3.3 shows images for PMAs from a control (Figure 3.3 A-C) and a STZ-LI rat (Figure 3.3 E-G) immunolabeled for both β-tubulin III and peripherin. Only the thicker β-tubulin III-IR nerve fibers were strongly peripherin-IR in PMAs from control and STZ-HI rats (not shown). By contrast, in the PMAs from the STZ-LI rats, there was an increase in peripherin-IR in the finer nerve fibers (c.f. Figure 3.3 B, F). Figure 3.3 also shows representative line profiles used for quantifying the β-tubulin III-IR and peripherin-IR nerve plexus (see below) collected from the images (Figure 3.3 D, H).
CHAPTER 3 DIABETES INCREASES PERIPHERIN IN SYMPATHETIC AXONS INNERVATING PMAS

A. Tyrosine Hydroxylase

B. kDa MW Control STZ-HI STZ-LI

C. β-tubulin III

D. kDa MW Control STZ-HI STZ-LI

E. Peripherin

F. kDa MW Control STZ-HI STZ-LI
**Figure 3.2.** Tyrosine hydroxylase (TH), β-tubulin III and peripherin protein levels in control, STZ-HI and STZ-LI rats. There were no detectable differences in TH and β-tubulin III protein expression levels between the PMAs from control rats (n = 13) and STZ-HI (n = 12) or STZ-LI (n = 12) rats. Peripherin protein expression in PMAs from STZ-LI rats was greater than that in PMAs from control and STZ-HI rats. (A, C, D) Protein expression for TH (A), β-tubulin III (C) and peripherin (E) was measured relative to loading control β-actin. In A and C, the medians and interquartile ranges are indicated whereas in E the means and SEMs are indicated. (B, D, F) Representative Western blots for TH (B), β-tubulin III (D) and peripherin (F) in PMAs from a control, a STZ-HI and a STZ-LI rat. The predicted molecular weight (MW) for TH, β-tubulin III, peripherin and β-actin were approximately 60, 50, 57 and 45 kDa, respectively. Initial comparisons between the control, STZ-HI and STZ-LI groups were made using a one-way ANOVA or the Kruskal-Wallis tests. In C, pairwise comparisons between the groups were made with Dunn’s tests (ns = not significant; *P < 0.05).
β-tubulin III content in PMAs was not changed by diabetes

Three bands were identified by streptavidin detection on the Western blots for β-tubulin III (Figure 3.2 D). The first two bands (from the top) had much higher molecular weights than that expected for β-tubulin III (~120 kDa and ~75 kDa). These bands were also present on the membranes where no primary antibody or biotinylated antibody had been applied and are therefore most likely due to streptavidin binding to endogenous biotin or a biotin-like protein present in the samples (McKay et al., 2008).

The third band at approximately 50 kDa is consistent with the predicted molecular weight of β-tubulin III (Figure 3.2 D). There was no difference in β-tubulin III content between PMAs from control, STZ-HI and STZ-LI rats (Kruskal-Wallis test, P = 0.14; Figure 3.2 C).

The thickness of the β-tubulin III immunoreactive nerve fibers and the area of the vessel surface covered by these fibers were increased in PMAs from STZ-LI rats

The percentage area of the vessel surface covered by the β-tubulin III-IR nerve plexus and the integrated β-tubulin III-IR immunofluorescence per 100 µm² of the vessel surface were significantly larger (~20% and ~45%, respectively) in PMAs from STZ-LI rats compared to their controls (Figure 3.4 A). In PMAs from STZ-LI rats, the frequency of the β-tubulin III-IR intercepts along the line profiles did not differ significantly from those of their controls but the widths of the intercepts were ~30% larger (Figure 3.4 A). There was also a tendency for the peak β-tubulin III-IR immunofluorescence of the intercepts to be increased in PMAs from STZ-LI rats (Figure 3.4 A) but this effect did not reach the level of statistical significance (P = 0.06). For the PMAs from STZ-HI animals, measured β-tubulin III-IR nerve plexus parameters did not differ from those of their controls (Figure 3.4 B).
Peripherin content was significantly increased in PMAs from STZ-LI rats

Western blots for peripherin had a single protein band located at a position slightly greater than 50 kDa (Figure 3.2 F) and this is consistent with the predicted molecular weight for peripherin (57 kDa). There was a significant difference between the peripherin content of PMAs from control, STZ-HI and STZ-LI rats (Figure 3.2 E, F; one-way ANOVA; P = 0.02). Post-hoc comparisons using Tukey’s tests revealed that the peripherin protein expression levels were increased in PMAs from STZ-LI rats compared to both control (P = 0.04) and STZ-HI (P = 0.02) rats. There was no significant difference between the peripherin content of PMAs from control and STZ-HI rats (P = 0.96).

The density of peripherin-IR nerve fibers was increased in PMAs from STZ-LI rats

Compared to PMAs from control rats, there was an approximately 25% increase in the frequency of peripherin-IR intercepts along the line profiles in PMAs from STZ-LI rats (Figure 3.4 C). In addition, the percentage area of the vessel surface covered by the peripherin-IR nerve plexus and the integrated peripherin-IR fluorescence per 100 µm² of the vessel surface were both increased by ~60% in PMAs from STZ-LI rats compared to their controls (Figure 3.4 C). The widths of the intercepts along the line profiles were also increased (~15%) in PMAs from STZ-LI rats but their peak immunofluorescence did not differ from that of their controls (Figure 3.4 C). For the PMAs from STZ-HI animals, peripherin-IR nerve plexus parameters did not differ from those of their controls (Figure 3.4 D).
CHAPTER 3 DIABETES INCREASES PERIPHERIN IN SYMPATHETIC AXONS INNERVATING PMAS
Figure 3.3. β-tubulin III immunoreactive (magenta) and peripherin immunoreactive (green) nerve fibers in the PMA of a control and STZ-LI rat. In comparison with the PMA from a control rat, the perivascular β-tubulin III immunoreactive (IR) nerve fibers were thickened in the PMA from a STZ-LI rat. In addition, there was an increase in the number of fine peripherin-IR fibers in the PMA from the STZ-LI rat. Images (A-C and E-G) of the perivascular nerve plexus of a control PMA (A-D) and STZ-LI PMA (E-H) immunolabeled for β-tubulin III (magenta; A, E), peripherin (green; B, F) and the combination of both proteins (C, G). These images are collected with a x63 objective. D and H show line profiles for β-tubulin III (magenta) and peripherin (green) collected along the orange lines indicated on C and G, respectively. The scale bar in A indicates 50 µm and applies to all images and line profiles.
CHAPTER 3 \textbf{Diabetes increases peripherin in sympathetic axons innervating PMAs}

(A) \textbf{$\beta$-Tubulin III Plexus Measurements in PMAs of STZ-LI Rats}

(B) \textbf{$\beta$-Tubulin III Plexus Measurements in PMAs of STZ-HI Rats}

(C) \textbf{Peripherin Plexus Measurements in PMAs of STZ-LI Rats}

(D) \textbf{Peripherin Plexus Measurements in PMAs of STZ-HI Rats}
CHAPTER 3 DIABETES INCREASES PERIPHERIN IN SYMPATHETIC AXONS INNERVATING PMAS

Figure 3.4. The percent area of the vessel surface covered by the immunolabeled nerve plexus (% area), integrated fluorescence/100 μm² of vessel surface (integrated fluorescence), and the frequency, width, and peak fluorescence of intercepts along line profiles of PMAs immunolabeled for β-tubulin III (A, B) or peripherin (C, D) from STZ-LI (A, C) and STZ-HI (B, D) rats expressed as a percentage of these measures in paired control tissues (i.e. terminated on the same day). For both the β-tubulin III-IR (n = 8 pairs) and the peripherin-IR (n = 13 pairs) nerve plexus of PMAs from STZ-LI rats there were increases in the % area, the integrated fluorescence and the widths of the intercepts along the line profiles. In addition, in PMAs from STZ-LI rats there was an increase in the frequency of peripherin-IR intercepts along the line profiles. The peak fluorescence of the intercepts along the line profiles was not different from control for either the β-tubulin III-IR or the peripherin-IR nerve plexus in PMAs from STZ-LI rats. None of the measures of the β-tubulin III-IR or peripherin-IR nerve plexus of PMAs from STZ-HI rats differed from their controls. The data are presented as means and SEMs. Statistical comparisons were made with one-sample t-tests (*P < 0.05, **P < 0.01).
Increased peripherin protein expression levels were correlated with the percentage of glycosylated hemoglobin in blood.

The correlations between peripherin protein expression levels in the PMAs and the % glycosylated hemoglobin in the blood of the rat from which they were obtained were assessed by Pearson’s product-moment correlation analysis (Figure 3.5). For this analysis, data from all three experimental groups (i.e. control, STZ-LI and STZ-HI) were included, and both the protein expression levels and the % of glycosylated hemoglobin levels were confirmed to be normally distributed using Kolmogorov-Smirnov tests (peripherin protein expression levels P = 0.62; % glycosylated hemoglobin levels P = 0.85). There was a significant positive correlation between the peripherin protein expression level in the PMAs and the % glycosylated hemoglobin in the blood of the rat from which they were obtained ($r = 0.509$, $n = 37$, $P < 0.001$).

**Figure 3.5.** Relationship between peripherin protein expression in plantar metatarsal arteries (PMAs) and the % glycosylated hemoglobin in the blood of the rat from which they were obtained. The data are for PMAs and blood obtained from control ($n = 13$), STZ-LI ($n = 12$) and STZ-HI ($n = 12$) rats. The Pearson product-moment correlation test indicated that peripherin protein expression was positively correlated with the % glycosylated hemoglobin level in blood.
CHAPTER 3 DIABETES INCREASES PERIPHERIN IN SYMPATHETIC AXONS INNERVATING PMAS

PMAs from STZ-LI rats labeling for peripherin was increased in fibers that were co-labeled for NPY

To determine whether the diabetes-induced increase in peripherin-IR occurred in sympathetic nerve terminals, line profiles were used to estimate the percentage of peripherin-IR intercepts that were also NPY-IR (a sympathetic nerve marker), and vice versa, in PMAs from control and STZ-LI rats. Figure 3.6 shows images for PMAs from a control (Figure 3.6 A-C) and a STZ-LI rat (Figure 3.6 E-G) immunolabeled for both peripherin and NPY, and representative line profiles for peripherin-IR and NPY-IR collected from these images (Figure 3.6 D, H). In control PMAs, the larger diameter fibers that were peripherin-IR showed little or no NPY-IR (see the green fibers in the merged image; Figure 3.6 C). In comparison with controls, STZ-LI PMAs had an increase in the number of fibers that were co-labeled for peripherin and NPY; expression of NPY and peripherin increased in the thicker and finer nerve fibers, respectively (Figure 3.6 E-G). There was also a generalized increase in NPY-IR in the nerve plexus of STZ-LI PMAs but this was not further investigated (c.f. Figures 3.6 B and F). In PMAs from STZ-LI rats, the percentage of intercepts along the line profiles for NPY-IR that were co-labeled for peripherin-IR was increased compared to that in PMAs from control rats (Figure 3.7 A). Similarly, the percentage of peripherin-IR intercepts that were NPY-IR increased in these vessels (Figure 3.7 B). These findings indicate that peripherin protein expression increased in NPY expressing (i.e. sympathetic) nerve fibers.

