Investigating the relative contribution of obesity and glucose in the development of β-cell dysfunction

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Insulin resistance and impaired insulin secretion are hallmark features that contribute to the development of hyperglycaemia in type 2 diabetes (T2D) and other complications such as kidney failure, stroke and cardiovascular disease. Obesity is considered to be one of the main drivers in expediting hyperglycaemia by inducing insulin resistance in the liver, muscle and fat. These defects place additional stress on the β-cell to increase insulin output to compensate for the prevailing glucose and over time, can result in declined β-cell function and T2D. Pre-clinical and clinical studies investigating interventions that reduce obesity in pre-diabetes have shown that the incidence of T2D can be attenuated by preserving β-cell function through enhanced insulin sensitivity. However, as obesity clearly induces hyperglycaemia, it has become inherently difficult to dissociate the relative contribution of each in the progression of impaired glucose tolerance (IGT) to T2D. Accordingly, the scope of this thesis was to use dietary and pharmacological interventions to determine the contribution of obesity and glucose in the development of metabolic defects associated with T2D, namely glucose intolerance, insulin resistance and β-cell dysfunction. The overall hypothesis was that both obesity and excess glucose contribute to these defects.

In order to address the overall aim of this thesis, it was necessary to firstly characterise a pre-clinical model that does not rely on the presence of obesity to drive hyperglycaemia and subsequently, insulin resistance and β-cell dysfunction. We therefore utilised the phosphoenolpyruvate carboxykinase (PEPCK) transgenic rat, which is characterised by a 2-3 fold induction of PEPCK in the liver and kidney that leads to the impaired suppression of endogenous glucose production. Through the use of in vivo and in vitro experimental techniques, we show that the PEPCK transgenic rat develops defective glucose-stimulated insulin secretion in parallel with the worsening of glucose tolerance at 14 weeks of age, and that this is primarily due to the significant reduction in β-cell Glut2 gene expression and the inability of the constituents that make up the K⁺ATP channel, Sur1 and Kir6.2, to function properly. This defect in insulin secretion progressively worsens by 20 weeks due to the combination of β-cell dysregulation and reduced β-cell mass.
We next investigated the potential of the selective glucose-lowering SGLT2 inhibitor, dapagliflozin, in preventing the progression of insulin resistance and β-cell dysfunction in the PEPCK transgenic rat. We show that in older animals with established insulin resistance and β-cell dysfunction, dapagliflozin treatment for 6 weeks resulted in lower body weight gain despite the compensatory increase in food intake due to energy loss from the urine, reduced plasma glucose and insulin levels, and improvements in glucose tolerance which was associated with enhanced insulin sensitivity and glucose uptake in muscle and fat. In addition, dapagliflozin treatment in PEPCK transgenic rats significantly improved GLUT4 protein content in fat while adipocyte number was increased and the size reduced. A subset of PEPCK transgenic rats were also calorie-restricted in order to prevent further weight gain so that they could be used to account for any potential weight-induced insulin-sensitising benefits seen with dapagliflozin treatment. The prevention of weight gain in these PEPCK transgenic rats greatly enhanced peripheral insulin sensitivity to levels comparable with dapagliflozin treatment. Interestingly, dapagliflozin treatment did not preserve β-cell mass or improve the insulin secretory response to glucose. These data suggest that dapagliflozin elicits its effects on the β-cell in an indirect manner by increasing insulin sensitivity and providing an islet β-cell sparring effect.

To dissociate the relative contribution of obesity and glucose per se in IGT and β-cell dysfunction, obesity and glucose excess were prevented by commencing calorie-restriction and dapagliflozin treatment prior to any metabolic defects in 5 week-old PEPCK transgenic rats. Our findings show that preventing either obesity or glucose improves glucose tolerance but does not directly increase the insulin secretory capacity when assessed by a hyperglycaemic clamp or static incubation of isolated islets. These results support the hypothesis that both obesity and glucose per se contribute to the development of glucose intolerance, insulin resistance and β-cell dysfunction in the PEPCK transgenic rat and that enhancing insulin sensitivity with either intervention could effectively prevent the decline in β-cell function with age.
DECLARATION

This is to certify that:

(i) the thesis comprises only my original work towards the PhD except where indicated in the Preface,

(ii) due acknowledgement has been made in the text to all other materials used,

(iii) the thesis is less than 100,000 words in length, exclusive of tables, figures, bibliographies and appendices.

..............................

Christos Nikita Joannides

November 2016
PREFACE

Haematoxylin and eosin staining of epididymal adipose tissue was performed at the Monash Histology Platform, Department of Anatomy and Developmental Biology, Monash University (Clayton, Victoria, Australia).

Insulin immunohistochemistry of pancreatic sections was performed at Anatomical Pathology, Department of Medicine, University of Melbourne (Parkville, Victoria, Australia).

Digital images of microscopic sections were obtained through the Austin Health, Victorian Cancer Biobank Slide Scanning service (Heidelberg, Victoria, Australia).

Catheterisation of animals was performed by Associate Professor Sofianos Andrikopoulos.

Inflation of pancreata with collagenase was performed by Dr Maria Stathopoulos and Ms Mengjie Huang.

Assistance with the one-step hyperglycaemic, basal turnover and hyperinsulinaemic-euglycaemic clamp was provided by Dr Salvatore Mangiafico and Mr Matthew Waters, where necessary.
PUBLICATIONS

Published Peer-reviewed Journal Articles

Joannides C.N., Mangiafico S., Water M.F., Lamont B.J., Andrikopoulos S. Dapagliflozin improves insulin resistance and glucose intolerance in a novel transgenic rat with chronic glucose overproduction and glucose toxicity. Accepted manuscript online at Diabetes Obes Metab, February 28th 2017


Fam B.C., Joannides C.N., Andrikopoulos S. The liver: Key in regulating appetite and body weight. Adipocyte 1(4)259-264, 2012


Journal Articles in Preparation

Published Abstracts and Presentations

Joannides C.N., Stathopoulos M., Mangiafico S.P., Lamont B.J., Andrikopoulos S. Sodium glucose co-transporter 2 is not expressed in rat pancreatic islets. *Australian Diabetes Society & Australian Diabetes Educators Association Annual Scientific Meeting – Melbourne 2014* (Poster presentation)

Joannides C.N., Lamont B.J., Stathopoulos M., Mangiafico S.P., Fam B.C., Proietto J., Andrikopoulos S. Glucose toxicity causes a defect in insulin secretion via the $K_{ATP}^+$ channel. *International Diabetes Federation World Diabetes Congress – Melbourne 2013* (Poster presentation)

Joannides C.N., Lamont B.J., Stathopoulos M., Mangiafico S.P., Fam B.C., Proietto J., Andrikopoulos S. Glucose toxicity causes a defect in insulin secretion via the $K_{ATP}^+$ channel. *American Diabetes Association 73rd Scientific Sessions – Chicago, USA 2013* (Poster presentation)


Joannides C.N., Stathopoulos, M., Mangiafico S.P., Fam B.C., Proietto J., Andrikopoulos S. Glucose toxicity causes a defect in insulin secretion via the $K_{ATP}^+$ channel. *5th Australian Islet Study Group Meeting – Melbourne 2012* (Poster presentation)

Joannides C.N., Stathopoulos, M., Mangiafico S.P., Fam B.C., Proietto J., Andrikopoulos S. Glucose toxicity causes a defect in insulin secretion via the $K_{ATP}^+$ channel. *4th Australian Islet Study Group Meeting – Sydney 2011* (Poster presentation)

Joannides C.N., Stathopoulos, M., Mangiafico S.P., Fam B.C., Proietto J., Andrikopoulos S. Primary insulin resistance causes beta-cell failure associated with a defect in the $K_{ATP}^+$ channel. *Australian Diabetes Society & Australian Diabetes Educators Association Annual Scientific Meeting – Sydney 2010* (Poster presentation)
Educators Association Annual Scientific Meeting – Perth 2011 (Oral presentation – Pincus Taft Young Investigator Session)


Invited Speaker Presentations

Joannides C.J. Weight loss improves insulin sensitivity but does not enhance insulin secretion in rats with chronic glucose overproduction, Festschrift for Professor Joseph Proietto, April 17th 2015
ACKNOWLEDGEMENTS

The amount of emotional, intellectual and physical energy that goes into a PhD can only be appreciated by those who are in the inner sanctum of the candidate. It is for this reason that I would like to acknowledge the following people whose willingness to guide and support me along the way, made everything that little bit more easier.

First and foremost, I would like to offer my sincerest gratitude to my supervisors: Associate Professor Sofianos Andrikopoulos, Dr Benjamin Lamont and Dr Barbara Fam.

Sof: I am indebted to you for giving me the opportunity to conduct interesting research in your laboratory. Thank you for providing me with the resources necessary to quench my thirst for knowledge. I would like to reiterate what I wrote in my acknowledgements section of my Honours thesis, as it still resonates with me today. “You have taken a student with very little direction in life and given him the drive he needed to pursue a science career – for this I truly thank you”.

Ben: Your arrival back to Melbourne from Toronto in 2011 was a blessing in disguise for me and my PhD project. You have always taken time out of your busy schedule to offer assistance in interpreting data and to discuss any upcoming experiments that I may have. I would also like to thank you for your friendship throughout my PhD.

Barbara: Thank you for your ongoing support throughout my PhD. To say that it has been a rollercoaster ride would be an understatement. You were there every bit of the way to ensure that my spirits never waned. Every student should have a supervisor just like you.

Although not an “official” supervisor, I would like to take this opportunity to acknowledge the efforts of Professor Joseph Proietto who has been pivotal as a mentor throughout my PhD candidature. I always looked forward to presenting and discussing new data with you at lab meetings.
To the members of my supervisory committee: Associate Professor Tony Verberne and Dr Michael Hildebrand, thank you for your suggestions throughout my PhD and for your advice in pursuing a post-doctoral position overseas. To the PhD coordinator Jo Mayall, thank you for ensuring that the administrative side of things ran smoothly at all times. You definitely made life as a PhD student easier.

To the staff at the Austin Health BioResources Facility: Joshua Lorimer, Samantha O’Dea, Cleo Christodoulou, Hayley Sleep, Betty Nguyen and Julia Fyfe, thank you for maintaining the breeding and looking after the PVG/c and PEPCK colonies.

A big thank you to all of the past and present members of the Andrikopoulos lab who have each in their own unique way contributed to my PhD experience. Zheng Ruan – you are the only person that I know who can die today and be canonised tomorrow. Thank you for teaching me molecular biology techniques and for introducing me to cycling. Dr Salvatore Mangiafico – thank you for assisting me during those long days and nights in the animal house, especially when we used to do basal glucose turnover, hyperinsulinaemic-euglycaemic clamp and 2DG studies. I am forever grateful. Matthew Waters – I consider you as one of my good friends. Thank you for helping me in the lab and for your ongoing support and encouragement while I was writing this thesis.

To all of the other members of the lab, Dr Nicole Wong, Steve Weng, Dr Maria Stathopoulos, Mengjie Huang, Michael Pichler, Jessie Yang, Diane Vu, Chrysovalantou Xirouchaki, Viktoria Ntouma and Christian Haralambous, thank you for your friendship and for making the laboratory an enjoyable place.

To my colleagues at the Monash Research Office, Monash University, specifically Dr Ursula Manuelpillai, Dr Assunta Pelosi, Maks Sipowicz, Dennis Batson, Dr James Chan and Vetha Srinivasan, thank you for your constant support, words of encouragement and for keeping me accountable when I made hard deadlines.

There’s an age-old saying that goes “You can’t choose your family, but you can choose your friends”. Indeed, I am blessed to be part of a family whose unconditional love and support has helped me to get through this PhD. To my parents Chris and Shona, thank you for instilling in me the confidence to chase my dreams. To my siblings Paris, Sofie
and Clair and their respective partners Mikey and Kane, you have all played a significant part in my PhD journey and I am extremely fortunate to have you in my life.

I would like to particularly thank my older sister Clair and my brother-in-law Kane, who allowed me to move into their home so that I could focus on putting together this thesis. I will never forget the generosity that you have showed me.
DEDICATIONS

How fragile, precious and fleeting our lives are.
We take for granted that “tomorrow” will always come.

I would like to dedicate this PhD thesis to my late brother, Robert Thomas William Bell Wade, whose life was sadly cut short.

Robert has left us with the realisation that our own “tomorrows” may never arrive either, and that we must SEIZE THE DAY every day.