There were no detectable changes in protein expression levels for tyrosine hydroxylase, β-tubulin III and peripherin in the proximal and distal tail artery

Table 3.4 summarizes the protein expression values for tyrosine hydroxylase, β-tubulin III and peripherin in both the proximal and distal segments of the tail artery from STZ-LI, STZ-HI and control rats. The levels of the 3 proteins (relative to β-actin) did not differ between the experimental groups in either the proximal or distal artery segments (Table 3.4).
CHAPTER 3 DIABETES INCREASES PERIPHERIN IN SYMPATHETIC AXONS INNERVATING PMAS
**Figure 3.6.** Peripherin-IR (green) and NPY-IR (magenta) nerve fibers in the PMA of a control and STZ-LI rat. In comparison with the PMA from a control rat, the number of peripherin-IR fibers that were NPY-IR increased in the PMA from a STZ-LI rat. In addition, there was an increase in the thickness and immunofluorescence of the NPY-IR fibers in the PMA from the STZ-LI rat. (A-C, E-G) Perivascular nerve plexus of a control PMA (A-D) and STZ-LI PMA (E-H) immunolabeled for peripherin (green; A, E), NPY (magenta; B, F) and the combination of both molecules (C, G). These images are collected with a x63 objective. D and H show line profiles for peripherin (green) and NPY (magenta) collected along the orange lines indicated on C and G, respectively. The scale bar in A indicates 50 µm and applies to all images and line profiles.


Figure 3.7. Percentage of fibers that were co-labeled for peripherin and NPY-IR in the perivascular plexus of PMAs from control and STZ-LI rats. In comparison with PMAs from control rats (n = 8), those from STZ-LI rats (n = 8) had an increase in the number of NPY-IR intercepts along the line profiles that were peripherin-IR and vice versa. (A) Shows the percentage of NPY immunolabeled intercepts along the line profiles that were co-labeled for peripherin. (B) Shows the percentage of peripherin immunolabeled intercepts along the line profiles that were co-labeled for NPY. The data are presented as means and SEMs. Statistical comparisons were made with unpaired t-tests (* P < 0.01).
### Table 3.4. Tyrosine hydroxylase, β-tubulin III and peripherin protein expression levels relative to β-actin, in the distal and proximal tail arteries from control, STZ-HI and STZ-LI rats.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Distal Tail</th>
<th></th>
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<th>Proximal Tail</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>STZ-HI</td>
<td>STZ-LI</td>
<td>Control</td>
<td>STZ-HI</td>
<td>STZ-LI</td>
</tr>
<tr>
<td>Tyrosine Hydroxylase</td>
<td>0.93 ± 0.10</td>
<td>0.66 ± 0.095</td>
<td>0.74 ± 0.11</td>
<td>0.40 ± 0.062</td>
<td>0.29 ± 0.076</td>
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<tr>
<td>n = 13</td>
<td>n = 12</td>
<td>n = 12</td>
<td></td>
<td>n = 13</td>
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<td></td>
</tr>
<tr>
<td>ANOVA P = 0.18</td>
<td></td>
<td></td>
<td></td>
<td>ANOVA P = 0.45</td>
<td></td>
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</tr>
<tr>
<td>β-tubulin III</td>
<td>1.36 (0.53 - 1.57)</td>
<td>1.01 (0.96 - 1.52)</td>
<td>1.53 (0.75 - 3.43)</td>
<td>1.91 (0.98 - 4.01)</td>
<td>1.54 (0.94 - 1.89)</td>
<td>1.88 (1.14 - 2.45)</td>
</tr>
<tr>
<td>n = 9</td>
<td>n = 10</td>
<td>n = 10</td>
<td></td>
<td>n = 9</td>
<td>n = 10</td>
<td></td>
</tr>
<tr>
<td>Kruskal Wallis test P = 0.61</td>
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<td></td>
<td></td>
<td>Kruskal Wallis test P = 0.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripherin</td>
<td>0.09 (0.05 - 0.62)</td>
<td>0.10 (0.03 - 0.19)</td>
<td>0.14 (0.05 - 0.30)</td>
<td>0.45 ± 0.09</td>
<td>0.32 ± 0.07</td>
<td>0.59 ± 0.11</td>
</tr>
<tr>
<td>n = 13</td>
<td>n = 12</td>
<td>n = 12</td>
<td></td>
<td>n = 13</td>
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</tr>
<tr>
<td>Kruskal Wallis test P = 0.60</td>
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<td>ANOVA P = 0.14</td>
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</tr>
</tbody>
</table>

Data are presented as means and SEMs or medians and interquartile ranges (in parentheses). Statistical comparisons between the control, STZ-HI and STZ-LI groups were made using one-way ANOVA or the Kruskal Wallis test.
CHAPTER 3 DIABETES INCREASES PERIPHERIN IN SYMPATHETIC AXONS INNERVATING PMAs

Discussion

This study shows that diabetes increases both the thickness of perivascular nerve fibers and the expression of peripherin, a putative marker for nerve regeneration, in the perivascular nerve plexus of PMAs. As the increase in peripherin immunolabeling occurred in nerve fibers that were also NPY-IR, they are sympathetic nerve terminals. The diabetes-induced increases in peripherin protein expression levels in PMAs were positively correlated with the levels of glycosylated hemoglobin in the blood of the rat from which they were isolated. Furthermore, no changes in structure of the perivascular nerve plexus or in peripherin protein expression or immunolabeling were observed in PMAs from STZ-HI rats. Together these findings suggest that the effects observed in the PMAs from STZ-LI rats were due to hyperglycemia. While there was an increase in the integrated immunofluorescence for both TH (see Chapter 2) and β-tubulin III in the perivascular plexus of PMAs from STZ-LI rats, the expression levels for both these proteins assessed using Western blots were not significantly changed by diabetes. This suggests that the increased integrated immunofluorescence for TH and β-tubulin III is primary due to the diabetes-induced changes in the structure of the nerve fibers rather than an increase in their protein expression levels (i.e. relative to that of β-actin). Diabetes did not produce a change in the levels of peripherin protein in either the proximal or distal regions of the tail artery, suggesting that the sympathetic nerves controlling the PMAs may be particularly vulnerable to the effects of hyperglycemia.

Diabetes-induced changes in β-tubulin III and TH immunoreactive nerve plexus in PMAs

In Chapter 2 I reported that diabetes produces thickening of the sympathetic nerves fibers within the perivascular nerve plexus of PMAs. In this vessel, even the smallest nerve fibers are composed of more than one axon, and therefore it is not
possible to quantify the thickness of individual axons. The integrated immunofluorescence per 100 μm² of vessel surface of the pan-neuronal marker, β-tubulin III, was increased in PMAs from STZ-LI rats relative to those from control rats. As the area of the vessel surface covered by the β-tubulin III-IR nerve plexus increased, this change provides the simplest explanation for the increase in the integrated fluorescence. There was also a tendency for the peak fluorescence intensity of the β-tubulin III-IR intercepts along the line profiles to be increased but this did not quite reach the level of statistical significance (P = 0.06).

As reported in Chapter 2, both the peak intensity of the TH-IR fluorescent intercepts along the line profiles and the integrated TH-IR fluorescence per 100 μm² of vessel surface were higher in PMAs from STZ-LI rats compared to controls. As the vast majority of the nerve fibers within the nerve plexus are TH-IR (see Chapter 2), the change in integrated fluorescence can be explained, at least in part, by the increased area of the vessel surface covered by the nerve plexus. As the Western blots showed that TH protein levels were not different in PMAs from STZ-LI rats compared to those from control rats, the increased fluorescence of the TH-IR intercepts along the line profiles does not appear to be due to an increase in their TH content (measured relative to β-actin). Similarly, in the pineal gland of diabetic rats where sympathetic axons were swollen, TH protein levels, assessed by Western blotting, were not detectably changed (Tsai et al., 2008). It is possible that the apparent increase in the fluorescence of the TH-IR intercepts is produced by axon thickening. The diameter of sympathetic nerve terminal varicosities is typically about 1 μm, and the inter-varicose segments are <0.5 μm. The optical sections acquired with the x63 objective on the confocal microscope were 0.8 μm thick, and it is possible that the increase in immunofluorescence of the intercepts is an artifact produced by axon thickening (i.e. increasing the fluorescence signal acquired in each voxel). However, it is worth noting that the measured β-actin protein levels were derived from the whole tissue, whereas
that of TH was derived from the nerve terminals, which represent only a small fraction of the total tissue. Therefore it is possible that changes in TH protein levels in the nerve terminals cannot be detected when expressed relative to total tissue content of β-actin. Ideally the measurements of protein expression in the nerve terminals should have been made relative to a neuron-specific housekeeper protein, but I was unable to identify a neuron-specific protein that could be used for this purpose.

**Changes in peripherin-IR nerve plexus in PMAs**

In PMAs from STZ-LI rats there was an increase in the density of peripherin-IR nerve fibers within the perivascular plexus, with an increase in the frequency of fine fibers that were immunolabeled. As peripherin levels have been reported to increase in sensory neurons during axon regeneration (see Introduction), the increase in peripherin in STZ-LI rats might reflect ongoing regeneration within the nerve terminals in PMAs from STZ-LI rats. Previous studies have suggested that sympathetic fibers undergo cycles of degeneration, sprouting and regeneration (Stewart *et al.*, 1992; Schmidt, 2002), which are accelerated by diabetes (Schmidt, 2002). I did not observe any evidence of the growth cone-like structures within the nerve plexus at the light microscope level, but they have been observed in the mesenteric paravascular nerves supplying the distal ileum and colon of diabetic rats using electron microscopy (Schmidt *et al*., 1981).

**Lack of effects of diabetes on protein levels in the tail artery**

Diabetes had no effects on the levels of TH, β-tubulin III and peripherin protein in either the proximal or distal tail artery. Therefore it appears that diabetes differentially affects peripherin protein expression in sympathetic axons supplying arteries in different vascular beds. In Chapter 2 I demonstrated that diabetes impaired sympathetic neurovascular transmission and changed the perivascular nerve plexus in PMAs but not in mesenteric arteries, and postulated that the nerve terminals of
sympathetic neurons with long axons were more susceptible to the effects of diabetes. In the present study, the cell bodies of sympathetic neurons supplying the hind paw are located in the L1-L4 chain ganglia (Baron et al., 1988) whereas those supplying the tail artery are located in the sacral sympathetic chain ganglia S1-Co1 (Sittiracha & McLachlan, 1986). While the length of the sympathetic axons supplying the proximal tail artery is likely to be similar to those supplying the PMAs, those supplying the distal tail artery are longer. Therefore the differential effects of diabetes on peripherin protein expression in PMAs and the tail artery are unlikely to be explained by the difference in axon length. Both PMAs and the tail artery supply blood to the skin and play a role in thermoregulation, so their function is similar. A possible explanation for the increased susceptibility of the perivascular nerves that supply arteries located superficially on the plantar surface of the hind paw (where PMAs are located) is the effects of biomechanical stress from weight-bearing and locomotion (Clarke, 1995; Mak et al., 2010).

**Peripherin and diabetes**

Autoimmune antibodies directed against peripherin have been identified in the non-obese diabetic (NOD) mouse (Boitard et al., 1992), a well-established genetic model of type I diabetes (Driver et al., 2011). For this reason peripherin expressing nerve fibers within the pancreatic islets have been suggested to be a primary target for autoantibodies produced by islet-infiltrating B lymphocytes in type I diabetes (Puertas et al., 2007; Carrillo et al., 2008; DiLorenzo, 2011). Indeed, it has been hypothesized that the production of these autoantibodies by islet-targeted B lymphocytes is triggered by an inflammation-induced increase in peripherin expression by nerve fibers within the pancreas (Puertas et al., 2007). Antibodies reactive against peripherin have also been identified in serum from patients with small fiber neuropathy (including patients with type I diabetes), 73% of which had autonomic dysfunction, but not in age-matched healthy controls (Chamberlain et al., 2010). Serum from patients with
CHAPTER 3 DIABETES INCREASES PERIPHERIN IN SYMPATHETIC AXONS INNERVATING PMAs

uncomplicated type I diabetes also did not contain peripherin autoantibodies (Chamberlain et al., 2010). Chamberlain et al. (2010) demonstrated the presence of peripherin immunoreactivity in nerves within the pancreas and other endocrine organs from mice and suggested that these fibers may provide an initial target for autoimmune attack in multiple forms of endocrine autoimmunity including type I diabetes. Furthermore, they suggested the possibility that peripherin autoimmunity might be a cause of autonomic and sensory dysfunction. Strom et al. (2010), however, reported that peripherin autoantibodies were detected in blood from both healthy and diabetic humans and non-diabetic mice strains. They suggested that these may be natural autoantibodies which aid in the removal of protein from aging and/or dying nerves. The study of Strom et al. (2010) does not exclude the possibility of the involvement of peripherin in the pathogenesis of type I diabetes but the presence of peripherin-IgG in control subjects requires further investigation. Autoimmunity to peripherin does not contribute to the induction of diabetes by STZ but there is a possibility that it contributes to the nerve dysfunction observed in this animal model.

Peripherin in peripheral neurodegenerative disorders

Peripherin has been implicated in the pathogenesis of peripheral neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS; reviewed in Perrot & Eyer, 2009) and more recently Charcot-Marie-Tooth type 2B (CMT2B) disease (Cogli et al., 2013). Peripherin aggregates are found within inclusion bodies of motor neurons in ALS (Corbo & Hays, 1992; Migheli et al., 1993; He & Hays, 2004). In a mouse model of ALS, overexpression of peripherin resulted in degeneration of axons in the ventral roots and the formation of peripherin-containing inclusion bodies in soma and axons of somatic motor neurons similar to those seen in human patients (Beaulieu et al., 1999a). Genetic mutations in the peripherin gene (Gros-Louis et al., 2004; Leung et al., 2004; Corrado et al., 2011) and altered ratios of the different peripherin protein
CHAPTER 3 Diabetes increases peripherin in sympathetic axons innervating PMAs

isoforms have been suggested to shift the normal role of this protein in injury-mediated neuroregeneration to neurodegeneration (McLean et al., 2010). CMT2B disease is characterized by a loss of sensory and motor nerve function resulting in loss of sensation in the feet, progressive weakness of the lower extremities combined with an increased risk of the development of foot ulceration. CMT2B results from genetic mutations in the RAB7 gene (Verhoeven et al., 2003). The RAB7 protein has been identified as a GTPase involved in the anterograde transport of the nerve growth factor receptor, TrkA, in motor and sensory neurons (Saxena et al., 2005; Deinhardt et al., 2006; BasuRay et al., 2013). RAB7a, a subtype of the RAB7 protein, has been demonstrated to be important in regulating peripherin assembly and function (Cogli et al., 2013). It was therefore proposed that mutations in RAB7a alter its interaction with peripherin and that this may explain neurodegenerative processes seen in CMT2B (Cogli et al., 2013). These findings further raise the possibility that changes in peripherin expression and/or function contribute to diabetic neuropathy.

Role of cytokines in the regulation of peripherin expression

Peripherin expression can be induced by the cytokines, interleukin-6 (IL-6) (Sterneck et al., 1996) and leukemia inhibitory factor (Lecomte et al., 1998; Beaulieu et al., 2002), whereas tumor necrosis factor α (TNF-α), together with peripherin overexpression, mediates nerve cell death (Robertson et al., 2001). Increased serum levels of IL-6 and TNF-α have been associated with the development of diabetic retinopathy in children with type I diabetes (Mysliwiec et al., 2008). Young type I diabetic patients with good glycemic control and without overt signs of vascular complications (Targher et al., 2001; Snell-Bergeon et al., 2010) have increased plasma levels of IL-6. Additionally, activation of the TNF-α system has been associated with the development of diabetic complications such as neuropathy (Gonzalez-Clemente et al., 2005) and retinopathy (Gustavsson et al., 2008). Acute hyperglycemia in type I diabetic
patients produced increases in plasma levels of IL-6 and TNF-α (Gordin et al., 2008). Serum levels of IL-6 and TNF-α are also increased in STZ-treated rats made diabetic for 14 weeks (Jain et al., 2009). Therefore, it is possible that increased peripherin expression in diabetic rats is due, at least in part, to increases in systemic levels of IL-6 and TNF-α.

**Conclusion**

Diabetes increased peripherin immunoreactivity in NPY-IR axons innervating the PMAs of STZ-LI rats suggesting that sympathetic fibers were undergoing increased levels of regeneration. This study also shows that peripherin protein levels are dependent on hyperglycemia and thus might reflect nerve damage and altered cytokine signaling. Autoantibodies to peripherin have been identified in diabetic animals and humans and have been suggested to be a cause of autonomic and sensory neuropathy. Changes in peripherin expression/function have also been implicated in the etiology of ALS and CMT2B. On the basis of these findings the potential role of peripherin in diabetic neuropathy and other neuropathies warrants further investigation.
CHAPTER 4 Expression of selected genes in sympathetic ganglia do not explain changes in the perivascular nerve plexus of plantar metatarsal arteries from streptozotocin-induced diabetic rats
CHAPTER 4 UNALTERED GENE EXPRESSION IN DIABETIC SYMPATHETIC GANGLIA

Abstract

Twelve weeks of streptozotocin (STZ)-induced diabetes in rats produced an increase in nerve fiber thickness and expression of peripherin protein (a putative marker for nerve regeneration) within the perivascular sympathetic nerve plexus of plantar metatarsal arteries (PMAs). The purpose of this study was to investigate whether these diabetes-induced changes were associated with altered gene expression in parent cell bodies located within the L1-L4 sympathetic ganglia of STZ-treated rats receiving a low dose of insulin (hyperglycemic) compared to those from STZ-treated rats receiving a high dose of insulin (normoglycemic) and vehicle treated controls (n = 10 for each group). Using quantitative reverse transcription-PCR, mRNA expression of selected genes of interest involved in neurotransmission (tyrosine hydroxylase, dopamine β-hydroxylase, norepinephrine transporter and synaptophysin), axon structure (neurofilament-heavy, neurofilament-medium, neurofilament-light and β-tubulin III), plasticity (peripherin, growth associated protein-43, Wnt5a), neurotrophin signaling (TrkA and p75NTR receptors) and stress (activating transcription factor-2, activating transcription factor-3 and c-Jun) were measured. Gapdh and Ppia were identified as stable housekeeper genes to which expression levels of all genes studied were normalized. No changes in mRNA expression levels for the genes of interest were detected suggesting that the changes seen in the terminal axons innervating the PMAs from diabetic rats did not involve changes in their transcription. The findings also suggest that for peripherin the increased protein expression level in the nerve terminals is most likely due to a post-transcriptional change.
CHAPTER 4 UNALTERED GENE EXPRESSION IN DIABETIC SYMPATHETIC GANGLIA

Introduction

Chapter 2 demonstrated reduced sympathetic nerve-mediated neurovascular transmission (NVT) of plantar metatarsal arteries (PMAs) in streptozotocin (STZ)-induced type I diabetic rats given no insulin (STZ-NI). This effect of diabetes could potentially be explained, at least in part, by a reduction in noradrenaline release from the nerve terminals or an increase in the clearance of this neurotransmitter by reuptake into the nerve terminals by the neuronal norepinephrine transporter (NET). Noradrenaline is synthesized locally within the sympathetic axon terminals. Key enzymes involved in the noradrenaline synthesis pathway include tyrosine hydroxylase (TH), the rate-limiting enzyme in the pathway that converts tyrosine to 3,4-dihydroxypheynylanil, and dopamine β-hydroxylase, which hydroxylates dopamine to form noradrenaline. While no changes in NET function were detected in PMAs from STZ-NI rats (Chapter 2), the perivascular sympathetic axon plexus density was decreased and fluorescence intensity of TH immunolabeled nerve fibers was increased. The decrease in nerve fiber density may produce a decrease in the number of sympathetic neuromuscular junctions in the PMAs. In STZ-treated rats given a low dose of insulin (STZ-LI), which remained hyperglycemic, changes in NVT and nerve fiber density were not detected but TH immunolabeling was still increased.