# TABLE OF CONTENTS

ABSTRACT ..................................................................................................................... i

DECLARATION ........................................................................................................... iii

PREFACE ...................................................................................................................... iv

PUBLICATIONS ............................................................................................................ v

ACKNOWLEDGEMENTS ............................................................................................. vii

DEDICATIONS ........................................................................................................... viii

TABLE OF CONTENTS .............................................................................................. ix

LIST OF TABLES ....................................................................................................... xiv

LIST OF FIGURES ...................................................................................................... xv

ABBREVIATIONS ..................................................................................................... xvii

Chapter 1 Literature Review ......................................................................................... 1

Type 2 Diabetes Mellitus .............................................................................................. 1

Definition and Prevalence ........................................................................................ 1

Normal Metabolic Physiology ...................................................................................... 2

Glucose Homeostasis ................................................................................................ 2

Endogenous Glucose Production .............................................................................. 2

Peripheral Glucose Utilisation .................................................................................. 3

Glucose Counter-Regulation .................................................................................... 5

Regulation of Insulin ................................................................................................ 7

Insulin Action ......................................................................................................... 18

Pathophysiology of Type 2 Diabetes Mellitus ............................................................ 21

Obesity .................................................................................................................... 21

Insulin Resistance ................................................................................................... 22

β-cell Dysfunction .................................................................................................. 24
Lipotoxicity ............................................................................................................ 30
Glucagon Dysregulation ......................................................................................... 32
Therapeutic Options for Type 2 Diabetes Mellitus .................................................... 32
Dietary Intervention ................................................................................................ 33
Pharmacological Interventions ............................................................................... 34
PEPCK Transgenic Rat .......................................................................................... 50
Study Rationale ........................................................................................................... 51
Overall Hypothesis ................................................................................................... 51
Overall Aim ................................................................................................................ 52
Chapter 2 General Materials and Methods .......................................................... 53
Materials ..................................................................................................................... 53
Molecular Biology .................................................................................................... 53
Cell Biology .............................................................................................................. 54
Animal Physiology .................................................................................................... 55
Equipment ................................................................................................................... 55
Animal Source & Maintenance ................................................................................ 57
Breeding and Maintenance ....................................................................................... 57
General Protocols ....................................................................................................... 58
Isolation of Pancreatic Islets ................................................................................... 58
Quantitative Real-Time PCR .................................................................................. 58
Western Blot: Detecting total GLUT4 on Protein Gels .......................................... 61
Physiological Studies ............................................................................................... 63
Calorie-restriction .................................................................................................... 63
Dapagliflozin Administration .................................................................................. 64
Characterisation of PEPCK transgenic rats ........................................................... 64
General Surgical Procedures ................................................................................... 64
Chapter 3 Characterisation of the PEPCK transgenic rat as a suitable model of progressive β-cell dysfunction

Introduction

Chapter Aims

Results

PEPCK transgenic rats become obese due to over-eating.

PEPCK transgenic rats develop fasting hyperglycaemia and hyperinsulinaemia.

PEPCK transgenic rats develop glucose intolerance with age.

PEPCK transgenic rats have impaired glucose-stimulated insulin secretion.

The down-regulation of Glut2 may contribute to the initial impairment in glucose-stimulated insulin secretion in the PEPCK transgenic rat.

PEPCK transgenic rats have larger but fewer pancreatic islets.

Discussion

Summary

Chapter 4 Dapagliflozin reduces insulin resistance but does not improve the insulin secretory capacity of the β-cell in the PEPCK transgenic rat

Introduction

Chapter Aims

Results

Calorie-restriction and dapagliflozin treatment reduces weight gain in PEPCK transgenic rats.
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Table of Contents</td>
<td></td>
</tr>
<tr>
<td></td>
<td>The effect of calorie-restriction and dapagliflozin treatment on fasting glucose and insulin levels in PEPCK transgenic rats.</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>Calorie-restriction and dapagliflozin treatment improves glucose tolerance in PEPCK transgenic rats.</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>Dapagliflozin treatment does not restore glucose-stimulated insulin secretion in glucose intolerant PEPCK transgenic rats.</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>Dapagliflozin treatment does not alter islet size or islet number in glucose intolerant PEPCK transgenic rats.</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>Calorie-restriction and dapagliflozin treatment improves insulin sensitivity in glucose intolerant PEPCK transgenic rats.</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td>Calorie-restriction and dapagliflozin treatment improves insulin sensitivity in skeletal muscle and adipose tissue in glucose intolerant PEPCK transgenic rats.</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>Calorie-restriction and dapagliflozin treatment reduces adipocyte size in glucose intolerant PEPCK transgenic rats.</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>Discussion</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>Summary</td>
<td>143</td>
</tr>
<tr>
<td></td>
<td><strong>Chapter 5</strong> The effects of obesity and chronic mild excess glucose in the progression of β-cell dysfunction in the PEPCK transgenic rat.</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>Chapter Aims</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>Results</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>Early intervention with calorie-restriction or dapagliflozin treatment causes reduced weight gain in PEPCK transgenic rats.</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>The effect of early intervention with calorie-restriction or dapagliflozin treatment on fasting glucose and insulin levels in PEPCK transgenic rats.</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>Early intervention with calorie-restriction or dapagliflozin improves glucose tolerance in PEPCK transgenic rats.</td>
<td>150</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1.1: Clinical efficacy of dapagliflozin monotherapy in patients with type 2 diabetes ........................................................................................................................... 45

Table 3.1: Body weight, food intake and white adiposity mass in 5, 14 and 20 week-old control and PEPCK transgenic rats ................................................................................ 77

Table 3.2: Change in fasting glucose and insulin levels in control and PEPCK transgenic rats with age ........................................................................................................ 79

Table 3.3: Quantitative insulin immunohistochemical staining of pancreatic sections of 5, 14 and 20 week-old control and PEPCK transgenic rats ........................................... 95

Table 4.1: The effects of treatment on growth parameters and adiposity in PEPCK transgenic rats ............................................................................................................... 110

Table 4.2: The effects of treatment on fasting biomarkers in PEPCK transgenic rats. 112

Table 4.3: Quantitative insulin immunohistochemical staining of pancreatic sections from PEPCK transgenic rats fed ad-libitum or treated with dapagliflozin for 6 weeks starting from 14 weeks of age................................................................. 120

Table 5.1: The effects of preventative treatment on growth parameters and adiposity in PEPCK transgenic rats ......................................................................................................... 147

Table 5.2: The effect of early intervention on fasting biomarkers in PEPCK transgenic rats .............................................................................................................................. 149

Table 5.3: Quantitative insulin immunohistochemical staining of pancreatic sections from PEPCK transgenic rats fed ad-libitum, calorie-restricted or treated with dapagliflozin for 9 weeks starting from 5 weeks of age..................................................... 160
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Abundance and distribution of endocrine cells in mouse and human pancreatic islets</td>
<td>9</td>
</tr>
<tr>
<td>1.2</td>
<td>Insulin synthesis</td>
<td>10</td>
</tr>
<tr>
<td>1.3</td>
<td>Glucose-stimulated insulin secretion in pancreatic β-cells</td>
<td>12</td>
</tr>
<tr>
<td>1.4</td>
<td>Percentile lines for the relationship between insulin sensitivity and first-phase insulin in response to glucose (AIRglucose) based on data from 93 normal subjects</td>
<td>25</td>
</tr>
<tr>
<td>1.5</td>
<td>The localisation of SGLT1 and SGLT2 in the kidneys</td>
<td>38</td>
</tr>
<tr>
<td>1.6</td>
<td>SGLT2 inhibition reduces renal glucose reabsorption and increases urinary glucose excretion</td>
<td>42</td>
</tr>
<tr>
<td>3.1</td>
<td>Intraperitoneal glucose tolerance tests in 5, 14 and 20 week-old control and PEPCK transgenic rats</td>
<td>81</td>
</tr>
<tr>
<td>3.2</td>
<td>One-step hyperglycaemic clamp in 14 and 20 week-old control and PEPCK transgenic rats</td>
<td>85</td>
</tr>
<tr>
<td>3.3</td>
<td>Glucose and secretagogue-stimulated insulin secretion in isolated pancreatic islets from 14 and 20 week-old control and PEPCK transgenic rats</td>
<td>87</td>
</tr>
<tr>
<td>3.4</td>
<td>Gene expression analysis of Glut2, Gck, Sur1 and Kir6.2 in isolated pancreatic islets from 5, 14 and 20 week-old control and PEPCK transgenic rats</td>
<td>90</td>
</tr>
<tr>
<td>3.5</td>
<td>Insulin immunohistochemical staining of the pancreas of 5, 14 and 20 week-old control and PEPCK transgenic rats</td>
<td>94</td>
</tr>
<tr>
<td>4.1</td>
<td>Intraperitoneal glucose tolerance test in PEPCK transgenic rats fed ad-libitum, calorie-restricted or treated with dapagliflozin for 6 weeks starting from 14 weeks of age</td>
<td>114</td>
</tr>
<tr>
<td>4.2</td>
<td>One-step hyperglycaemic clamp in PEPCK transgenic rats fed ad-libitum or treated with dapagliflozin for 6 weeks starting from 14 weeks of age</td>
<td>117</td>
</tr>
<tr>
<td>4.3</td>
<td>Insulin immunohistochemical staining of the pancreas of PEPCK transgenic rats fed ad-libitum or treated with dapagliflozin for 6 weeks starting from 14 weeks of age</td>
<td>119</td>
</tr>
</tbody>
</table>
Figure 4.4: Insulin sensitivity in control and PEPCK transgenic rats fed *ad-libitum*, calorie-restricted or treated with dapagliflozin for 6 weeks starting from 14 weeks of age ................................................................. 125

Figure 4.5: Peripheral insulin sensitivity in control and PEPCK transgenic rats fed *ad-libitum*, calorie-restricted or treated with dapagliflozin for 6 weeks starting from 14 weeks of age ................................................................. 128

Figure 4.6: Histology of epididymal adipose tissue with H&E staining and morphological analyses in control and PEPCK transgenic rats fed *ad-libitum*, calorie-restricted or treated with dapagliflozin for 6 weeks starting from 14 weeks of age..... 131

Figure 5.1: Intraperitoneal glucose tolerance test in PEPCK transgenic rats fed *ad-libitum*, calorie-restricted or treated with dapagliflozin for 9 weeks starting from 5 weeks of age ........................................................................ 151

Figure 5.2: One-step hyperglycaemic clamp in PEPCK transgenic rats fed *ad-libitum*, calorie-restricted or treated with dapagliflozin for 9 weeks starting from 5 weeks of age ......................................................................... 155

Figure 5.3: Glucose-stimulated insulin secretion in isolated pancreatic islets from PEPCK transgenic rats fed *ad-libitum*, calorie-restricted or treated with dapagliflozin for 9 weeks starting from 5 weeks of age ................................................................. 157

Figure 5.4: Insulin immunohistochemical staining of the pancreas of PEPCK transgenic rats fed *ad-libitum*, calorie-restricted or treated with dapagliflozin for 9 weeks starting from 5 weeks of age ................................................................. 159

Figure 6.1: Fluid intake in 10-12 week-old female PEPCK transgenic rats given either water or dapagliflozin over 20 days ................................................................. 232

Figure 6.2: Gene expression analysis of *Pck1* in tissues from 4 week-old fed control and PEPCK transgenic rats ................................................................. 233

Figure 6.3: Intravascular glucose tolerance tests in control and PEPCK transgenic rats fed *ad-libitum*, and PEPCK transgenic rats treated with dapagliflozin .......... 234
ABBREVIATIONS

2DG 2-deoxy-D-[1-14C]-glucose
A260 Absorbance at 260 nm
A280 Absorbance at 280 nm
ad-libitum as much as one likes
ADP Adenosine diphosphate
AIRglucose First-phase insulin in response to glucose
Akt Protein kinase B
AMV Avian myeloblastosis virus
ANOVA Analysis of variance
APS Ammonium persulphate
ATP Adenosine triphosphate
Bq Becquerel
BSA Bovine serum albumin
cAMP Cyclic adenosine monophosphate
CANVAS Canagliflozin Cardiovascular Assessment Study
Cbl Casitas B-lineage lymphoma
cDNA complementary DNA
CR Calorie-restricted
db Diabetes mutation
DEPC Diethylpyrocarbonate
DNA Deoxyribonucleic acid
DNase Deoxyribonuclease
dNTP Deoxyribose nucleotide triphosphates
dpm Disintegrations per minute
DPP-4 Dipeptidyl peptidase-4
DTT Dithiothreitol
e.g. exempli gratia, for example
ECL Enhanced chemiluminescence
EDTA Ethylenediaminetetraacetic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGP</td>
<td>Endogenous glucose production</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>EMA</td>
<td>European Medicines Agency (Europe)</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>F</td>
<td>Rate of $[6^{-3}\text{H}]-\text{glucose}$ infused</td>
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<td>$Fbp1$</td>
<td>Fructose 1,6-bisphosphatase 1</td>
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<td>FBPase</td>
<td>Fructose 1,6-bisphosphatase</td>
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<td>FDA</td>
<td>Food and Drug Administration (USA)</td>
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<tr>
<td>FFAs</td>
<td>Free fatty-acids</td>
</tr>
<tr>
<td>FOXO1</td>
<td>Forkhead box protein O1</td>
</tr>
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<td>FPG</td>
<td>Fasting plasma glucose</td>
</tr>
<tr>
<td>G6Pase</td>
<td>Glucose-6-phosphatase</td>
</tr>
<tr>
<td>$G6pc$</td>
<td>Glucose-6-phosphatase</td>
</tr>
<tr>
<td>Gab-1</td>
<td>GRB2-associated binding protein-1</td>
</tr>
<tr>
<td>GCK</td>
<td>Glucokinase</td>
</tr>
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<td>GDP</td>
<td>Guanosine diphosphate</td>
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<td>GIP</td>
<td>Glucose-dependent insulinotropic polypeptide</td>
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<td>GIR</td>
<td>Glucose infusion rate</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide 1</td>
</tr>
<tr>
<td>GLP-1RA</td>
<td>GLP-1 receptor agonists</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GPRs</td>
<td>G protein-coupled receptors</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor bound protein 2</td>
</tr>
<tr>
<td>GSIS</td>
<td>Glucose-stimulated insulin secretion</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Glycated haemoglobin</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HEPES</td>
<td>Hydroxyethyl piperazineethanesulfonic</td>
</tr>
<tr>
<td>HGP</td>
<td>Hepatic glucose production</td>
</tr>
<tr>
<td>HNF-1α</td>
<td>Hepatocyte nuclear factor-1α</td>
</tr>
<tr>
<td>HSP90</td>
<td>Heat shock protein 90</td>
</tr>
</tbody>
</table>
Abbreviations

i.e.  id est; that is
i.p.  Intraperitoneal
iAUC  Incremental area under the curve
IGT  Impaired glucose tolerance
iNOS  Inducible form of NO synthase
IPGTT  Intraperitoneal glucose tolerance test
IRS  Insulin receptor substrate
IVGTT  Intravascular glucose tolerance test
K$_{ATP}$ channel  ATP-sensitive K$^+$ channel
kDA  kilo Daltons
Kir6.2  Potassium inward rectifier 6.2
K$_m$  Michaelis constant
KRBB  Krebs-ringer bicarbonate buffer
MafA  V-maf musculoaponeurotic fibrosarcoma oncogene homologue
MAPK  Mitogen-activated protein kinase
mRNA  messenger RNA
N.S.  not significant
NEFA  Non-esterified fatty-acid
NGT  Normal glucose tolerance
NO  Nitric oxide
NP40  Nonidet P40
NZO mouse  New Zealand obese mouse
PBS  Phosphate-buffered saline
Pck1  Phosphoenolpyruvate carboxykinase 1
PCR  Polymerase chain reaction
PDX-1  Pancreas duodenum homeobox-1
PenStrep  Penicillin 100 U/mL, streptomycin 100 µg/mL
PEPCK  Phosphoenolpyruvate carboxykinase
PI(3,4)P$_2$ or PIP$_2$  Phosphatidylinositol (3,4)-bisphosphate
PI(3,4,5)P$_3$ or PIP$_3$  Phosphatidylinositol (3,4,5)-trisphosphate
PI3K  Phosphatidylinositol 3-kinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>PI3P or PIP</td>
<td>Phosphatidylinositol 3-phosphate</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride membrane</td>
</tr>
<tr>
<td>PVG/c</td>
<td>Piebald Virology Glaxo control</td>
</tr>
<tr>
<td>Rₐ</td>
<td>Rate of glucose appearance</td>
</tr>
<tr>
<td>rDNase</td>
<td>Recombinant deoxyribonuclease</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNasin</td>
<td>Ribonuclease inhibitor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>RPMI medium</td>
<td>Royal Park Memorial Institute medium</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SA</td>
<td>Specific activity of glucose</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SGLT</td>
<td>Sodium-glucose linked transporter</td>
</tr>
<tr>
<td>Shc</td>
<td>Src homology 2 domain-containing</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>SUR1</td>
<td>Sulfonylurea receptor 1</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetic acid EDTA</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGA</td>
<td>Therapeutics Goods Administration (Australia)</td>
</tr>
<tr>
<td>Tₘ</td>
<td>Renal transport maximum</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>wt/wt</td>
<td>weight/weight</td>
</tr>
<tr>
<td>Rcf</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>ZDF rat</td>
<td>Zucker diabetic fatty rat</td>
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Chapter 1 Literature Review

Type 2 Diabetes Mellitus

Definition and Prevalence

Diabetes mellitus is characterised by high blood glucose (hyperglycaemia) due to the body not producing enough insulin and because cells do not respond to the insulin being produced. There are three main types of diabetes mellitus:

1. Type 1 diabetes (T1D) mellitus arises as a result of the autoimmune destruction of the pancreatic β-cells which causes hyperglycaemia due to an absolute deficiency of plasma insulin (Bekris, Kavanagh, & Lernmark, 2006). It is also known as insulin-dependent diabetes mellitus as patients depend on external administration of insulin for survival. This form of diabetes generally presents in children and adolescents but can also occur in adults.

2. Type 2 diabetes (T2D) mellitus is characterised by hyperglycaemia, which results from impaired insulin secretion due to pancreatic β-cell dysfunction (S. E. Kahn, 2003). Peripheral insulin resistance (a reduced capacity of insulin to stimulate glucose uptake into skeletal muscle and adipose tissue) and impaired hepatic insulin action (a reduced ability of insulin to suppress liver glucose production) are also important contributors to the development of T2D (DeFronzo, Simonson, & Ferrannini, 1982). These hallmark features are influenced by a combination of dietary and lifestyle factors in addition to genetic predispositions. If poorly controlled or left untreated, T2D results in microvascular and macrovascular complications including retinopathy, nephropathy, neuropathy, cardiovascular disease, peripheral vascular disease and stroke (Klein, 1995; Stratton et al., 2000).

3. Gestational diabetes is characterised by hyperglycaemia during pregnancy and is considered a risk factor for the development of T2D later on in life (Gilmartin, Ural, & Repke, 2008).
Normal Metabolic Physiology

Glucose Homeostasis
The regulation of plasma glucose is essential for maintaining normal cellular function. In humans, the concentration of circulating glucose over a 24 hour period averages 5.5-6 mmol/L with a maximal concentration usually not exceeding 9 mmol/L after meal ingestion and a minimum of 3 mmol/L following prolonged fasting (Gerich, 1993). The maintenance of a constant supply of glucose in the circulation without exceeding the normal physiological range of 3.5-5.5 mmol/L is essential for two major reasons. The first is because the brain relies on glucose as a major fuel source, as it can neither produce nor store glucose. If blood glucose falls significantly below the normal physiological range (hypoglycaemia) even for a short period of time, severe and sometimes irreversible changes in brain function may occur (Auer, Wieloch, Olsson, & Siesjo, 1984). The second reason is too much glucose in the blood (hyperglycaemia) may initially lead to symptoms such as polyuria, polydipsia and dehydration (Jones, 1994), and if poorly controlled or left untreated, can result in glucose-induced oxidative damage (also known as glucotoxicity). This juggling act of keeping glucose within the normal physiological range is achieved by a dynamic equilibrium between endogenous glucose production (EGP) mainly from the liver and to a lesser extent, the kidney, and glucose utilisation by peripheral tissues. Both of these processes are primarily regulated by two key pancreatic hormones: insulin and glucagon.

Endogenous Glucose Production
In the post-absorptive state (overnight fasting), the majority of tissue glucose uptake takes place in insulin independent tissues, primarily the brain, which accounts for approximately 50 % of basal glucose disposal (Eastman et al., 1990), with the remainder being 25 % in the splanchnic tissue (DeFronzo, Gunnarsson, Bjorkman, Olsson, & Wahren, 1985) and 25 % in skeletal muscle and other peripheral tissues such as adipose and red blood cells (DeFronzo, 1988). The basal rate of glucose utilisation is perfectly matched by the rate of EGP which allows fasting plasma glucose (FPG) concentrations to be maintained within a very narrow range. Thus, EGP in the basal state is considered to be the primary determinant of FPG levels.
EGP is the result of two concerted, tightly regulated processes. Hepatic glucose is initially produced by glycogenolysis (the breakdown of glycogen stores to glucose), but as glycogen stores become depleted, e.g., during the early stage of fasting, the de novo synthesis of glucose from non-carbohydrate precursors such as pyruvate, lactate and amino-acids occurs; a process known as gluconeogenesis. Gluconeogenesis is limited to the liver, kidney and the small intestine as these are the only organs to contain appreciable levels of glucose-6-phosphatase (G6pase), a key enzyme involved in catalysing the last biochemical reaction of gluconeogenesis (Foster, Pederson, & Nordlie, 1997; Mithieux, 1997; Rajas, Bruni, Montano, Zitoun, & Mithieux, 1999). The other two rate-limiting enzymes involved in gluconeogenesis include phosphoenolpyruvate carboxykinase (PEPCK) and fructose 1,6-bisphosphatase (FBPase). In response to elevated blood glucose during feeding, insulin is released from the pancreatic β-cells whereby it suppresses EGP by negatively regulating the gluconeogenic enzymes and suppressing glycogenolysis.

Peripheral Glucose Utilisation
The plasma membrane is highly impermeable to polar molecules (water-soluble) such as glucose. For this reason, glucose uptake into peripheral tissues can only be achieved through membrane-associated protein transporters that bind and transfer it across the lipid bilayer. There are two distinct classes of glucose transporters that exist in mammalian organisms. The first class are the sodium-glucose linked transporters (SGLT), a family of proteins that actively transport glucose across cell membranes against a concentration gradient, using the energy provided by co-transport of Na⁺ ions (Hediger & Rhoads, 1994). There are currently six different genes that encode for SGLT that have been isolated in humans but only SGLT1 and SGLT2 have been well characterised (Wright & Turk, 2004). The SGLT1 is a high-affinity, low-capacity SGLT with a sodium-to-glucose coupling of 2:1, and is expressed mainly in the distal segment of the proximal tubule in the kidney and intestine (Wright, Loo, & Hirayama, 2011). In contrast, SGLT2 is a low-affinity, high-capacity SGLT with a sodium-to-glucose ratio of 1:1 and is found in high abundance in the renal proximal tubule where it is responsible for the bulk (90%) of renal glucose reabsorption (Wright et al., 2011). Due to its significant role in regulating glucose reabsorption in the kidney, the inhibition of SGLT2 has been flagged as an attractive therapeutic option for glycaemic control in
diabetic subjects. The other class is the facilitative glucose transporters (GLUT), a family of proteins that passively facilitate glucose movement from the extracellular to the intracellular space along its chemical gradient. To date, there are at least 13 members of the GLUT family described with all showing a high degree of sequence homology, although they differ in their substrate specificity, kinetic characteristics, tissue and subcellular distribution, and their response to extracellular stimuli (Joost et al., 2002).

The effect of insulin on glucose transport into skeletal muscle and adipose tissue was first documented in the 1950’s when several groups demonstrated that insulin was able to accelerate the rate of glucose uptake (Levine & Goldstein, 1958; C. R. Park & Johnson, 1955). The principle glucose transporter that mediates this uptake is GLUT4, and is considered to be the primary cellular mechanism for disposal of an exogenous glucose load, e.g., after caloric ingestion, from the circulation where it is either stored as glycogen (in skeletal muscle) or oxidised to produce energy following the transport step (Huang & Czech, 2007). Skeletal muscle and adipose tissue also express a selective cohort of other glucose transporters. For example, in skeletal muscle, GLUT1, GLUT5 and GLUT12 may also contribute to glucose uptake (Stuart, Wen, Gustafson, & Thompson, 2000; Stuart et al., 2006), while in adipose tissue, GLUT8 and GLUT12 are also expressed (Ibberson, Uldry, & Thorens, 2000; Wood, Hunter, & Trayhurn, 2003). Unlike these glucose transporters, GLUT4 is unique in that it is sequestered from an intracellular membrane pool to the plasma membrane upon insulin stimulation or exercise. This has been confirmed through a variety of experimental techniques such as subcellular fractionation (Deems et al., 1994; Marette, Burdett, Douen, Vranic, & Klip, 1992; Pilch et al., 1993), electron and fluorescence microscopy (Voldstedlund, Tranum-Jensen, & Vinten, 1993) and the use of exogenously expressed GLUT4 fusion proteins labelled with various epitope tags (Czech et al., 1993; Dawson, Aviles-Hernandez, Cushman, & Malide, 2001; Dobson, Livingstone, Gould, & Tavare, 1996; Kanai et al., 1993). These studies have demonstrated that in non-stimulated cells, < 5 % of the total GLUT4 pool reside at the plasma membrane with the remainder found within intracellular membranes.
GLUT2 is the major glucose transporter of the liver and pancreatic β-cell, and therefore plays an important role in glucose metabolism in hepatocytes and glucose-stimulated insulin secretion (GSIS) from β-cells. GLUT2 has a uniquely high $K_m$ for glucose of 17 mmol/L, making it the highest among the known members of the GLUT family (Johnson, Newgard, Milburn, Lodish, & Thorens, 1990). This ensures a high transport capacity between the extracellular space and the cell cytosol even under diabetes-associated glycaemic levels. Interestingly, the rate of glucose metabolism is not controlled by modifications to the glucose transporter activity, albeit only if its reduction is sufficient to limit glucose entry into β-cells during diabetic hyperglycaemia. Instead, once glucose is transported into the cell, glucose utilisation is dependent on the glucose phosphorylation step catalysed by the enzyme, glucokinase (GCK).