The intensity of TH labeling in the nerve terminals has been suggested to be a good indicator of the level of sympathetic nerve activity (Burgi et al., 2011). This suggestion is based on the demonstration of increased immunolabeling for TH in the perivascular nerve plexus of arterial vessels from spontaneous hypertensive rats (SHRs) compared to those from normotensive Wistar Kyoto rats (WKYs) (Burgi et al., 2011). In SHRs, an increase in vasoconstrictor nerve activity is believed to be the primary cause of elevated blood pressure (Minsøn et al., 1996). Increased immunolabeling for TH has been interpreted as indicating an increase in the concentration and activity of this
enzyme in the sympathetic nerve terminals. In accord with this suggestion, Burgi et al. (2011) demonstrated increased noradrenaline content in sympathetically innervated tissues from the SHR rats compared to those from WKYs. In rats with STZ-induced diabetes, increased noradrenaline content has been demonstrated in the tail artery (Morrison et al., 2004), penile tissues (Morrison et al., 2006; Morrison et al., 2007a; Morrison et al., 2007b) and heart (Morrison et al., 2001). In the corpus cavernosum, there was also an increase in fluorescence intensity of the TH immunolabeled axons (not assessed in the other tissues) (Morrison et al., 2007b). This change could also reflect an increase in sympathetic nerve activity. In Chapter 3, Western blotting did not reveal a significant increase in TH protein content in the PMAs, but it remains possible that Western blotting is not sensitive enough to detect a relatively small change in total tissue content of this enzyme (see Discussion in Chapter 3). It has been demonstrated that mRNA levels for TH in cell bodies of postganglionic sympathetic neurons are increased by nerve activity (Biguet et al., 1989). Therefore, it is possible that TH content is increased in the nerve terminals by diabetes, and that this is produced by an increase in gene expression for this enzyme in the cell bodies of the sympathetic neurons that supply the PMAs.

Gene expression studies on sural nerve biopsies from patients with long-term diabetes have identified upregulation of genes involved in axonogenesis (Hur et al., 2011). In Chapters 2 and 3 it was demonstrated that diabetes caused thickening of the sympathetic nerve fibers within the perivascular nerve plexus of PMAs from both STZ-NI and STZ-LI rats and it was suggested that this was due to remodeling of the nerve terminal axons. Increased protein expression levels of peripherin, growth associated protein 43 (GAP-43) and β-tubulin III are associated with axon plasticity and regeneration in neurons (Skene et al., 1986; Oblinger et al., 1989b; Moskowitz & Oblinger, 1995). In Chapter 3, an increase in peripherin protein content, but no detectable changes in β-tubulin III protein expression levels, was demonstrated in
Chapter 4 Unaltered Gene Expression in Diabetic Sympathetic Ganglia

PMAs from STZ-LI rats. No changes in protein expression levels of GAP-43 were detected in sympathetic ganglia from diabetic rats and humans (Schmidt et al., 1991), but this may be concealed by high expression levels under normal conditions (Stewart et al., 1992). Peripherin is an intermediate filament protein shown to be functionally interdependent with neurofilament-heavy (NF-H), neurofilament-medium (NF-M) and neurofilament-light (NF-L) (Yuan et al., 2012), which are major components of the cytoskeleton that maintains axon shape (Helfand et al., 2003). It is possible that changes in the expression of these neurofilament proteins also contribute to the altered structure of the sympathetic nerve terminals produced by diabetes.

In the PMAs, the diabetes-induced changes in sympathetic nerve terminals might involve altered nerve growth factor (NGF) signaling. NGF is produced by the target organ and plays an important role in maintaining the normal function of adult sympathetic neurons via actions mediated through both TrkA and p75 <sup>NTR</sup> receptors (Cowen & Gavazzi, 1998). The levels of NGF in a range of sympathetically innervated tissues are decreased in chronic STZ-induced diabetes (Hellweg & Hartung, 1990). In addition, STZ-treated rats with 8 weeks of diabetes have reduced retrograde transport of NGF in the sciatic nerve, which contains a mixed population of sympathetic and sensory nerves (Hellweg et al., 1994; Delcroix et al., 1997). However, axon dystrophy of sympathetic neurons supplying the distal ileum of STZ-induced diabetic rats has been suggested to be due to increased neuronal uptake of NGF from the hypertrophied intestine (Schmidt et al., 2000). NGF is involved in regulating the expression of axon structural proteins including peripherin (Aletta et al., 1988), GAP-43 (Leslie et al., 1995) and the neurofilament triplet proteins (Lee et al., 1982). In addition, NGF also regulates the expression of TH (Otten et al., 1977; Toma et al., 1997) and dopamine β-hydroxylase (Otten et al., 1977) in sympathetic neurons. The secreted signaling protein, Wnt5a, has been found to be a downstream mediator of the effects of NGF on axonal branching and growth during development of
sympathetic neurons (Bodmer et al., 2009). Whether this protein also plays a role in the regeneration of sympathetic axons is currently unknown.

In dorsal root ganglia, diabetes increases the number of sensory neurons expressing the stress markers activating transcription factor-3 (ATF-3) and phosphorylated c-jun N-terminal kinase (Wright et al., 2004; Middlemas et al., 2006). In STZ-treated diabetic rats, diabetes elevates retrograde axonal transport of activated (phosphorylated) c-jun N-terminal kinases and activating transcription factor-2 (ATF-2) in the sciatic nerve (Middlemas et al., 2003). NGF modulates axonal transport of ATF-2 in the sciatic nerve (Delcroix et al., 1999) and therefore the changes observed in transport of ATF-2 in diabetic rats may be due to changes in NGF availability. In cultured sympathetic neurons, NGF withdrawal leads to activation of transcription factors, c-Jun and ATF-2, which precedes cell death (Towers et al., 2009). Although there is no evidence of sympathetic neuron loss in diabetes (Schmidt & Plurad, 1986; Schmidt, 2001), increased expression of ATF-2, ATF-3 and c-Jun may provide an indication that sympathetic neurons are stressed by diabetes.

This study tested the hypothesis that in L1-L4 sympathetic chain ganglia diabetes produces changes in gene expression for proteins associated with neurotransmission (TH, dopamine β-hydroxylase, NET and synaptophysin), axon structure (NF-H, NF-M, NF-L and β-tubulin III), plasticity (peripherin, GAP-43, Wnt5a), neurotrophin signaling (TrkA and p75NTR receptors) and stress (ATF-2, ATF-3 and c-Jun). L1-L4 ganglia were chosen for this study because they contain the cell bodies of sympathetic neurons projecting to the hind paw.
CHAPTER 4 UNALTERED GENE EXPRESSION IN DIABETIC SYMPATHETIC GANGLIA

Materials and Methods

Animals

Rats were treated as described in Chapter 3. At termination, sympathetic ganglia were dissected from STZ-treated rats given a high dose (STZ-HI) or a low dose of insulin (STZ-LI) and their age-matched controls, in 10 replicates.

Isolation of L1-L4 Sympathetic Chain Ganglia

The L1-L4 region of the sympathetic chain ganglia on both sides of the spinal column were selected for this study because the sympathetic axons innervating the medial plantar artery, which gives rise to the PMAs, arise from the saphenous and sciatic nerve trunks (Bentzer et al., 1997). The neuronal cell bodies of the sympathetic axons in the saphenous nerve arise from the T13-L3 region of the sympathetic chain ganglia (Baron et al., 1988). The sciatic nerve gives rise to the sural and tibial nerves which supply the hind paw (Greene, 1936) and sympathetic axons in these nerves originate from neurons located between the L2-L5 and L1-L5 regions, respectively, of the sympathetic chain (Baron et al., 1988). The L1 sympathetic ganglion was identified as the first sympathetic chain ganglia transversing the tendons of the dorsal crura of the diaphragm (Baron et al., 1988). During dissection, the ganglia were kept moist with cold phosphate buffered saline (PBS) containing 1:1000 phenylmethylsulfonyl fluoride (PMSF). Immediately after the L1-L4 sympathetic ganglia were dissected they were snap frozen in liquid nitrogen.

RNA Extraction

From each rat, RNA was extracted from L1-L4 sympathetic chain ganglia using PureLink™ RNA Mini Kit (Cat. No. 12183-018A, Life Technologies, Victoria, Australia). The kit-based protocol was followed. Briefly, each sample was immersed in 300 µl of Lysis Buffer with 1% β-mercaptoethanol and was homogenized using a sonicator.
(speed 6; XL2000, Misonix, NY, USA). Samples were centrifuged at 12,000 g for 2 minutes. The supernatant was removed and placed in a 1:1 volume of 70% EtOH, briefly vortexed and transferred into a spin cartridge attached to a collection tube. The spin cartridge was centrifuged at 12,000 g for 15 seconds to allow the filter at the bottom of spin cartridge to collect RNA while allowing the liquid to collect into the collection tube. This flow through was discarded. The filter was washed by placing 350 µl Wash Buffer 1 on top of the filter followed by centrifugation at 12,000 g for 15 seconds, and the flow through was again discarded. After the PureLink DNase mixture was reconstituted as per the manufacturer’s instruction, 80 µl was added to the filter of the spin column and incubated for 15 minutes at room temperature. This step was done to ensure the removal of DNA present on the filter. The filter was subsequently washed with Wash Buffer 1 once and Wash Buffer 2 (with 80% (v/v) of absolute ethanol added) twice. After the addition of each buffer to the spin column, the samples were centrifuged at 12,000 g for 15 seconds and the flow through was discarded. Following the last wash, the remaining wash buffer was removed by centrifugation at 12,000 g for 1 minute. To each spin column, 30 µl of RNase-free water was pipetted onto the filter and the isolated RNA was eluted into the recovery tube by centrifugation at 12,000 g for 1 minute.

RNA quality and quantity were measured using a T009 NanoDrop 1000 (Thermo Fisher Scientific Australia Pty Ltd, Victoria, Australia). RNA purity, measured by the ratio of absorbance at 260 nm and 280 nm (OD$_{260/280}$), for all samples was in the range 2.03-2.09. According to the manufacturer’s protocol, an OD$_{260/280}$ of >1.8 indicates the isolated RNA sample has very little protein and UV chromophore contaminants that may interfere with downstream applications or RNA stability during storage. RNA samples had concentrations between 272-548 ng/µl and were stored at -80°C.
CHAPTER 4 UNALTERED GENE EXPRESSION IN DIABETIC SYMPATHETIC GANGLIA

Reverse Transcription PCR

For each sample, 1 µg of RNA was reverse transcribed into complementary DNA (cDNA) using the SuperScript® III First-Strand Synthesis System (Cat. No. 18080-051, Life Technologies, Victoria, Australia). A sample containing 1 µg of RNA was made up to 16 µl with RNase-free water, to which 2 µl of 50 µM oligo deoxynuclemine (dT) 20-base primer and 2 µl of 10 mM deoxyribonucleotide triphosphate (dNTP) mix was added. Samples were incubated at 65°C for 5 minutes and subsequently placed on ice for at least 1 minute. To each sample, the following solutions were added: 4 µl of 10x reverse transcriptase (RT) buffer, 8 µl of 25 mM MgCl₂, 4 µl of 0.1 M dithiothreitol (DTT), 1 µl of RNaseOUT™ (40 U/μl), 1 µl of SuperScript™ III RT (200 U/μl). Control samples negative for reverse transcriptase had 1 µl of RNase-free water instead of 1 µl of SuperScript™ III RT added. Using a PCR machine (GeneAmp® PCR System 9700, Life Technologies, Victoria, Australia), all samples were incubated for 50 minutes at 50°C, 85°C for 5 minutes and terminated at 4°C. RNA was removed by adding 1 µl of RNase H to each sample and incubating for 20 minutes at 37°C. cDNA samples were aliquoted in 20 µl volumes and stored at -20°C.

Quantitative Real Time PCR

TaqMan gene expression assays (Life Technologies, Victoria, Australia) that were used are summarized in Table 4.1. Using 384 well plates (Cat. No. 4309849, Life Technologies, Victoria, Australia), gene expression analysis for each sample was performed in triplicate. A bulk mastermix was made up for each triplicate as follows: 12.5 µl of 2x TaqMan master mix, 1.25 µl of 20x Gene expression assay and 10.3 µl of Nuclease-free water. For each triplicate, 1 µl of sample cDNA was added, mixed, centrifuged and 7 µl of the supernatant place into the 3 replicate wells. Up to three genes were studied for all the tissues per plate. After all the samples were loaded, each plate was covered with a MicroAmp® Optical Adhesive Film (Cat. No. 4311971,
CHAPTER 4 UNALTERED GENE EXPRESSION IN DIABETIC SYMPATHETIC GANGLIA

Life Technologies, Victoria, Australia). Plates were centrifuged at 3500 rpm for 10 minutes at 4°C (Rotor 11650, Sigma 4-16K Centrifuge, John Morris Scientific, Victoria, Australia) to ensure all the contents were at the bottom of each well. PCR amplification was performed and quantified using a 7900HT Fast Real-Time PCR System (Life Technologies, Victoria, Australia). A standard thermal cycler protocol was followed (total time 62 minutes): Stage 1 at 50°C for 2 minutes; Stage 2 at 95°C for 10 minutes; Stage 3 at 95°C for 15 seconds followed by 60°C for 1 minute (repeated for 40 cycles). Results were recorded and analyzed using the Sequence Detection System v2.4.1 Standard Edition program (Life Technologies, Victoria, Australia).

The cycle threshold value (Ct-value) refers to the number of thermal amplification cycles required for fluorescent intensity of the PCR product (i.e. gene of interest) to be above the background intensity during the exponential phase of the PCR. The Ct-value was measured for each sample/well and a mean Ct-value was calculated for each triplicate. Results were stored as .sds or .txt files for use by the Biogazelle’s program qbasePLUS.
Table 4.1. Summary of TaqMan gene expression assays investigated in the L1-L4 sympathetic ganglia of low and high dose insulin treated diabetic rats and their controls.

<table>
<thead>
<tr>
<th>Genes of Interest Subgrouped</th>
<th>Gene Name</th>
<th>Assay ID</th>
<th>Protein Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurotransmission</td>
<td>Tyrosine hydroxylase</td>
<td>Th Rn00562500_m1</td>
<td>Rate-limiting enzyme in noradrenaline production</td>
</tr>
<tr>
<td></td>
<td>Dopamine beta-hydroxylase (dopamine beta-monooxygenase)</td>
<td>Dbh Rn00565819_m1</td>
<td>Enzyme that converts dopamine into noradrenaline</td>
</tr>
<tr>
<td></td>
<td>Solute carrier family 6 (neurotransmitter transporter, noradrenaline), member 2</td>
<td>Slc6a2 Rn00580207_m1</td>
<td>Na(^+)/Cl(^-)-dependent transporter which re-uptakes noradrenaline</td>
</tr>
<tr>
<td></td>
<td>Synaptophysin</td>
<td>Syp Rn00561986_m1</td>
<td>Synaptic vesicle-specific membrane protein involved in neurotransmission</td>
</tr>
<tr>
<td>Structural</td>
<td>Tubulin, beta 3 class III</td>
<td>Tubb3 Rn01431594_m1</td>
<td>Neuron-specific structural marker</td>
</tr>
<tr>
<td>components</td>
<td>Neurofilament, heavy polypeptide</td>
<td>Nefh Rn00709325_m1</td>
<td>~200kDa subunit of the neurofilament protein triplet</td>
</tr>
<tr>
<td></td>
<td>Neurofilament, light polypeptide</td>
<td>Nefl Rn00582365_m1</td>
<td>~160kDa subunit of the neurofilament protein triplet</td>
</tr>
<tr>
<td></td>
<td>Neurofilament, medium polypeptide</td>
<td>Nefm Rn00566763_m1</td>
<td>~68kDa subunit of the neurofilament protein triplet</td>
</tr>
<tr>
<td>Neurotrophin</td>
<td>Nerve growth factor receptor</td>
<td>Ngfr Rn00561634_m1</td>
<td>P75(^{NTR}) that interacts with Trk receptors</td>
</tr>
<tr>
<td>signaling</td>
<td>Neurotrophic tyrosine kinase, receptor, type 1</td>
<td>Ntrk1 Rn00572130_m1</td>
<td>TrkA (high affinity nerve growth factor) receptor</td>
</tr>
</tbody>
</table>
## Chapter 4 Unaltered Gene Expression in Diabetic Sympathetic Ganglia

<table>
<thead>
<tr>
<th>Plasticity</th>
<th>Peripherin</th>
<th>Prph</th>
<th>Rn00688569_g1</th>
<th>type III neuronal intermediate filament</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth associated protein 43</td>
<td>Gap43</td>
<td>Rn01474579_m1</td>
<td>A major component in the formation of growth cones at the tips of elongating axons</td>
<td></td>
</tr>
<tr>
<td>Wingless-type MMTV integration site family, member 5A</td>
<td>Wnt5a</td>
<td>Rn01402000_m1</td>
<td>Signaling protein ligand for the seven transmembrane receptor frizzled-5 and the tyrosine kinase orphan receptor 2</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stress indicators</th>
<th>activating transcription factor 2</th>
<th>Atf2</th>
<th>Rn00578832_m1</th>
<th>A transcription factor subtype of the leucine zipper family of DNA binding proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>activating transcription factor 3</td>
<td>Atf3</td>
<td>Rn00563784_m1</td>
<td>One of the transcription factors involved in the mammalian activation transcription factor/cAMP responsive element-binding (CREB) protein family</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Candidate housekeeping genes</th>
<th>TATA box binding protein</th>
<th>Tbp</th>
<th>Rn01455646_m1</th>
<th>AP-1 transcription factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)</td>
<td>Sdha</td>
<td>Rn00590475_m1</td>
<td>Part of a protein complex which oxidizes succinate in the mitochondria</td>
<td></td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Gapdh</td>
<td>Rn01775763_g1</td>
<td>Enzyme involved in glycolysis</td>
<td></td>
</tr>
<tr>
<td>Hydroxymethylbilane synthase</td>
<td>Hmbs</td>
<td>Rn00565886_m1</td>
<td>Enzyme involved in the production of heme</td>
<td></td>
</tr>
</tbody>
</table>
## Chapter 4 Unaltered Gene Expression in Diabetic Sympathetic Ganglia

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Gene ID</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxanthine phosphoribosyltransferase 1</td>
<td><em>Hprt1</em></td>
<td>Rn01527840_m1</td>
<td>Enzyme which recycles purines</td>
</tr>
<tr>
<td>Peptidylprolyl isomerase A (cyclophilin A)</td>
<td><em>Ppia</em></td>
<td>Rn00690933_m1</td>
<td>Enzyme involved in protein cis-trans structure formation</td>
</tr>
<tr>
<td>Ubiquitin C</td>
<td><em>Ubc</em></td>
<td>Rn01789812_g1</td>
<td>Labels proteins for various cellular functions</td>
</tr>
<tr>
<td>Beta-2 microglobulin</td>
<td><em>B2m</em></td>
<td>Rn00560865_m1</td>
<td>Component of the MHC class I molecule</td>
</tr>
</tbody>
</table>
Selection of Housekeeping Genes

To the best of my knowledge, no studies have identified appropriate housekeeping genes in the adult rat L1-L4 sympathetic ganglia for use in quantitative reverse transcription PCR. The following were trialed as potential housekeeping genes: Tbp, Sdha, Gapdh, Hmbs, Hprt1, Ppia, Ubc, B2m (see Table 4.1 for further details). Multiple candidate housekeeping genes were analyzed to identify the most stably expressed genes. Ideally, the expression ratio between any two stably expressed housekeeping genes is the same in all tissues regardless of the experimental conditions or cell type (Vandesompele et al., 2002). Appropriate housekeeping genes were selected using Biogazelle’s program qbasePLUS (version Eclipse 3.7 for Mac OS X, Zwijnaarde, Belgium) (Hellemans et al., 2007). This program calculates for all the samples the relative expression value (RQ) for each candidate housekeeping gene using the following formula:

\[
RQ = 2^{(Ct_{\text{min}} - Ct_x)}
\]

where \(Ct_{\text{min}}\) is the smallest Ct value for all the samples and \(Ct_x\) is the Ct value for each sample. This formula accounts for the assumption that the amount of target DNA doubles every cycle during the exponential phase of cDNA amplification. The program then calculates a stability measure, the geNorm M-value, for each candidate housekeeper. This geNorm M-value is the mean of the standard deviations of \(\log_2\) transformed expression ratios (i.e. RQ ratios) between the candidate housekeeper gene and each of the other candidate genes derived for all of the samples. For two candidate housekeeper genes \(j\) and \(k\) for \(m\) samples the mean \((A_{jk})\) and standard deviation \((V_{jk})\) of the \(\log_2\) transformed expression ratios is calculated:

\[
A_{jk} = \frac{\log_2\left(\frac{RQ_{1j}}{RQ_{1k}}\right) + \log_2\left(\frac{RQ_{2j}}{RQ_{2k}}\right) + \ldots + \log_2\left(\frac{RQ_{mj}}{RQ_{mk}}\right)}{m}
\]

\[
V_{jk} = \sqrt{\frac{\sum_{i=1}^{m} (A_{ij} - A_{jk})^2}{m-1}}
\]
CHAPTER 4 UNALTED GENE EXPRESSION IN DIABETIC SYMPATHETIC GANGLIA

\[ V_{jk} = \text{st. dev} \left( A_{jk} \right) \]

The geNorm M-value is the mean \( M_j \) of the \( V_{jk} \) values for one candidate gene versus all the other candidate genes.

\[ M_j = \frac{\sum_{k=1}^{n} V_{jk}}{n-1} \]

The smaller the geNorm M-value, the more stable the gene expression (Vandesompele et al., 2002). This measure assumes that the candidate housekeeping genes are not co-regulated.