**Glucose Counter-Regulation**

Hypoglycaemia results from an imbalance between EGP and glucose utilisation by insulin-sensitive tissues due to either excessive glucose removal from the circulation, reduced glucose delivery into the circulation, or a combination of both. The glucose counter-regulatory response not only requires the dissipation of insulin but also involves, in order of importance, the key counter-regulatory factors glucagon, epinephrine, cortisol, growth hormone and neuronal regulation to restore euglycaemia if hypoglycaemia should occur. These counter-regulatory factors are discussed in more detail below.

**Glucagon**

Like the insulin-producing β-cell, the glucagon-secreting α-cell has the ability to sense low glucose concentrations directly. Glucagon exhibits its counter-regulatory actions to glucose exclusively on the liver where it stimulates both glycogenolysis and gluconeogenesis, and increases glucose output within minutes (Dunning, Foley, & Ahren, 2005). In addition, paracrine signals from pancreatic α-cells acting to suppress β-cell release of insulin allows glucose in the bloodstream to accumulate, thereby returning glycaemia to the normal range. During fasting or prolonged hypoglycaemia, glucagon can also stimulate the liver to release ketone bodies as a substitute for glucose in meeting the energy requirements of the brain (Vons et al., 1991).
**Epinephrine**

The adrenomedullary hormone epinephrine is also secreted in response to hypoglycaemia where it stimulates liver EGP and limits glucose utilisation. These actions occur through indirect and direct mechanisms. The indirect actions of epinephrine include $\alpha$-adrenergic suppression of insulin secretion and augmentation of glucagon secretion. The direct effect of epinephrine interacting with $\beta_2$-adrenergic receptors is the production of increased gluconeogenic substrate, i.e., glycerol from lipolysis (the breakdown of lipids) and pyruvate from glycolysis (the breakdown of glucose), and a reduction in glucose utilisation (Sprague & Arbelaez, 2011).

**Cortisol and Growth Hormone**

In contrast to the rapid effects, i.e., a few minutes, of glucagon and epinephrine on glucose regulation, the effects of cortisol and growth hormone on hypoglycaemia occur over a period of hours (MacGorman, Rizza, & Gerich, 1981). Both hormones work similarly to glucagon by antagonising the effects of insulin to mediate glucose utilisation and hepatic glucose production (HGP). However, the contribution to glucose counter-regulation is relatively small when compared to glucagon as patients who lack both hormones have similar recovery rates to healthy control subjects after prolonged hypoglycaemia in adulthood (Boyle & Cryer, 1991).

**Neuronal Regulation**

The regulation of blood glucose was generally thought to be under the sole control of the endocrine system. However, work over the past decade has established that a complex neural circuitry exists, with several neurons located in the hypothalamus playing an important role in the glucose counter-regulatory response (Briski & Sylvester, 2001; Cai et al., 2001; Moriguchi, Sakurai, Nambu, Yanagisawa, & Goto, 1999). There are three peripheral glucose-sensing sites, the liver, pancreas and adrenal gland that act to maintain glucose homeostasis via signals from sympathetic and parasympathetic neurons located in the brainstem and spinal cord. These central glucose-sensing neurons impart their actions by communicating via the hepatic portal vein, vagal and sympathetic afferents, intestinal vagal glucose-sensors and the carotid body. These then pass the integrated signals to effector systems involved in the regulation of peripheral glucose metabolism so that the secretion of insulin, glucagon,
epinephrine, cortisol and growth hormone can be adjusted accordingly (Verberne, Sabetghadam, & Korim, 2014).

**Regulation of Insulin**

Insulin was first discovered in 1921 by Fred Banting and Charles Best when they carried out experiments in pancreatectomised dogs in their quest to find a possible treatment for T2D (Banting, 1926). Insulin is a hormone that is secreted to remove excess glucose from the blood. It does this by promoting glucose uptake and utilisation in peripheral tissues such as skeletal muscle and adipose tissue. The counter-regulatory hormones glucagon and epinephrine counteract the action of insulin by stimulating HGP via glycogenolysis and gluconeogenesis, thus raising the blood glucose concentration.

**The Pancreas**

The pancreas comprises both exocrine and endocrine components. The exocrine part is involved in the synthesis and release of digestive juices. The endocrine portion of the pancreas, also known as Islets of Langerhans, comprise of at least five types of cells, including the insulin-producing β-cells, the glucagon-releasing α-cells, the somatostatin-producing δ-cells, the pancreatic polypeptide-containing PP cells and the ghrelin-containing ε-cells.

- **β-cells**: The most abundant population of cells in pancreatic islets, as identified by electron microscopy, account for 40-70 % per human islet and 80-90 % per rodent islet (Cabrera et al., 2006). These cells are the most important in the lowering of blood glucose because they control insulin synthesis and its subsequent release.
- **α-cells**: Human α-cells make up between 33-46 % and are one of the main endocrine cell populations that co-exist in the pancreatic islet along with β-cells. These cells produce and secrete glucagon which counteracts hypoglycaemia and opposes the effects of insulin, e.g., stimulating HGP, to increase blood glucose concentrations.
- **δ-cells**: Pancreatic δ-cells synthesise and secrete somatostatin. These cell types are also present in the hypothalamus, central nervous system, peripheral neurons and the gastrointestinal tract (Arimura, Sato, Dupont, Nishi, & Schally, 1975; Hokfelt et al., 1975). This hormone can decrease the rate of gastric emptying by
suppressing the release of gastrointestinal hormones such as gastrin, cholecystokinin, vasoactive intestinal peptide and gastric inhibitory polypeptide. In addition, somatostatin has been shown to inhibit insulin and glucagon secretion through several mechanisms, one of which being the inhibition of adenylate cyclase activity, thereby decreasing cyclic adenosine monophosphate (cAMP) levels and protein kinase A (Schuit, Derde, & Pipeleers, 1989).

- PP-cells: Human PP-cells produce and secrete the hormone pancreatic polypeptide. This particular hormone stimulates the secretion of gastric and intestinal enzymes, and inhibits intestinal motility. Unlike β-, α- and δ-cells which are distributed in a similar fashion throughout the Islet of Langerhans, i.e., in single cells and small clusters to large islets throughout the pancreas, human studies have found that PP-cells represent < 2 % of the islet cell population in humans (Stefan et al., 1982), with the majority of these cells being confined to the head region of the pancreas (X. Wang et al., 2013).

- ε-cells: Despite these cells only being detected in substantially low amounts in the pancreas compared to the stomach (Ariyasu et al., 2001), human pancreatic ε-cells produce and secrete the only currently known hunger hormone ghrelin (Wierup, Svensson, Mulder, & Sundler, 2002). Interestingly, immunohistochemistry with antiserum against ghrelin have shown overlap in both β-cells (Volante et al., 2002) and α-cells (Date et al., 2002) respectively.

It is important to note that there are marked differences in the abundance and distribution of endocrine cells that exist between human and rodent pancreatic islets. Rodent pancreatic islets are arranged with a mantle of δ- and α-cells surrounding β-cells, with these β-cells being in contact with other β-cells, but not with other endocrine cells. By contrast, human pancreatic islets possess an even distribution of the three endocrine cell types and most β-cells are in contact with δ- and α-cells (Cabrera et al., 2006). The arrangement and proportion of endocrine cells in the human and rodent pancreatic islet are illustrated in Figure 1.1.
Figure 1.1: Abundance and distribution of endocrine cells in mouse and human pancreatic islets. Immunohistochemical staining of insulin (red), glucagon (green) and somatostatin (blue) in (A) mouse and (B) human pancreatic islets. As can be observed by the high abundance of red staining, β-cells account for 80-90 % of islet endocrine cells in rodents whereas only 40-70 % are found in human islets. Note that PP- and ε-cells were not stained and are therefore not shown (Cabrera et al., 2006).

Insulin Synthesis

The primary stimulus for insulin synthesis and release is the β-cell’s response to changes in ambient glucose concentrations (Coore & Randle, 1964). The production of insulin within the β-cell involves the synthesis of proinsulin at the endoplasmic reticulum followed by the proteolytic cleavage via the proteases, prohormone convertases 1 and 2, in the Golgi apparatus. Mature insulin and a cleavage peptide known as C-peptide are generated and stored in secretory granules, ready to be released in response to increased blood glucose levels (Orci, 1985) (Figure 1.2). This synthesis pathway is tightly regulated by glucose at several steps, including at transcription from the insulin gene (Nielsen, Welsh, Casadaban, & Steiner, 1985), mRNA stabilisation (M.
Welsh, Nielsen, MacKrell, & Steiner, 1985), translation (M. Welsh, Scherberg, Gilmore, & Steiner, 1986), and at the processing of proinsulin to insulin (Nagamatsu, Bolaffi, & Grodsky, 1987).

Figure 1.2: Insulin synthesis. Insulin production involves many intermediate steps. Initially, pre-proinsulin is secreted into the endoplasmic reticulum. Post-translational processing clips the N-terminal sequence allowing for chain A and chain B to form disulphide bridges. Lastly, the polypeptide is clipped at two positions by prohormone convertases 1 and 2 (not shown) to release chain C. This C-peptide and the active form of insulin are then packaged into secretory vesicles for storage (Cartailler, 2004).

**Glucose-Stimulated Insulin Secretion**

Despite numerous investigations, the exact metabolic signals that stimulate insulin release in response to increases in blood glucose concentrations still remain relatively unclear. GSIS in vivo typically follows a biphasic nature over time (Barbosa et al., 1998). Shortly after the blood glucose concentration is elevated, a rapid (2-5 minute
duration) and transient stimulation of insulin secretion is observed. This is referred to as the first-phase of insulin secretion. This initial rise in insulin secretion acts to suppress EGP and primes insulin-sensitive tissues for glucose disposal (Pratley & Weyer, 2001). The second-phase, characterised by the gradual increase in insulin secretion 10-20 minutes following glucose exposure, can be sustained until glucose is cleared from the blood. There are at least two signalling pathways that appear to be involved in the biphasic secretion of insulin in response to glucose. The first is the $K^{+}_{ATP}$ channel-dependent pathway and accounts for the release of $< 5$% insulin granules from the readily releasable pool (Rorsman et al., 2000). This was first identified when cell-attached membrane patches from isolated rat pancreatic $\beta$-cells showed that ATP-sensitive $K^{+}$ channel ($K^{+}_{ATP}$ channel) activity is responsible for glucose-induced depolarisation events in the $\beta$-cell (Ashcroft, Harrison, & Ashcroft, 1984). This pathway involves the widely accepted sequence of events for GSIS: GLUT2, the rate-limiting glycolytic enzyme GCK, cell-surface $K^{+}_{ATP}$ channels and voltage-sensitive $Ca^{2+}$ channels (Figure 1.3).
Figure 1.3: Glucose-stimulated insulin secretion in pancreatic β-cells. (A) Following the uptake of glucose via GLUT2 and its metabolism by GCK, (B) a rise in intracellular ATP:ADP ratio results in the closure of K^+_{ATP} channels, (C) depolarisation of the cell membrane and subsequent opening of voltage-dependent Ca^{2+} channels. (D) The increase in cytosolic Ca^{2+} concentration triggers the exocytotic machinery to release insulin (De Leon & Stanley, 2007).

Following oral ingestion or intravenous administration of glucose, blood glucose levels are elevated resulting in the entry of glucose into β-cells via GLUT2. Once in the β-cell, glucose is phosphorylated by GCK and continues through glycolysis within the cytosol. Following its phosphorylation, the β-cell enzymatic machinery metabolises glucose resulting in the net generation of ATP from several sources, e.g., glycolysis, mitochondrial glucose oxidation and active shuttling of reducing equivalents from the cytosol to the mitochondrial electron transport chain. This increase in the ATP:ADP ratio results in the subsequent closure of the K^+_{ATP} channel, a complex composed of four potassium inward rectifier 6.2 (Kir6.2) subunits and four sulfonylurea receptor 1 (SUR1) molecules (Aguilar-Bryan et al., 1998). The closure of the K^+_{ATP} channel complex results in plasma membrane depolarisation and the opening of voltage-
dependent L-type Ca\textsuperscript{2+} channels which facilitates extracellular Ca\textsuperscript{2+} influx into the β-cell. The consequent rise in intracellular Ca\textsuperscript{2+} triggers exocytosis of pre-formed and de novo insulin secretory granules (Hao et al., 2005; Newgard & McGarry, 1995; Rorsman & Renstrom, 2003).