The program then determines the minimum number of genes required to derive a normalization factor (NF), a correction factor which accounts for relative housekeeper gene expression levels between samples (Vandesompele et al., 2002). Initially the NF for each tissue is calculated using the geometric mean of the RQ values of the two most stably expressed housekeeping genes (NF\(_n\); i.e. the two genes with the smallest geNorm M-values). For each tissue:

\[ NF_n = \sqrt[2]{RQ_1 \times RQ_2} \]

To determine whether use of more than two housekeeper genes would produce a more accurate NF, the standard deviation (geNorm V-value) of the ratios between NF\(_n\) and a recalculated NF including the RQ value of the candidate gene with the next lowest geNorm M-value (NF\(_{n+1}\)) for each tissue is calculated. For each tissue:

\[ NF_{n+1} = \sqrt[3]{RQ_1 \times RQ_2 \times RQ_3} \]

A geNorm V-value >0.15 indicates that the addition of a particular gene to the recalculated NF value has a significant effect and should be included in the NF calculation (Hellemans et al., 2007). Conversely, if the geNorm V-value is <0.15, the additional candidate housekeeping gene does not have a significant effect and can be
CHAPTER 4 UNALTERED GENE EXPRESSION IN DIABETIC SYMPATHTIC GANGLIA

excluded from the calculation of a reliable NF. The program repeats these calculations sequentially to assess the effects of adding each candidate housekeeper gene to the calculation of the NF for each tissue.

Calculating Gene Expression Levels

For each gene the relative expression value (RQ) was calculated for each tissue using the formula indicated above. These values were then normalized to the level of housekeeper gene expression by calculating the normalized relative expression value (NRQ):

$$NRQ_x = \frac{RQ_x}{NF_x}$$

where the $RQ_x$ is relative expression of the gene of interest, and $NF_x$ is relative expression of housekeeper genes, for each tissue.

Using the NRQ values for each gene of interest, differences in gene expression between the three treatment groups (control, STZ-HI, STZ-LI) were evaluated using a one-way ANOVA or Kruskal-Wallis test. The Kruskal-Wallis test was used when the variance differed significantly between the test groups (assessed with a Levene’s test). When the one-way ANOVA indicated a significant difference, Tukey’s honest significance test was used to make multiple pairwise comparisons between the three treatment groups.

Results

*Gapdh and Ppia were the most stable housekeeping genes in L1-L4 sympathetic chain ganglia*

One of the control samples was excluded from all analyses because there was little or no amplification of the PCR product for any of the genes analyzed. *Gapdh* and *Ppia* were identified as the two most stably expressed housekeeping genes in the L1-L4
sympathetic ganglia, both having geNorm M-values ≤0.2 (Figure 4.1). Additionally, the optimal number of reference genes calculated was 2 (i.e. Gapdh and Ppia), because adding additional candidate housekeeper genes did not have a significant effect on the calculated NF values with all geNorm V-values <0.15 (Figure 4.2). These findings indicate that an accurate NF could be calculated using RQ values for Gapdh and Ppia.

**Figure 4.1.** Determining most stably expressed housekeeper genes in L1-L4 sympathetic chain ganglia under diabetic and control conditions using qBasePlus Program. The stability measure geNorm M of candidate housekeeping genes derived from averaging of the standard deviation of logarithmically transformed relative expression ratios between the candidate housekeeper gene and each of the other candidate genes for all tissues. *Gapdh* and *Ppia* were identified as the two most stable housekeeping genes in the L1-L4 sympathetic chain ganglia.
Figure 4.2. Determination of the optimal number of housekeeper genes required to calculate an accurate normalization factor (NF). This was calculated by comparing the geometric mean of the ratios between the calculated normalization factor with and without an additional candidate gene for all tissues (geNorm V-value). A geNorm V-value >0.15 indicates an additional gene is required to obtain an accurate normalization factor. The 2 most stably expressed housekeeper genes Gapdh and Ppia provided an accurate NF.
Hyperglycemia-induced changes in gene expression were not detected in L1-L4 sympathetic chain ganglia.

Another control sample was excluded from the overall statistical analyses because its values were consistently higher than that of the other control samples (i.e. outlier). The relative gene expression values are presented in Table 4.2. For all genes of interest except Nefh, no statistically significant changes in expression levels were detected between L1-L4 sympathetic ganglia samples from control, STZ-HI and STZ-LI rats (Table 4.2). The one-way ANOVA indicated a difference in the expression levels between the treatment groups for the gene Nefh (P = 0.023), which encodes the structural protein neurofilament-heavy. Post-hoc pair-wise comparisons revealed that ganglia from STZ-HI rats (i.e. normoglycemic) had increased Nefh expression levels compared to those from STZ-LI rats (Tukey test P = 0.017). However, the expression levels for Nefh in ganglia from both these insulin-treated groups did not differ from those in control ganglia (P ≥ 0.34).
Table 4.2. Summary of one-way ANOVA comparisons in expression levels of genes of interest between low and high dose insulin treated diabetic rats and their controls.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Mean relative gene expression</th>
<th>P-values between groups</th>
<th>Test of homogeneity of variances P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>High dose insulin</td>
<td>Low dose insulin</td>
</tr>
<tr>
<td><strong>Atf2</strong></td>
<td>0.46 ± 0.07</td>
<td>0.78 ± 0.10</td>
<td>0.61 ± 0.09</td>
</tr>
<tr>
<td><strong>Atf3</strong></td>
<td>0.43 ± 0.04</td>
<td>0.47 ± 0.07</td>
<td>0.57 ± 0.07</td>
</tr>
<tr>
<td><strong>Tubb3</strong></td>
<td>0.80 ± 0.08</td>
<td>0.80 ± 0.08</td>
<td>0.90 ± 0.06</td>
</tr>
<tr>
<td><strong>Jun</strong></td>
<td>0.65 ± 0.08</td>
<td>0.54 ± 0.05</td>
<td>0.61 ± 0.07</td>
</tr>
<tr>
<td><strong>Dbh</strong></td>
<td>0.89 ± 0.06</td>
<td>1.02 ± 0.14</td>
<td>0.84 ± 0.04</td>
</tr>
<tr>
<td><strong>Gap43</strong></td>
<td>0.68 ± 0.06</td>
<td>0.71 ± 0.06</td>
<td>0.77 ± 0.05</td>
</tr>
<tr>
<td><strong>Nefh</strong></td>
<td>0.84 ± 0.05</td>
<td>0.96 ± 0.08</td>
<td>0.72 ± 0.04</td>
</tr>
<tr>
<td><strong>Nefl</strong></td>
<td>0.71 ± 0.08</td>
<td>0.94 ± 0.08</td>
<td>0.78 ± 0.06</td>
</tr>
<tr>
<td><strong>Nefm</strong></td>
<td>0.66 ± 0.09</td>
<td>0.73 ± 0.09</td>
<td>0.71 ± 0.07</td>
</tr>
<tr>
<td><strong>Slc6a2</strong></td>
<td>0.50 ± 0.09</td>
<td>0.65 ± 0.09</td>
<td>0.66 ± 0.10</td>
</tr>
<tr>
<td><strong>Ngfr</strong></td>
<td>0.81 ± 0.07</td>
<td>0.91 ± 0.09</td>
<td>0.75 ± 0.04</td>
</tr>
<tr>
<td><strong>Syp</strong></td>
<td>0.79 ± 0.06</td>
<td>0.93 ± 0.12</td>
<td>0.83 ± 0.06</td>
</tr>
<tr>
<td><strong>Ntrk1</strong></td>
<td>0.64 ± 0.07</td>
<td>0.73 ± 0.06</td>
<td>0.72 ± 0.04</td>
</tr>
<tr>
<td><strong>Prph</strong></td>
<td>0.84 ± 0.06</td>
<td>0.97 ± 0.11</td>
<td>0.93 ± 0.04</td>
</tr>
<tr>
<td><strong>Th</strong></td>
<td>0.97 (0.88 - 1.00)</td>
<td>1.04 (0.91 - 1.35)</td>
<td>1.04 (0.94 - 1.05)</td>
</tr>
<tr>
<td><strong>Wnt5a</strong></td>
<td>0.37 (0.17 - 0.40)</td>
<td>0.31 (0.26 - 0.45)</td>
<td>0.43 (0.19 - 0.78)</td>
</tr>
</tbody>
</table>

Data are presented as means and SEMs or medians and interquartile ranges (in parentheses). Multiple comparisons were made using one-way ANOVA or Kruskal-Wallis tests when the variances were unequal between groups (assessed with Levene’s tests).
The remaining six candidate housekeeper genes were analyzed as genes of interest and statistical comparisons of gene expression levels are summarized in Table 4.3. There were no differences in expression levels of Tbp, Sdha, Hmbs, Ubc and B2M between ganglia from control, STZ-HI and STZ-LI rats (Table 4.3). There was, however, a difference in the expression levels of Hprt1 between the three groups of rats (ANOVA P = 0.007). Post-hoc pair-wise comparisons indicated that Hprt1 expression levels were significantly higher in ganglia from STZ-HI compared to those from STZ-LI rats (P = 0.035) and controls (P = 0.009). There were no differences in expression levels for Hprt1 between ganglia from STZ-LI and control rats (P = 0.737).

Table 4.3. Summary of one-way ANOVA comparisons in expression levels of the remaining candidate housekeeping genes between low and high dose insulin treated diabetic rats and their controls.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Mean relative gene expression</th>
<th>One-way ANOVA P-values between groups</th>
<th>Test of homogeneity of variances P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td><strong>High dose insulin</strong></td>
<td><strong>Low dose insulin</strong></td>
<td></td>
</tr>
<tr>
<td>Tbp</td>
<td>0.77 ± 0.07</td>
<td>1.08 ± 0.13</td>
<td>0.87 ± 0.05</td>
</tr>
<tr>
<td>Sdha</td>
<td>0.78 ± 0.09</td>
<td>1.07 ± 0.16</td>
<td>0.84 ± 0.05</td>
</tr>
<tr>
<td>Hmbs</td>
<td>0.85 ± 0.04</td>
<td>0.88 ± 0.05</td>
<td>0.86 ± 0.04</td>
</tr>
<tr>
<td>Hprt1</td>
<td>0.74 ± 0.04</td>
<td>1.01 ± 0.08</td>
<td>0.80 ± 0.03</td>
</tr>
<tr>
<td>Ubc</td>
<td>1.10 ± 0.04</td>
<td>1.21 ± 0.09</td>
<td>1.12 ± 0.05</td>
</tr>
<tr>
<td>B2m</td>
<td>0.93 ± 0.05</td>
<td>1.02 ± 0.10</td>
<td>0.91 ± 0.05</td>
</tr>
</tbody>
</table>

Data are presented as means and SEMs. Multiple comparisons were made using one-way ANOVA.
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Discussion

This study investigated whether changes in gene expression in sympathetic neurons supplying the PMAs may explain the observed changes in the perivascular nerve plexus described in Chapters 2 and 3. *Gapdh* and *Ppia* were identified as stable housekeeper genes in L1-L4 sympathetic ganglia with minimal differences in the expression ratio for these two genes in all the tissues studied from both control and STZ-treated rats. After the expression levels for the genes of interest were normalized to *Gapdh* and *Ppia*, hyperglycemia did not produce detectable changes in gene expression in the sympathetic ganglia from STZ-LI rats (i.e. compared to those from control rats). These results suggest that after 3 months of STZ-induced diabetes, the changes detected in the nerve terminals were not accompanied by detectable altered expression of the genes of interest studied.

Although *Gapdh* is a widely used housekeeper gene and has been selected for the present study, Kanwar and Kowluru (2009) demonstrated a decrease in *Gapdh* gene and protein expression levels in retinas from rats with STZ-induced diabetes given a low dose of insulin for 1 year (i.e. that were hyperglycemic). These authors also showed that Gapdh protein levels were not reduced in retinas from diabetic rats treated with a dose of insulin that maintained normal blood glucose levels (they did not assess gene expression levels). Another study showed that, in adipocytes isolated from rats 1 week post-STZ induction, there was a reduction in *Gapdh* mRNA expression levels and that gene expression levels for this protein increased after insulin supplementation (Alexander-Bridges *et al.*, 1992). *B2m* was used as a housekeeping gene by Kanwar and Kowluru (2009) but they did not provide an explanation for how or why it was chosen. In L1-L4 sympathetic ganglia in the present study, *B2m* expression was relatively stable (see Figure 4.1) when assessed as a candidate housekeeping gene but *Gapdh* and *Ppia* were more stably expressed. Other studies
have also reported that diabetes produces no changes in Gapdh gene or protein expression levels. For instance, 3 months of uncontrolled STZ-induced diabetes did not produce a detectable change in Gapdh gene expression in rat glomeruli (Jung et al., 2008). Similarly, Gapdh protein expression in the brains of rats with 5-6 months of uncontrolled STZ-induced diabetes was unchanged (Ball et al., 2011). Taken together, these studies highlight the need to carefully select suitable housekeeper genes because it is likely that they will differ depending on the tissue type studied.

Although I did not detect changes in expression of the genes of interest in L1-L4 ganglia, this does not exclude the possibility that gene expression in the postganglionic neurons supplying the plantar arterial vasculature is selectively affected by diabetes. This is because the cell bodies of these neurons represent only a small proportion of those present in the L1-L4 sympathetic ganglia, and changes could be masked if the majority of neurons in these ganglia have no diabetes related changes. Evidence for diabetes selectively affecting a subset of sympathetic ganglia comes from a study which demonstrated 2-6 weeks of STZ-induced diabetes increased gene expression levels for TH and synapsin, a synaptic vesicular protein involved in regulating neurotransmitter release, in sympathetic neurons located in the prevertebral superior mesenteric and celiac ganglia but not in those located in the paravertebral superior cervical ganglia (Carroll et al., 2004). This study parallels the demonstration that STZ-induced diabetes produces structural abnormalities in the superior mesenteric and celiac ganglia but not in superior cervical ganglia (Schmidt & Plurad, 1986). However, neurons in the superior cervical ganglia innervate the pineal gland and STZ-induced diabetes produces swelling of their axon terminals (Tsai et al., 2008). Therefore, at least some sympathetic neurons with cell bodies within the superior cervical ganglia are affected by diabetes. Swelling of sympathetic axons in the paravascular mesenteric nerves is not seen until >3 months following the induction of diabetes (Schmidt &
Plurad, 1986) and it is not known if gene expression levels for TH and synapsin remain elevated in the superior mesenteric and celiac ganglia with this duration of diabetes.

Twelve weeks of STZ-induced diabetes may not have been an appropriate time point to study changes in gene expression in L1-L4 sympathetic ganglia. In L4-L5 dorsal root ganglia (DRG) that contain sensory neurons projecting to the hind paw, STZ-induced diabetes reduced mRNA levels for the TrkC neurotrophin receptor at 12 weeks, but not at 6 weeks (Fernyhough et al., 1998). These studies indicate that in sensory neurons supplying the hind limb, 6-12 weeks post-STZ is sufficient to detect changes in gene expression. To my knowledge there has been no investigation of longitudinal changes in gene expression in sympathetic ganglia following the induction of diabetes.

The gene expression levels for both Nefh and Hprt1 in L1-L4 sympathetic ganglia were increased in STZ-HI rats compared to those from STZ-LI rats but expression of this gene in both these groups did not differ from that in control ganglia. These results suggest that insulin treatment by itself may play a role in modifying gene expression for selected genes. This has been demonstrated in cultured adipocytes where insulin simulates Gapdh gene expression, which is an enzyme involved in glycolysis (Alexander-Bridges et al., 1992). These investigators also demonstrated in STZ-treated rats that Gapdh mRNA expression was markedly reduced in adipocytes 1 week post-treatment. This finding may be explained by insulin activating an insulin-responsive DNA binding protein, IRP-A, which binds to an element of the 5’-flanking region of the Gapdh gene increasing Gapdh gene expression (Alexander-Bridges et al., 1992). Although there appear to be no studies reporting increases in gene expression levels for Nefh and Hprt1 in insulin treated diabetic animals, it is possible that insulin is involved in increasing gene expression levels seen in L1-L4 sympathetic ganglia of STZ-HI rats.
In conclusion, the results of this study show that $\text{Gapdh}$ and $\text{Ppia}$ are suitable housekeeper genes in L1-L4 sympathetic ganglia of the STZ-induced type I diabetic rat model despite reports that STZ-induced diabetes can change $\text{Gapdh}$ mRNA expression in other tissues. Although hyperglycemia did not change mRNA expression levels of the genes of interest, sympathetic ganglia from STZ-HI rats had increased $\text{Nefh}$ and $\text{Hprt1}$ gene expression levels compared to those from STZ-LI rats, perhaps suggesting insulin itself affects gene expression in L1-L4 sympathetic ganglia. These experiments cannot exclude the possibility of altered expression of the chosen genes in sub-populations of neuron cell bodies located in the L1-L4 sympathetic ganglia sympathetic neurons. However, these observations could also suggest that the diabetes-induced changes in the morphology of perivascular sympathetic axons innervating PMAs do not involve increased transcription of any of the selected genes of interest. Additionally, the increase in peripherin protein content within the perivascular nerve fibers innervating the PMAs (Chapter 3) does not appear to be due to an increase in its gene expression. This finding suggests that diabetes produces post-transcriptional changes in peripherin mRNA expression and/or alters the distribution of this protein within these sympathetic neurons.
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Summary of thesis findings

There is growing clinical evidence that diabetic patients, with no overt signs of diabetic complications, have impaired sympathetic nerve-mediated vasoconstriction in plantar arteries (Chapter 1). In this thesis I documented impaired sympathetic nerve-mediated neurovascular transmission (NVT) in the plantar metatarsal arteries (PMAs) of streptozotocin (STZ)-induced type I diabetic rats, a well-studied model of human type I diabetes (Chapter 2). These changes were accompanied by a reduction in the density and thickening of perivascular sympathetic fibers (Chapter 2). Despite unchanged protein content of tyrosine hydroxylase and β-tubulin III, increased peripherin protein expression was detected in perivascular sympathetic axons innervating the PMAs of diabetic rats (Chapter 3). Structural changes in perivascular sympathetic axons innervating PMAs were not associated with changes in gene expression of parent cell bodies located within the L1-L4 sympathetic chain ganglia (Chapter 4). As discussed in detail in the Discussion sections of the relevant chapters, some of these results are paradoxical.

Advantages and limitations of the streptozotocin-induced type I diabetic rat model

The STZ-induced type I diabetes rat model was used in this thesis because it has been the most extensively used model for studying experimental diabetic neuropathy. This model provides researchers with the ability to manipulate the administration of insulin (i.e. effects of glycemic variability) and study its consequent physiological effects (Rees & Alcolado, 2005). These studies are highly relevant to human type I diabetics since they require insulin supplementation. STZ diabetic rats demonstrate impaired sensory neuropathy in the hind paws (Westerman et al., 1988), which is consistent with loss of sensory nerve function in the feet of type I diabetics (Vinik et al., 1995). Interestingly, while there are numerous clinical studies demonstrating a loss of sympathetic nerve-mediated vasoconstriction in arterial vessels supplying blood to
plantar skin of the foot (see Chapter 1, section 3.1), prior to my studies there had not been any reports of impaired sympathetic NVT in plantar arteries from animal models of diabetes.

Type II diabetes is the most common type of diabetes; one limitation of the STZ-induced diabetes rat model is that it may not accurately represent the potential pathophysiological mechanisms of neuropathy produced by type II diabetes. To date, there is little evidence for an appropriate animal model of type II diabetes that can be used to study diabetic sympathetic neuropathy. In peripheral sensory neuropathies, including diabetes-induced neuropathies, neurons with the longest axons are thought to be affected first, producing the “glove and stocking” distribution of sensory abnormalities. Like type I diabetics, type II diabetic patients demonstrate impaired sympathetic NVT prior to the development of diabetic foot suggesting sympathetic nerves in the foot are particularly vulnerable to the effects of both types of diabetes. However, this thesis provides evidence that the location of arteries may be more important than the length of the axons innervating them (see discussion of Chapter 3 and below). The findings of this thesis strongly argue that the STZ-induced type I diabetic rat model is useful for understanding sympathetic neuropathy described in type I and II human diabetics.