The other signalling pathway that appears to be involved in GSIS is the K\textsuperscript{+}_{ATP} channel-independent pathway (Gembal, Gilon, & Henquin, 1992; Y. Sato, Aizawa, Komatsu, Okada, & Yamada, 1992). The preservation of insulin secretion without the need of depolarising events triggered by the closing of the K\textsuperscript{+}_{ATP} channel has been elegantly shown in a multitude of ways. These include studies using treatments that completely bypass the K\textsuperscript{+}_{ATP} channel, e.g., the use of a depolarising concentration of K\textsuperscript{+} (30 mmol/L) or the K\textsuperscript{+}_{ATP} channel opener, diazoxide (Gembal et al., 1992; Komatsu et al., 1997), and genetically-modified mice with complete ablation of Sur1, a constituent that makes up part of the K\textsuperscript{+}_{ATP} channel (Shiota et al., 2002). Moreover, mice with homozygous knockout of Kir6.2 have reduced GSIS, whereas heterozygous (Kir6.2\textsuperscript{+/+}) mice show enhanced glucose tolerance and increased GSIS (Remedi et al., 2006).

The second and sustained-phase of GSIS requires the regulation of K\textsuperscript{+}_{ATP} channels as an initiating event. However, the K\textsuperscript{+}_{ATP} channel-dependent pathway does not explain the amplification of insulin secretion that starts about 10-20 minutes after glucose exposure (Jensen et al., 2008). Therefore, the only logical explanation for this sustained insulin secretory response is the interplay of the K\textsuperscript{+}_{ATP} channel-independent pathway that acts in synergy with the K\textsuperscript{+}_{ATP} channel-dependent pathway, thus establishing a clear hierarchy between the two pathways whereby the independent pathway remains functionally silent as long as the dependent pathway has not depolarised the membrane and raised cytosolic Ca\textsuperscript{2+} (Aizawa, Komatsu, Asanuma, Sato, & Sharp, 1998; Taguchi, Aizawa, Sato, Ishihara, & Hashizume, 1995). With that said, the precise mechanisms involved in the K\textsuperscript{+}_{ATP} channel-independent pathway in relation to GSIS still requires further investigation.

**Non Glucose-Stimulated Insulin Secretion**

In addition to glucose, insulin secretion can be regulated by several other factors such as amino-acids, free fatty-acids (FFAs), K\textsuperscript{+} ions, incretins, e.g., glucagon-like peptide-1 (GLP-1), neurotransmitters, e.g., acetylcholine and hypoglycaemic drugs, e.g.,
sulphonylureas. Some of these agents, like sulphonylureas, are able to stimulate insulin release in the absence of physiological levels of glucose as they directly bind to and close the $K^{+}\text{ATP}$ channel, thus depolarizing the $\beta$-cell. In contrast, amino-acids, fatty-acids, incretins and neurotransmitters usually require the presence of basal glucose levels in order to stimulate insulin release. When glucose is present, these agents act to potentiate insulin secretion. Some of the key non-glucose secretagogues along with their mechanism of action are outlined below.

**Amino-acids:** Only a relatively small number of amino-acids have the capability of promoting or synergistically enhancing insulin secretion (Fajans, Floyd, Knopf, & Conn, 1967; McClenaghan, Barnett, O'Harte, & Flatt, 1996). The mechanisms by which amino-acids enhance insulin secretion are varied. Arginine, for example, is thought to induce insulin secretion by elevating the cytosolic $Ca^{2+}$ concentration as result of its electrogenic transport into the $\beta$-cell via the amino-acid transporter mCAT2A (Herczel, Lebrun, Boschero, & Malaisse, 1984; Newsholme, Brennan, Rubi, & Maechler, 2005). Alternatively, arginine can also be catalysed to glutamate via the urea cycle where it has been proposed to participate as an additive factor in the amplifying pathway of GSIS (Maechler & Wollheim, 1999). Thus, arginine-induced insulin secretion is a useful tool in dissecting the insulin secretory pathway; however, as it is found in very low physiological concentrations in the plasma, its relevance *in vitro* has often been questioned (Newsholme, Bender, Kiely, & Brennan, 2007).

Alanine and glutamine are two of the most abundant amino-acids found in the plasma and extracellular fluid, and therefore may play a more relevant role in non GSIS than arginine (Newsholme, Gaudel, & McClenaghan, 2010; Nolan & Prentki, 2008). Studies using alanine have consistently shown that it is metabolised by both primary and secondary $\beta$-cell lines (Dixon, Nolan, McClenaghan, Flatt, & Newsholme, 2003; Newsholme et al., 2010; Salvucci, Neufeld, & Newsholme, 2013) to increase insulin secretion through a variety of mechanisms, some of which include the increase in ATP production (Brennan et al., 2002; Sener & Malaisse, 2002) and membrane depolarisation due to the co-transport of $Na^{+}$ ions along with the amino-acid on entry into the $\beta$-cell (McClenaghan, Barnett, & Flatt, 1998; Salvucci et al., 2013).
Unlike arginine and alanine, leucine is a branched-chain amino-acid which cannot be manufactured in humans and must be supplied in the diet. Leucine acutely stimulates insulin secretion from pancreatic β-cells via two mechanisms. One is by regulating K\(^{\text{ATP}}\) channel activity which results in the increase of cytosolic Ca\(^{2+}\) and the triggering of insulin granules via mechanisms involving activation of some protein kinases and protein acylation (Straub & Sharp, 2007). Whilst the other involves leucine acting as an allosteric activator for glutamate dehydrogenase to enhance glutaminolysis, a process that increases the entry of glutamine carbons into the tricarboxylic (TCA) cycle (Bryla, Michalik, Nelson, & Erecinska, 1994; Fahien, MacDonald, Kmiotek, Mertz, & Fahien, 1988; Sener & Malaisse, 1980). The importance of this latter mechanism has been extensively shown by the discovery of a dominant form of congenital hyperinsulinism associated with a mutation in glutamate dehydrogenase (MacMullen et al., 2001; Stanley et al., 1998). Affected subjects have increased β-cell responsiveness to leucine and are susceptible to hypoglycaemia due to uncontrolled insulin secretion following a protein-rich meal (Hsu et al., 2001). In summary, the metabolism of key amino-acids is essential for producing the appropriate regulatory signals that contribute to insulin secretion and normal β-cell function.

**Free fatty-acids:** Studies have shown that FFAs do not initiate insulin release in the absence of glucose, but instead act to amplify GSIS upon acute exposure (Crespin, Greenough, & Steinberg, 1969; Parker, Moore, Johnson, & Poitout, 2003; Roduit et al., 2004). The mechanisms to explain FFAs effect on GSIS is incompletely understood. One suggestion is the “trident model” which encompasses TCA/malonyl-CoA metabolic signalling, glycerolipid/non-esterified fatty-acid (NEFA) cycling and the direct activation of G protein-coupled receptors (GPRs) (Nolan, Madiraju, Delgingaro-Augusto, Peyot, & Prentki, 2006). Briefly, in the presence of excess energy, e.g., NEFAs and high levels of glucose, the accumulation of lipids in the cytosol can enhance insulin secretion by altering the activity of regulatory ion channel proteins, increasing Ca\(^{2+}\) influx and facilitating the fusion of insulin secretory granules with the plasma membrane. The cycling of glycerolipid/NEFAs in β-cells can also influence insulin exocytosis by enhancing key vesicle priming and docking proteins such as mammalian unc13 and synaptosomal-associated protein 25 via lipid signalling molecules (Deeney et al., 2000; Newsholme et al., 2010; Nolan, Leahy, et al., 2006; Rorsman & Braun, 2013).
There has also been evidence of a FFA specific receptor on the β-cell membrane which triggers insulin release. The identification of the fatty-acid receptor GPR40 has enabled researchers to test a novel mechanism of action of fatty-acids on insulin secretion. Through loss of function studies using small interfering RNA (Itoh & Hinuma, 2005; Itoh et al., 2003; Schnell, Schaefer, & Schofl, 2007; Shapiro, Shachar, Sekler, Hershfinkel, & Walker, 2005), antisense oligonucleotides (Salehi et al., 2005), pharmacological inhibitors (Briscoe et al., 2006), or gene deletion in the mouse (Latour et al., 2007; Steneberg, Rubins, Bartoov-Shifman, Walker, & Edlund, 2005), fatty-acid potentiation of GSIS is inhibited. Conversely, both transgenic over-expression of Gpr40 in β-cells (Nagasumi et al., 2009) and administration of GPR40 agonists (Doshi et al., 2009; Lin et al., 2011; Tsujihata et al., 2011) enhance insulin secretion. These findings reveal a physiological role for fatty-acid potentiation of GSIS that involves, at some level, GPR40.

Potassium ions (K⁺): A high extracellular K⁺ concentration results in the influx of K⁺ ions into the β-cell. This depolarises the β-cell membrane and mediates insulin secretion through Ca²⁺ influx along L-type Ca²⁺ channels as previously discussed (Roenfeldt, Safayhi, & Ammon, 1992). One such example of a high extracellular K⁺ agent is KCl and is often used as an insulin secretagogue in isolated pancreatic islets and transformed β-cell lines.

Incretins: Incretin hormones such as GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) are peptide hormones secreted from the gut after a meal that potentiate insulin secretion. Extensive research has been conducted in this area as both hormones exert their insulinotropic effects through distinct GPRs highly expressed on both islet β-cells and non-islet cells, e.g., brain, liver, skeletal muscle, white and brown adipose tissue, stomach and intestine (Campbell & Drucker, 2013). The precise mechanisms by which GLP-1 and GIP stimulate insulin secretion at elevated levels of plasma glucose remain unclear. Both incretins interact with their respective receptors and subsequently stimulate protein kinase A-dependent cAMP, a process which phosphorylates proteins associated with Ca²⁺-induced exocytosis (Ding, Renstrom, Rorsman, Buschard, & Gromada, 1997; Holz, 2004; Wheeler et al., 1995).
Proteins Involved in Glucose-Sensing

GLUT2 glucose transporter
As previously described, GLUT2 is important in the β-cell’s uptake of glucose from the extracellular space. Reduced protein and mRNA levels of GLUT2 have been observed in a number of hyperglycaemic animal models (Milburn, Ohneda, Johnson, & Unger, 1993; Thorens, Weir, Leahy, Lodish, & Bonner-Weir, 1990; Thorens, Wu, Leahy, & Weir, 1992); however, whether this under-expression is the cause or consequence of β-cell dysfunction in these animals remains unresolved. Early studies of normoglycaemic transgenic mice expressing reduced β-cell GLUT2 levels revealed no defect in GSIS (Tal et al., 1992). In contrast, generation of GLUT2 null mice led to the loss of first-phase insulin secretion, with the second-phase being dependent on glucose metabolism (Guillam, Dupraz, & Thorens, 2000; Guillam et al., 1997). These conflicting studies have created much debate surrounding the importance of GLUT2 in insulin secretion.

Glucokinase
The conversion of glucose into glucose-6-phosphate is the first step in glucose metabolism. This reaction is catalysed by the enzyme, GCK, which is often termed the “glucose-sensor” of the β-cell (Liang et al., 1994). As a rate-limiting enzyme in GSIS, defective GCK expression has been shown to cause diabetes in both humans and rodent models. In humans, heterozygous point mutations have been linked to at least three syndromes inherited in an autosomal dominant manner: persistent hyperinsulinaemic hypoglycaemia, neonatal diabetes and maturity-onset diabetes of the young (Byrne et al., 1994; Froguel et al., 1993; Gidh-Jain et al., 1993; Glaser et al., 1998; Stoffel et al., 1993). Some of the phenotypes which characterise these three syndromes have also been observed in heterozygous null mutant mice (Bali et al., 1995; Grupe et al., 1995). They include reduced GSIS and excessive glucose production by the liver under hyperglycaemic and hyperinsulinaemic conditions. These data clearly indicate that an impairment in GCK in the β-cell decreases glycolytic flux and can result in defective insulin secretory function and diabetes.

ATP-sensitive K⁺ channel
While other ATPase transporters couple ATP hydrolysis for the movement of molecules against a concentration gradient, the K⁺ATP channel does not require ATP hydrolysis to
power K\(^+\) ion movement. Instead, SUR1 controls the flux of K\(^+\) ions through the Kir6.2 pore which is driven by the difference between K\(^+\) gradient potential and the membrane potential (Aguilar-Bryan, Bryan, & Nakazaki, 2001). The K\(^+\)\(_{\text{ATP}}\) channel plays a significant role in connecting glucose metabolism to the exocytosis of insulin (Ashcroft, 2005). Studies in mice utilising complete deletions of SUR1 have shown defects in both first- and second-phase GSIS (Nakazaki et al., 2002; Seghers, Nakazaki, DeMayo, Aguilar-Bryan, & Bryan, 2000; Shiota et al., 2002), whereas the specific deletion of Kir6.2 in the pancreatic β-cell leads to persistent hyperinsulinism in neonates followed by markedly reduced GSIS when they reach adulthood (Miki et al., 1997). The exact reason for the difference in GSIS between SUR1 and Kir6.2 knockout mice is not currently known. Further to these knockout models, our laboratory has recently shown the importance of the K\(^+\)\(_{\text{ATP}}\) channel in GSIS by using the New Zealand obese (NZO) mouse which is characterised by defective GSIS when tested using islets \textit{ex vivo} (Larkins & Martin, 1972; Veroni, Proietto, & Larkins, 1991). By generating transgenic mice expressing the C57BL/6J \textit{Sur1}/Kir6.2 genes on the NZO background, we were able to show significant improvements in insulin secretion in response to either glucose or the sulphonylurea, tolbutamide (Andrikopoulos et al., 2016).

**Insulin Action**

Insulin is an anabolic hormone and is considered pivotal in regulating cellular energy supply in the fed state. Upon release from pancreatic β-cells, insulin binds to its plasma membrane receptor where a number of signalling cascade events take place to stimulate glucose, protein and lipid metabolism, as well RNA and DNA synthesis, by modifying the activity of enzymes and transport processes (C. R. Kahn & White, 1988; Rosen, 1987). Some examples of insulin’s metabolic actions include increasing the rate of glucose transport into skeletal muscle and adipose tissue by stimulating the translocation of internal GLUT4 to the plasma membrane, decreasing gluconeogenesis in the liver and kidney by inhibiting key enzyme activities, and decreasing lipolysis in adipose tissue thereby reducing the efflux of FFAs into the bloodstream. The net effect of these actions, amongst others not listed, is to increase glucose uptake and storage, and to reduce circulating glucose levels. The insulin receptor and subsequent signalling events that take place are described in more detail under their appropriate headings below.
Insulin Receptor
The actions of insulin at the cellular level are mediated by insulin binding to its plasma membrane receptor (Cuatrecasas, 1972; Freychet, Roth, & Neville, 1971). The insulin receptor is present on virtually all mammalian tissues, although the concentration varies from as few as 40 receptors on erythrocytes to more than 200,000 receptors in insulin-responsive tissues like liver, skeletal muscle and adipose tissue (C. R. Kahn & White, 1988). The complete insulin receptor is a heterotetramer of α- and β-subunits linked by disulphide bonds to give a β-α-α-β structure (Kasuga, Hedo, Yamada, & Kahn, 1982; Massague, Pilch, & Czech, 1980) and was first characterised in rat liver membranes by Freychet and colleagues in 1971 (Freychet et al., 1971). Affinity labelling using radioactively labelled insulin and bifunctional cross-linking agents have shown that the α-subunit is entirely extracellular and houses the insulin binding site (Kasuga, Hedo, et al., 1982; Massague et al., 1980) and the β-subunits are transmembrane proteins. Knowledge of this transmembrane protein and its involvement in intracellular signalling was first highlighted through the use of labelled $[^{32}\text{P}]$orthophosphate with the finding that the β-subunit of the receptor was an insulin-stimulated protein kinase capable of phosphorylating itself and other substrates on tyrosine residues (Kasuga, Zick, Blithe, Crettaz, & Kahn, 1982). This in turn would initiate insulin’s biological effects through intracellular signalling events (Hedo & Simpson, 1984).

Insulin Signalling
Insulin binds to the receptor’s extracellular α-subunit, resulting in a conformational change which enables ATP to bind to the intracellular component of the β-subunit (Patti & Kahn, 1998). ATP binding in turn triggers phosphorylation of multiple substrates at tyrosine residues including four insulin receptor substrates IRS-1, IRS-2, IRS-3 and IRS-4 (White, 1998), and others such as p60dok, Gab-1, Cbl and Shc (Pessin & Saltiel, 2000). Transgenic rodent models suggest that IRS-1 and IRS-2 are the main regulators of insulin’s effects that then mediate signalling events through either the phosphatidylinositol 3-kinase (PI3K) or mitogen-activated protein kinase (MAPK) pathways (Saltiel & Kahn, 2001; White, 1998). The degree of pathway activation entirely depends on extracellular and intracellular cues, e.g., the strength of the stimulus and the state of positive feed-forward and negative feed-back loops (Mendoza, Er, & Blenis, 2011).
**Phosphatidylinositol 3-kinase pathway:** The activation of the PI3K pathway is important for many of insulin’s actions, e.g., glucose uptake (Okada, Kawano, Sakakibara, Hazeki, & Ui, 1994), glycogen (Shepherd, Nave, & Siddle, 1995), lipid (Okada et al., 1994) and protein synthesis (Weng et al., 1995). The enzyme PI3K exists as a heterodimer, consisting of a catalytic 110 kDa subunit and a regulatory subunit for which there are several isoforms. The most highly expressed regulatory subunit is p85α. Once PI3K is activated through the binding of p85 to phosphotyrosine residues, PI3K catalyses the phosphorylation of phosphoinositides at the D3 position of the inositol ring to produce PI3P or PIP, PI(3,4)P2 or PIP2 and PI(3,4,5)P3 or PIP3. The generated phosphoinositides act as intracellular messengers to activate PI-dependent kinases 1 and 2, and some atypical forms of protein kinases (Kido, Nakae, & Accili, 2001; Ruderman, Kapeller, White, & Cantley, 1990).

One of the major targets of PI3K is the downstream effector protein kinase B, also known as Akt (Bellacosa, Testa, Staal, & Tsichlis, 1991). The Akt kinases are activated by the phosphorylation at serine and threonine residues (Bellacosa et al., 1998; Kohn, Takeuchi, & Roth, 1996). Akt exists as three different isoforms, with Akt2 being enriched in insulin-responsive tissues and is the most critical for the sequestration of GLUT4 to the plasma membrane for insulin-stimulated glucose uptake. Akt also has the ability to phosphorylate other target sites that regulate glycogen synthase kinase (glycogen synthesis) (Cross, Alessi, Cohen, Andjelkovich, & Hemmings, 1995), mammalian target of rapamycin (protein synthesis) (Scott, Brunn, Kohn, Roth, & Lawrence, 1998) and transcription factor FOXO1 (inhibits gluconeogenesis) (Nakae, Kitamura, Ogawa, Kasuga, & Accili, 2001).

**Mitogen-activated protein kinase pathway:** Aside from the PI3K pathway, insulin signalling can also activate the MAPK pathway which is important for insulin’s mitogenic effects including cell growth, proliferation, differentiation and death. The activation of the MAPK pathway by insulin involves the tyrosine phosphorylation of IRS proteins, Shc and phosphotyrosine-binding domains, e.g., Grb2. The most well characterised route of Ras activation is via the recruitment of son of sevenless from the cytosol to the plasma membrane where it facilitates the exchange of GDP bound to Ras by GTP that is required for a positive regulation of Ras activity. Upon activation, Ras is
able to stimulate, in a step-wise manner, Raf, MAP kinase-kinase and the MAPK’s extracellular signal-regulated kinase 1/2. Regulation of these target effectors, especially Ras and Raf, is crucial for the proper maintenance of cellular proliferation and differentiation, as activating mutations can lead to the formation of cancer (Karnoub & Weinberg, 2008).

Pathophysiology of Type 2 Diabetes Mellitus
T2D mellitus is a complex disease that results from a combination of dietary and lifestyle factors in addition to genetic predispositions that compromise insulin action on tissues and insulin secretion in the β-cell. The hallmark features that contribute to hyperglycaemia in T2D are as follows:

1. **Hepatic and renal insulin resistance** – increased glucose production by the liver and kidney.
2. **Peripheral insulin resistance** – reduced glucose uptake and metabolism by skeletal muscle and adipose tissue.
3. **β-cell dysfunction** – impaired GSIS caused by a defect/s in the pancreatic β-cells.

While there is substantial evidence to suggest that the above abnormalities are present in most individuals before the onset of T2D, the sequence in which they develop and their relative contributions to the progression from NGT to IGT and T2D remain unclear.

Obesity
Obesity is commonly defined as an imbalance between the amount of energy consumed and the amount of energy expended, leading to increases in energy storage. The rising prevalence of obesity in a number of countries is reaching pandemic proportions. In Australia alone, the current incidence of overweight and obesity affects approximately 60 % of males and 56 % of women aged 20 years and over (Ng et al., 2014). Future predictions drawn from various cross-sectional national and state population surveys put this number at 83 % in males and 75 % in women by the year 2025 (Haby, Markwick, Peeters, Shaw, & Vos, 2012). This is of particular concern when one considers that obesity is implicated in a number of health complications associated with increased
morbidity and mortality, such as T2D, hypertension, coronary heart disease and even certain cancers (Kopelman, 2000). Since obesity is strongly linked to T2D, the term “diabesity” was given by Sims and colleagues back in the 1970’s to highlight the two conditions (Sims et al., 1973). The authors found that over-feeding young men with no family history of T2D for 6 months led to obesity, elevations in fasting glucose, insulin and triglyceride concentrations, and glucose intolerance (Sims et al., 1973). Some of the mechanisms that are driven by obesity to induce hyperglycaemia are outlined in the following sub-headings.

**Insulin Resistance**

Insulin resistance is defined as a condition in which a normal concentration of insulin elicits a suboptimal physiological response, particularly on the main target organs: liver, skeletal muscle and adipose tissue. The overload of fat storage from dietary excess has been shown to cause insulin resistance through multiple mechanisms, some of which include excess supply of NEFAs due to expansion of fat mass (Kraegen, Cooney, Ye, Thompson, & Furler, 2001; Leung et al., 2004), increased intracellular accumulation of triglycerides and fatty-acid derived metabolites, e.g., diacylglycerol and fatty acyl-CoA in insulin-sensitive tissues (Schmitz-Peiffer et al., 1997), and excessive production of pro-inflammatory cytokines (Hotamisligil, 2000). Chronic hyperglycaemia alone may also play an important role in the development of insulin resistance, particularly in the context of T1D (Vuorinen-Markkola, Koivisto, & Yki-Jarvinen, 1992; Yki-Jarvinen, Helve, & Koivisto, 1987). However, the exact mechanism has not been completely understood since there is contradicting evidence in the literature. For example, normalising blood glucose in rats with T1D by preventing glucose reabsorption with phlorizin reverses insulin resistance (B. B. Kahn, Shulman, DeFronzo, Cushman, & Rossetti, 1991; Rossetti, Smith, Shulman, Papachristou, & DeFronzo, 1987), whereas another study found that insulin is a stronger inducer of insulin resistance than hyperglycaemia in mice with T1D (H. Y. Liu et al., 2009). In any case, decreased insulin action results in the impaired suppression of glucose production from the liver and kidney, and a reduction in glucose utilisation by skeletal muscle and adipose tissue.
Hepatic Insulin Resistance

Hepatic insulin resistance is characterised by the incomplete suppression of HGP by insulin, leading to fasting and post-prandial hyperglycaemia. Although a strong correlation exists between obesity and insulin resistance as a whole, it appears that the distribution of body fat itself is a more critical determinant. Central obesity, i.e., fat centralised in the abdominal and chest regions, has been shown to cause hepatic insulin resistance (Fujimoto, Abbate, Kahn, Hokanson, & Brunzell, 1994). This is most likely due to the close proximity of intra-abdominal adipose to the liver where excess lipids and by-products of their metabolism, lipid intermediates, can accumulate. Transgenic mice with liver-specific over-expression of lipoprotein lipase (J. K. Kim et al., 2001) and patients with lipodystrophies (Reitman, Arioglu, Gavrilova, & Taylor, 2000) both exemplify this accumulation of hepatic lipid content and progress to develop severe hepatic insulin resistance without the presence of excess visceral fat. This was further demonstrated by Fabbrini and colleagues who tested whether a cause-and-effect relationship existed between excess visceral adipose tissue and hepatic insulin resistance (Fabbrini et al., 2010). The authors found that hepatic insulin resistance followed intrahepatic lipid accumulation and that this was not influenced by visceral fat mass since omentectomy in obese patients did not resolve hepatic insulin resistance (Fabbrini et al., 2010). Taken together, these data convincingly show that hepatic insulin resistance is mediated by the diversion of lipid products directly into the liver where they can act to inhibit enzymes pertinent to glycolysis and blunt insulin signalling from its receptor by inducing serine phosphorylation of IRS-1 and IRS-2, thus weakening the activation of PI3K and its downstream effectors.