**Denervation is an unlikely explanation for impaired sympathetic nerve-mediated neurovascular transmission in diabetes**

While it seemed likely that impaired sympathetic nerve-mediated NVT in PMAs of STZ-treated rats with uncontrolled diabetes might be due to a reduction in the density of perivascular sympathetic axons supplying this artery, histological evidence for reduced sympathetic innervation correlating with reduced sympathetic nerve-mediated NVT is weak. A recent study by Tripovic et al. (2013) demonstrated that removal of 50% of the sympathetic axons innervating the tail artery, another
cutaneous artery, did not reduce sympathetic nerve-mediated vasoconstriction of the vessel. Additionally, reports of impaired sympathetic nerve-mediated NVT in mesenteric and tail arteries isolated from STZ-induced diabetic rats (Hart et al., 1988; Ralevic et al., 1995) have not been correlated with a reduction in perivascular innervation of sympathetic fibers. It is possible, however, that prolonged periods of severe diabetes do lead to a loss of sympathetic axon fibers. Tack et al. (2002) have provided strong neurochemical evidence for sympathetic denervation in the feet of diabetic patients with painful neuropathy (see Chapter 1, section 3.2.1). As there is mounting evidence suggesting deficits in sympathetic nerve-mediated vasoconstriction of arteries supplying foot skin occurring prior to the development of complications including sensory neuropathy (see Chapter 1, section 3.1.1), it is likely that diabetes-induced impairments in NVT occur prior to the detectable loss of sympathetic nerve fibers.

Relative susceptibility of sympathetic axons to diabetes is not simply a matter of axon length

My studies showed that perivascular sympathetic axons innervating the PMAs appear to be more susceptible to the effects of diabetes compared to those innervating mesenteric and tail arteries. These data cast doubt on the contention that longer sympathetic axons are more likely to be affected by diabetes (Vinik et al., 2003), because those innervating the distal tail artery (which are longer than those innervating the PMAs) appear to be unaffected in terms of NVT (Speirs et al., 2006), innervation density (Speirs et al., 2006) and peripherin protein content (Chapter 3). Rather, it appears that PMAs are selectively affected, perhaps because these vessels are also subjected to the biomechanical stress of weight-bearing and locomotion (Clarke, 1995; Mak et al., 2010). Altered neural control of blood flow in plantar skin is not accompanied by changes in neural control of blood flow in palmar skin (Wilson et al., 1992; Benbow et al., 1995; Hilz et al., 2000).
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Effects of diabetes on peripherin expression in sympathetic axons innervating the PMAs

Chapter 3 provides the first evidence for a diabetes-induced increase in the intermediate filament protein, peripherin, in the perivascular sympathetic axons of PMAs from STZ-induced diabetic rats treated with a low dose of insulin. This finding provides an interesting insight into the effects of hyperglycemia on sympathetic nerve-mediated NVT because the thickness of perivascular sympathetic nerve fibers innervating PMAs was increased in STZ-induced diabetic rats with or without a low dose of insulin. On the one hand, there is growing evidence that peripherin plays important roles in maintaining nerve structure (Helfand et al., 2003) and in neuroregeneration after nerve injury (Oblinger et al., 1989a; Oblinger et al., 1989b; Wong & Oblinger, 1990; Reid et al., 2010). On the other hand, excess peripherin expression leads to defective axon transport (Millecamps et al., 2006), neurodegeneration (Perrot & Eyer, 2009) and cell death (Beaulieu et al., 1999a). On the basis of my findings, it is not possible to determine whether the elevated levels of peripherin protein detected in the sympathetic nerve terminals serve to promote neuroregeneration or to induce neurodegeneration. If increased peripherin expression plays a regenerative role in diabetes, further enhancement of sympathetic axon regeneration (i.e. administration of neurotrophins) could be therapeutic. Conversely, if peripherin protein expression plays a neurodegenerative role in diabetes, inhibiting peripherin expression (e.g. blocking cytokines which enhance peripherin expression) may prove beneficial in treating diabetic neuropathy.

Because changes in gene expression were not detected in sympathetic cell bodies of axons innervating the PMAs (Chapter 4), the changes in peripherin protein levels are most likely post-transcriptional. The transport of peripherin mRNA is selectively increased into axons in response to locally applied nerve growth factor or brain-derived neurotrophic factor (Willis et al., 2005). It is therefore possible that the
increased peripherin protein levels in the PMAs from diabetic rats is due to translation of increased levels of peripherin mRNA within the perivascular axons. Assuming peripherin is only expressed in neurons, mRNA expression levels of peripherin in the PMAs could be measured using quantitative reverse transcription PCR.

Protein synthesis is not necessarily proportional to mRNA expression levels because of post-transcriptional regulation by, for instance, microRNAs (miR; Park et al., 2013). The 3’ untranslated region of the rat peripherin mRNA is a predicted target for miR-216a and miR-342-3p (using the microrna.org website http://www.microrna.org/). If these miR (which would inhibit peripherin mRNA translation) were downregulated in sympathetic ganglia in diabetes, this would lead to upregulation of the peripherin protein without a change in mRNA levels. In order to demonstrate conclusively whether peripherin mRNA translation is increased in sympathetic axons innervating the PMAs of STZ-treated rats, the level of nascent protein production needs to be measured. A new method, described by Liu et al. (2012), demonstrated labeling of nascent polypeptide chains in vivo with an alkyne analog of puromycin, O-propargyl-puromycin (OP-puro). OP-puro-tagged polypeptide chains can be affinity-purified and analyzed by Western blot or, alternatively, in case peripherin antibodies fail to recognize the nascent polypeptides, by two-dimensional-SDS-PAGE and mass-spectrometry. The most attractive feature of this methodology is the ability to quantify nascent peripherin protein turnover in a non-genetically-modified animal model. Additional experiments could also determine whether the duration of diabetes impacts on the level of peripherin protein synthesis.

The many biological roles of insulin

It is likely that increased severity of diabetic autonomic neuropathy in diabetic patients corresponds to increased levels of hyperglycemia (Fleischer, 2012). Evidence presented in this thesis reinforces the importance of maintaining good glycemic
control as in rats given a high dose of insulin there were no diabetes-induced changes in sympathetic NVT and the structure of the perivascular nerve plexus of PMAs was also unaffected. Insulin is best-known for its role in controlling blood glucose levels, but it may play a more direct role in the biological function of arteries. Insulin predominantly mediates its effects via the insulin receptor which is present on vascular endothelial cells (Bottaro et al., 1989), smooth muscle (Johansson & Arnqvist, 2006) and sympathetic neurons (James et al., 1993; Karagiannis et al., 1997). Insulin appears to play a role in vascular endothelium by selectively increasing nitric oxide-mediated vasodilation of vascular smooth muscle (Laight et al., 1998). Insulin has also been suggested to have a trophic influence of sympathetic neurons although there appears to be no direct evidence for this action (James et al., 1993; Karagiannis et al., 1997). The physiological function of insulin on vascular smooth muscle is unknown (Johansson & Arnqvist, 2006). Uncontrolled STZ-induced diabetes produced functional deficits in NVT but not in endothelial function in PMAs of rats (Chapter 2), so the absence of insulin does not appear to have a strong influence on endothelial function in these cutaneous arteries. Loss of insulin signaling in vascular smooth muscle could contribute to the observed reduction in vascular reactivity but this is currently unknown. STZ-induced diabetic rats given a low dose of insulin (i.e. remained hyperglycemic) did not show a loss in NVT or a reduction in vascular muscle reactivity. Perhaps this is because a low level of insulin prevented this reduction in vascular reactivity from occurring.

In diabetic humans and rats, loss of insulin signaling also reduces systemic levels of insulin-like growth factor 1 (IGF-1) (Tan & Baxter, 1986; Ekstrom et al., 1989), a molecule structurally similar to insulin which also plays a neurotrophic role on peripheral neurons (for review see Rabinovsky, 2004). IGF-1, which is present in autonomic neurons (Hansson et al., 1988), mediates its effects via IGF-1 receptors that can form hybrid pairs with insulin receptors (Siddle et al., 1994; Seely et al., 1995). These hybrid IGF-1/insulin receptors are responsive to IGF-1, but not insulin, under
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physiological conditions (Soos et al., 1993). Co-localization of both receptors on autonomic nerves suggests the presence of the hybrid IGF-1/insulin receptors (Karagiannis et al., 1997). Brussee et al. (2004) found that intrathecal administration of a low dose of IGF-1 reversed functional and structural deficits in sensory axons. If this is the case, it is possible that the perivascular thickening seen in diabetic rats given a low dose or no insulin is due to reduced availability of IGF-1. Indeed, there is evidence that IGF-1 plays a role in diabetic sympathetic neuropathy because treatment of STZ-induced diabetic rats with IGF-1 ameliorates neuroaxonal dystrophy described in sympathetic axons of paravascular mesenteric nerves (Schmidt et al., 1999). It is unknown whether IGF-1 has an effect on peripherin protein expression but it would be interesting to investigate whether IGF-1 treatment can prevent and/or reverse hyperglycemia-induced nerve fiber thickening and normalize peripherin protein expression in perivascular sympathetic axons of PMAs.

Implications for diabetic foot

Human patients with long-term diabetes develop ulcers in the feet which result in amputation at the end-stage of the disease (i.e. diabetic foot). While there are no reports of endogenous ulcer formation in rats with STZ-induced diabetes, changes in sensory function of hind paw plantar skin (Westerman et al., 1988) have been correlated with decreases in the number of intra-epidermal nerve fibers in plantar skin (all of which are sensory) (Oltman et al., 2011). Furthermore, wound healing of skin is impaired by STZ-induced diabetes (Yavuz et al., 2005). There are also reports that STZ-induced diabetes produces thinning of rat skin and changes in its collagen content (Chen et al., 2010). These are all characteristic features of skin in diabetics that precede the more substantive complications of diabetic foot (Parkhouse & Le Quesne, 1988; Tahrani et al., 2012). Therefore the hind paws of STZ-treated rats provide a
suitable model for allowing a better understanding of factors contributing to the etiology of diabetic foot.

**Conclusion**

Combined, my studies showed that sympathetic nerves innervating the PMAs are functionally and morphologically affected by diabetes in STZ-treated rats. Additionally, this thesis provides evidence that sympathetic axons innervating the PMAs are selectively affected by diabetes compared to those innervating the mesenteric and tail arteries. These observations are consistent with studies in human diabetics where sympathetic nerve-mediated vasoconstriction has been demonstrated to be impaired in arteries supplying foot skin (Chapter 1, section 3) but not in the splanchnic vascular bed (Purewal *et al.*, 1995). Finally, my novel finding of increased peripherin protein expression in sympathetic nerve fibers innervating PMAs from diabetic animals may provide insight into the pathophysiological processes underlying diabetic autonomic neuropathy in PMAs. My studies provide the basis for further research investigating pathophysiological mechanisms underlying diabetic autonomic neuropathy using the STZ-treated rat model, which will contribute to the identification of therapeutic targets for prevention of vascular-related complications such as diabetic foot.
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