Peripheral Insulin Resistance

The disposal of glucose into skeletal muscle and adipose tissue is facilitated by the action of specific glucose transporters, namely GLUT4. The most common defect when peripheral insulin resistance is established is impaired insulin-stimulated glucose transport. In adipose tissue, GLUT4 mRNA and protein levels are down-regulated (Garvey et al., 1991). By contrast, skeletal muscle GLUT4 transcription and translation are unaffected (Garvey, Maianu, Hancock, Golichowski, & Baron, 1992; Pedersen et al., 1990), suggesting that the defect lies in the translocation or intrinsic activity of the glucose transporter itself. Impaired glucose transport is also closely linked with
reductions in glucose phosphorylation, glucose oxidation and glycogen synthesis, with all of these contributing to obesity-induced hyperglycaemia (Cusi et al., 2000; Karlsson & Zierath, 2007). Although the exact mechanism behind the development of the aforementioned defects are not yet fully understood, an inverse relationship between muscle insulin sensitivity and intramuscular triglyceride content using muscle biopsy studies exists (Kelley & Goodpaster, 2001). Additional evidence supporting the hypothesis that skeletal muscle insulin resistance occurs due to an increase in intramyocellular (inside the muscle fibre) fat content include enhancing skeletal muscle sensitivity by reducing intramuscular lipid content with the hypolipidaemic agent acipimox (Bajaj et al., 2005); and in mice, muscle-specific over-expression of lipoprotein lipase leads to the accumulation of lipid by-products such as fatty-acid CoA in muscle and severe muscle insulin resistance (J. K. Kim et al., 2001). Conversely, muscle-specific knockout mice are protected against high-fat diet induced skeletal muscle insulin resistance (H. Wang et al., 2009). These findings indicate that increases in intramyocellular lipids and other lipid metabolites may play a direct role in the pathogenesis of skeletal muscle insulin resistance.

**β-cell Dysfunction**

Insulin resistance and impaired insulin secretion are the two main mechanisms that contribute to hyperglycaemia in T2D. The relative contribution of both in the pathogenesis of T2D has been a major talking point for many decades, with growing evidence suggesting that hyperglycaemia does not occur without β-cell dysfunction. This concept is highlighted in a review article by Kahn in which data compiled from studies looking at first-degree relatives of patients with T2D, women with a history of gestational diabetes or polycystic ovarian disease, older subjects and subjects with IGT. Interestingly, although all subjects have impaired insulin sensitivity, they maintain normoglycaemia due to compensated insulin release. By contrast, patients with T2D showed impairments in measures for both insulin sensitivity and insulin response to glucose. The relationship between insulin sensitivity and first-phase insulin response for these groups are shown in Figure 1.4 and are hyperbolic in nature; that is, as insulin sensitivity falls insulin secretion must increase substantially in order to maintain normoglycaemia (S. E. Kahn, 2003). Therefore, it appears that in some insulin resistant individuals, the pancreatic β-cells are able to maintain normal plasma glucose by
increasing insulin production and secretion due to β-cell compensation. The exact mechanism/s in which β-cell compensation occurs is not yet fully understood; however, autopsy studies in humans (Butler et al., 2003) and rodents (Guillam et al., 1997) suggest that it is most likely due to an increase in β-cell mass. The presence of β-cell dysfunction is therefore central to the progression of T2D, with reduced β-cell mass, glucotoxicity and lipotoxicity all being implicated in this process.

Figure 1.4: Percentile lines for the relationship between insulin sensitivity and first-phase insulin in response to glucose (AIRglucose) based on data from 93 normal subjects. Mean data from six other studies are plotted. Fourteen subjects with a first-degree relative with T2D, eight women with gestational diabetes, eleven women with polycystic ovarian disease, thirteen healthy older subjects, twenty-one subjects with IGT and ten subjects with T2D. Note that as long as individuals are able to compensate for insulin resistance through an adaptive increase in insulin secretion, normoglycaemia will be maintained and T2D averted (S. E. Kahn, 2003).

β-cell Mass
During development in utero, β-cell mass is dramatically increased with an approximate doubling of the β-cell population each day starting from the sixteenth day post-conception in rats due to β-cell replication and islet neogenesis (McEvoy & Madson, 1980). With increasing age, β-cell mass is tightly regulated through a balance of β-cell
proliferation and apoptosis. Clinical observations in conjunction with work from type 2 diabetic animal models have shown that there are several changes that occur to β-cell mass during the progression from NGT to IGT and T2D. The first stage is the compensation stage, where insulin secretion increases in order to maintain glycaemia in the face of insulin resistance brought on by obesity and/or genetic factors. Much of this increase in insulin secretion occurs as a consequence of increased β-cell mass and is most likely attributed to by increased β-cell number and hypertrophy (Jetton et al., 2005; Steil et al., 2001). It has been postulated that during the early stages of insulin resistance, a signal is generated to stimulate genes responsible for maintaining β-cell growth, thereby prolonging the pre-diabetic period (Bleich, Jackson, Soeldner, & Eisenbarth, 1990).

The second stage occurs when the β-cells fail to secrete enough insulin to sustain blood glucose. At this point, genes associated with GSIS such Glut2 and Gck, are down-regulated and a decline in β-cell mass all contribute to the overall loss of first-phase insulin secretion, whilst the more sustained second-phase is preserved (Weir, Laybutt, Kaneto, Bonner-Weir, & Sharma, 2001). Insulin content is also preserved, suggesting that mechanisms important to β-cell function and not insulin synthesis are affected. The loss of first-phase GSIS is also accompanied by a loss of the β-cell phenotype, where highly expressed genes and proteins are down-regulated, and those that are normally suppressed are up-regulated (Weir & Bonner-Weir, 2004). A number of these up-regulated genes are associated with antioxidant and anti-apoptotic pathways, and are believed to aid in β-cell survival during hyperglycaemic conditions (Laybutt et al., 2002).

In T2D, the secretory capacity of the β-cell cannot meet the increasing demand caused by insulin resistance, and so β-cells decompensate with the loss of first-phase GSIS and defective second-phase GSIS (Hosker, Rudenski, Burnett, Matthews, & Turner, 1989). There remains, however, some level of β-cell function and mass which can be maintained for a period of time until the structural damage of the β-cell and the consequent marked decrease of β-cell mass occurs. This is considered the last stage of β-cell dysfunction and can be identified, at least in humans, by functional changes in the β-cell in the form of amyloid plaques (S. E. Kahn et al., 2000), lipid (Prentki & Corkey,
1996) and glycogen accumulation (Hellman & Idahl, 1969). Post-mortem studies on pancreatic tissues taken from people with T2D indicate at least a 50% decrease in β-cell mass and an increase in the rate of apoptosis when compared to healthy control subjects (Maclean & Ogilvie, 1955). These alterations could be due to the deleterious effects of glucotoxicity and lipotoxicity.

**Glucotoxicity**

The β-cell is extremely sensitive to the slightest change in blood glucose concentration. When these changes are of short duration and lie within the physiological range, such as a post-prandial event, insulin secretion occurs. However, when these changes are of longer duration and more pronounced in magnitude, such as the case with hyperglycaemia, long-term irreversible deterioration of β-cell function and death can occur. The pathogenesis of T2D has been reported to be associated with oxidative stress induced by reactive oxygen species (ROS) and a variety of pro-apoptotic pathways such as endoplasmic reticulum (ER) stress. Unger and Grundy were the first to introduce the concept of “glucotoxicity”, a toxic state that negatively influences β-cell function due to excessive glucose (Unger & Grundy, 1985). In their article, both authors put forward the idea that continuous over-stimulation of the β-cell by glucose would result in detrimental effects to insulin synthesis and secretion, leading to long-term irreversible deterioration of β-cell function and the occurrence of β-cell death (Unger & Grundy, 1985).

Given the challenges of studying human β-cells *in vivo*, much of our current understanding regarding the chain of events that underscore β-cell dysfunction due to glucotoxicity has been obtained from experiments using transformed β-cell lines, isolated pancreatic islets and animal models. Since glucose is the major physiological regulator of insulin gene transcription, it is not surprising that during the initial stages of glucotoxicity, the transcription of insulin and its subsequent secretion becomes defective. This was originally shown in the hamster insulinoma cell line HIT-T15, where cells were exposed to either low (0.8 mmol/L) or high (11.1 mmol/L) glucose concentrations after weekly splitting for 40 weeks (Robertson, Zhang, Pyzdrowski, & Walseth, 1992). Prolonged culturing of cells in low glucose maintained insulin gene expression, insulin content and GSIS, whereas bathing cells in high glucose drastically
reduced these parameters. These reductions were found to be associated with the reduction of mRNA and protein levels of pancreas duodenum homeobox-1 (PDX-1) and binding activity of V-maf musculoaponeurotic fibrosarcoma oncogene homologue A (MafA), both critical regulators of insulin promoter activity (Robertson et al., 1992). Similar observations with respect to insulin dysregulation and secretion have also been made in other commonly used transformed β-cell lines such as the INS-1 (Gohring et al., 2014; Pino et al., 2005), isolated islets from diabetic rodent models (Briaud, Rouault, Reach, & Poitout, 1999; Montana, Bonner-Weir, & Weir, 1993) and type 2 diabetic patient donors (Lupi et al., 2007). In addition, high glucose levels have also been shown to reduce the expression of several key genes involved in both glucose-sensing, e.g., *Glut2*, *Gck* (W. H. Kim et al., 2005; Ohneda, Inman, & Unger, 1995; Thorens et al., 1992), and the constituents that make up the K⁺_ATP channel, *Sur1* and *Kir6.2* (Moritz, Leech, Ferrer, & Habener, 2001), and in the exocytosis of insulin, e.g., SNARE complexes (Ostenson, Gaisano, Sheu, Tibell, & Bartfai, 2006). However, studies performed in islets from patients with T2D have only confirmed some of these changes. Interestingly, most of these alterations can be reversed through the use of the glucose-lowering agents’ phlorizin (Jonas et al., 1999) or troglitazone (Harmon et al., 1999; Higa et al., 1999), or by changing the media from high glucose to low glucose; however, the efficacy of these interventions are entirely time-dependent (Gleason, Gonzalez, Harmon, & Robertson, 2000).

As previously mentioned in this literature review, glucose enters the β-cell and is metabolised to generate electron donors for transfer to the mitochondria so that energy in the form of ATP can be produced (De Leon & Stanley, 2007). If more glucose is being oxidised, as in the case with glucotoxicity, more electron donors are generated and the mitochondrial capacity exceeded. These electron donors accumulate beyond the levels that the glycolytic enzymes can handle and are therefore shunted to other pathways that may form ROS. The pancreatic β-cell is highly susceptible to oxidative damage due to its intrinsically low levels of antioxidant enzyme expression and activity, namely superoxide dismutase 1 and 2, glutathione peroxidase 1 and catalase (Grankvist, Marklund, & Taljedal, 1981; Tiedge, Lortz, Drinkgern, & Lenzen, 1997; N. Welsh et al., 1995). A relationship between oxidative stress and glucotoxicity has been established in transformed β-cell lines (Tanaka, Gleason, Tran, Harmon, & Robertson,
Chapter 1  Literature Review

1999; Tanaka, Tran, Harmon, & Robertson, 2002), isolated islets (Kaneto et al., 2002; Tran et al., 2004) and diabetic animal models (Ihara et al., 1999; Tanaka et al., 1999). For example, administering compounds with antioxidant properties or over-expressing antioxidant enzyme genes in β-cells provide protection against the deleterious effects of ROS toxicity induced by high glucose levels on insulin gene expression, content, secretion and β-cell survival (Tanaka et al., 1999; Tanaka et al., 2002). These findings have been extrapolated to humans, where clinical studies have indicated that type 2 diabetic patients are also exposed to chronic oxidative stress and show increases in prooxidants and markers of oxidative stress (Rehman et al., 1999; Sakuraba et al., 2002).

The ER is where proteins are folded into their native conformation and post-translationally modified. Proteins that are folded correctly proceed to the Golgi apparatus so that they can be modified and packaged for use within the cell, whereas incorrectly folded proteins remain in the ER for either re-folding or degradation. Disruption of any of these processes can lead to ER stress and the activation of the unfolded protein response (J. Wu & Kaufman, 2006). Recent evidence suggests that oxidative stress and ER stress are closely linked in that ROS can increase the accumulation of misfolded proteins in the ER, thus amplifying ROS production which in turn further increases ER stress (Tu & Weissman, 2004). This vicious cycle disrupts insulin production and secretion, and eventually leads to β-cell death.

Finally, it is important to highlight a couple of sources of confusion in some of the findings gained from in vitro and in vivo models of β-cell glucotoxicity thus far. Firstly, the differences between in vitro and in vivo systems arise mainly from the variability of glucose concentrations that elicit glucotoxic effects on β-cell function; 10 mmol/L of glucose is optimal for rodent β-cell function and survival in vitro, but is considered harmful in vivo. On the other hand, supraphysiological concentrations of glucose in the range of 30-40 mmol/L induce glucotoxicity in vitro and are highly unlikely to occur in patients with T2D. These differences may result in part from the absence of key modulators such as amino acids, incretin hormones and neurotransmitters, which all play an important role in regulating GSIS. Another contentious issue that needs to be addressed is that glucose can affect human and rodent
islets differently. Over-stimulation with chronic glucose induces apoptosis of human pancreatic β-cells \textit{in vitro}, whereas chronic hyperglycaemia studies in rat islets have shown a decrease in apoptosis (Efanova et al., 1998) and an increase in β-cell formation (Lipsett & Finegood, 2002). Therefore, it is difficult to conclude with certainty whether β-cell dysfunction is brought on solely by glucotoxicity or a combination of factors.

\textbf{Lipotoxicity}

Prolonged exposure to elevated circulating NEFAs has been shown to have negative effects on β-cell function. This process is referred to as “lipotoxicity” and can lead to the toxic accumulation of intermediates of lipid metabolism that can directly and indirectly affect the β-cell (Unger, 1995). However, it is important to note that not all fatty-acids are equally detrimental, with evidence suggesting that both long-chain saturated and unsaturated (mono- and most poly-unsaturated) molecules are handled differently by the β-cell. The sustained incubation with long-chained saturated NEFAs can induce apoptotic events (Busch et al., 2005; Welters et al., 2006). In stark contrast, incubating transformed β-cells with unsaturated NEFAs for extended periods of time does not affect β-cell viability (Maedler, Oberholzer, Bucher, Spinas, & Donath, 2003). Interestingly, co-incubation with equimolar concentrations of a saturated and an unsaturated NEFA dramatically reduces any adverse events on the β-cell (Welters, Tadayyon, Scarpello, Smith, & Morgan, 2004). Another important factor to take into consideration is the chain length of saturated fatty-acids. Studies in the perfused rat pancreas have indicated that the chain length of fatty-acids used can greatly influence the amount of insulin that is secreted irrespective of nutrient status (Stein et al., 1997). This has also been observed in isolated perfused rodent islets where the amount of insulin secreted with increasing chain length of fatty-acids follows a bell-curve distribution (Opara, Garfinkel, Hubbard, Burch, & Akwari, 1994). Additional experiments using flow cytometry analysis as a tool to rank the toxicity of fatty-acids in two human leukemic cell lines have also shown that saturated molecules with a chain length in excess of C16 such as palmitate (16 carbons) or stearate (18 carbons) possess more cytotoxic potential than do shorter chain lengths such as laurate (12 carbons) (Lima, Kanunfre, Pompeia, Verlengia, & Curi, 2002). The precise mechanism/s by which the chain length of fatty-acids affect insulin secretion remain relatively unknown since the majority of investigators use either palmitate and/or oleate as they represent
the major species present in human serum and are therefore the most likely molecules to which β-cells are exposed to.

It is not known how lipotoxicity contributes to the decline in β-cell function and the pathogenesis of T2D. There is evidence to suggest that exposing the β-cell to NEFAs result in the inhibition of glucose metabolism. These findings were associated with the inhibition of pyruvate dehydrogenase; an important enzyme that links the TCA cycle with glycolysis, and phosphofructokinase; the key enzyme in the control of glycolysis (Y. Q. Liu, Tornheim, & Leahy, 1999). In addition to these findings, studies investigating gene expression levels in transformed β-cell lines and human islets incubated in palmitate and/or oleate have documented increments in genes pertinent to gluconeogenesis and fat oxidation, and decrements in genes associated with glucose transport, glycolysis and insulin production (Bikopoulos et al., 2008; Busch, Cordery, Denyer, & Biden, 2002; Gremlich, Bonny, Waeber, & Thorens, 1997; Xiao et al., 2001).

Aside from NEFAs directly affecting β-cell glucose metabolism and hence, altering β-cell function, there are a number of other candidates that have been flagged as potential mediators of lipotoxicity within the β-cell. One of these candidates is nitric oxide (NO), which has been implicated to play a role in the death of β-cells exposed to pro-inflammatory cytokines in T1D (H. E. Thomas, Darwiche, Corbett, & Kay, 2002). NO has been reported in high concentrations following the incubation of β-cells with fatty-acids in addition to elevated mRNA levels of the inducible form of NO synthase (iNOS) in some experiments (Y. Lee et al., 1997; Shimabukuro, Ohneda, Lee, & Unger, 1997). By preventing the upregulation of iNOS with specific inhibitors, e.g., nicotinamide or aminoguanidine, β-cell function was improved (Shimabukuro, Zhou, Levi, & Unger, 1998). The other candidate, sphingolipid ceramide, is generated from the use of palmitate and has been reported to lead to apoptosis, possibly via alterations in the disposition of plasma membrane survival (Y. Zhang et al., 2009) and death receptor signalling complexes (Gulbins, 2003). However, this finding has been challenged when a series of experiments in Chinese hamster ovary cells with palmitate together with a range of biochemical and genetic inhibitors of de novo ceramide synthesis showed that palmitate-induced apoptosis still occurred even though de novo
ceramide synthesis was completely prevented (Listenberger, Ory, & Schaffer, 2001). Of particular interest is that these experiments revealed that palmitate-induced apoptosis generated ROS production and that this was inhibited with antioxidants, suggesting a role of ROS in palmitate-induced apoptosis.

**Glucagon Dysregulation**

The presence of T2D is not only confined to the dysregulation of insulin action and secretion, but also to other hormones such as glucagon. More than 40 years ago, Unger and Orci proposed the bi-hormonal hypothesis to explain the pathogenesis of T2D (Unger & Orci, 1975). According to their hypothesis, T2D is the result of insulin deficiency/resistance along with an absolute excess of glucagon, which, as explained earlier, can raise glucose levels by stimulating HGP and reducing glucose utilisation. In human diabetes, the rate of HGP has been correlated with hyperglycaemia and the maintenance of this abnormality is associated with hyperglucagonaemia (Baron, Schaeffer, Shragg, & Kolterman, 1987; Del Prato, Castellino, Simonson, & DeFronzo, 1987). In addition to the ineffectiveness of insulin to act as a negative feedback regulator, e.g., switching off HGP, the secretory response of α-cells to low glucose concentrations is greatly impaired, increasing the risk of severe hypoglycaemic events. It is not currently clear how these impairments in α-cell regulation in the type 2 diabetic state occur as the current literature is somewhat confusing. For example, several groups have described abnormally elevated fasting glucagon levels in persons with IGT (Ahren & Larsson, 2001; Larsson & Ahren, 2000; Mitrakou et al., 1992), suggestive of early α- and β-cell dysfunction, whilst others have reported no changes in glucagon secretion in adolescents with T2D (Elder, Prigeon, Wadwa, Dolan, & D’Alessio, 2006; Tfayli, Bacha, Gungor, & Arslanian, 2010), raising the possibility that α-cell function may only decline with age. Some popular theories with respect to α-cell dysregulation include defective glucose-sensing, loss of β-cell function and hence, the potential decrease in paracrine regulation, insulin resistance and impaired neuronal regulation (Quesada, Tuduri, Ripoll, & Nadal, 2008).

**Therapeutic Options for Type 2 Diabetes Mellitus**

The overarching goal for the treatment of T2D is to firstly relieve the patients’ symptoms and then to prevent acute and chronic complications that are associated with
the disease from occurring such as neuropathy, nephropathy and cardiovascular disease. At the pre-diabetes stage, lifestyle measures such as diet, exercise and weight control are implemented. If glucose control is not achieved, patients then move onto drug treatments that vary in their mechanism of action, target tissue and time of administration. When thought of simply, these agents can be categorised in one of three ways to improving glycaemic control:

1. **Insulin secretagogues** – increasing insulin secretion.
2. **Insulin sensitisers** – increasing insulin action.
3. **Inhibitors of glucose absorption** – decreasing the need for insulin.

**Dietary Intervention**

Obesity is considered to be a major risk factor for T2D with its increased prevalence being paralleled to the concomitant increase in T2D. Obesity and the lack of physical activity can cause insulin resistance which places unnecessary stress on the β-cell in order to increase insulin production and secretion. Weight loss through dietary and exercise interventions increase insulin sensitivity and therefore reduce the workload on the β-cell. The United States Diabetes Prevention Program and The Finnish Diabetes Prevention Study are two of many randomised clinical trials which have interrogated whether lifestyle intervention can prevent or delay the onset of T2D in individuals with IGT. Both programs showed that achieving a respectable percentage of weight loss through diet and exercise regimens reduced the incidence of T2D by approximately 58% in patients with IGT (Knowler et al., 2002; Tuomilehto et al., 2001). The effect of lifestyle intervention on reducing the incidence of T2D was related to either preserved or improved β-cell function through enhanced insulin sensitivity, such that the hyperbolic relationship describing insulin sensitivity and secretion shown in Figure 1.4 was shifted to the right (de Mello et al., 2012; Kitabchi et al., 2005).

The use of dietary intervention has also been used with great effect in rodent models of IGT and T2D. For example, the C57BL/Ks-db/db mouse is characterised by significant alterations at the genetic and structural level of the β-cell which significantly impairs GSIS at 12 weeks of age. Long-term (< 3 months) calorie-restriction in these mice has been shown to significantly improve β-cell function through the restoration of key genes involved in GSIS and β-cell maintenance, e.g., *Glut2*, *Pdx-1* and *MafA*
(Sheng et al., 2016). Another study using the same duration of calorie-restriction but in a different model of diabetes, the Zucker diabetic fatty (ZDF) rat, have also shown similar findings in that reducing food intake can substantially prevent the loss of GSIS by maintaining β-cell Glut2 and β-cell mass respectively (Ohneda et al., 1995). Although both of these studies did not measure insulin sensitivity, there is enough evidence to suggest that like the clinical trials mentioned previously, the improvement in β-cell function is likely to be due to enhanced insulin sensitivity (Gazdag, Dumke, Kahn, & Cartee, 1999; S. Y. Park et al., 2005; Sharma et al., 2011; Sharma, Arias, Sequea, & Cartee, 2012).

Despite the positive effects that weight loss has on insulin-sensitive tissues and the β-cell, one of the major drawbacks with lifestyle-induced weight loss programs in general is that the differences in body weight between the lifestyle intervention and the control groups can become attenuated during follow-up. This is quite common and is outlined in a number of publications which report that maintaining weight loss for a period of time is difficult, with a large percentage of individuals reverting back to their pre-intervention body weight (Anderson, Konz, Frederich, & Wood, 2001; Sumithran et al., 2011). This relapse is due to the compensatory increase in circulating hormones associated with appetite that encourage weight gain (Sumithran et al., 2011). Therefore, although lifestyle intervention is readily used as a first-line therapy in slowing down the progression to T2D, the reality is that the majority fail to adhere to a diet and exercise regime, and consequently, fail to maintain weight loss over an extended period of time.

**Pharmacological Interventions**

There are currently eight classes of anti-diabetic drugs available for use in Australia. These include: α-glucosidase inhibitors, metformin, thiazolidinediones, sulphonylureas, dipeptidyl peptidase-4 (DPP-4) inhibitors, GLP-1 receptor agonists (GLP-1RAs), insulin and SGLT2 inhibitors. Since Chapters 4 and 5 of this thesis involves the use of the SGLT2 inhibitor, dapagliflozin, as a means to investigate the effects of glucose-lowering on insulin resistance and β-cell function, a review encompassing this drug class will be given.
Sodium-Glucose Linked Transporter 2 Inhibitors

The SGLT2 inhibitors are a class of oral hyperglycaemic drugs that work by inhibiting SGLT2, a protein that mediates approximately 90% of glucose reabsorption in the proximal renal tubule. The blockade of this particular transporter channel leads to the significant excretion of glucose via the urine and the subsequent lowering of blood glucose levels. Unlike the pharmacological interventions previously discussed in this section, the SGLT2 inhibitors present a novel therapeutic approach to treating T2D in that their mechanism of action is independent of insulin action and secretion, thereby making them an effective treatment option at any stage in the disease process and with any combination of other anti-hyperglycaemic agents. In order to fully appreciate the mechanism of action by which the SGLT2 inhibitor class of drugs work, a basic understanding of renal glucose handling is necessary and will be discussed below.

Renal Glucose Handling in the Normal and Type 2 Diabetic State

As previously mentioned in this literature review, the regulation of plasma glucose is essential for maintaining normal cellular function. Too little glucose in the blood can result in severe and sometimes irreversible damage to the brain, whilst too much glucose for extended periods of time can expedite both oxidative and ER damage in multiple tissues which can then contribute to insulin resistance and β-cell dysfunction. The kidneys involvement in glucose homeostasis was first described in the 1930’s through a series of experiments in rabbits (Bergman & Drury, 1938). Despite the large body of evidence amassed over the years, the kidney is still often overlooked as a key player in the regulation of glucose homeostasis. This organ is capable of increasing glycaemia through gluconeogenesis or reducing glucose from the blood by facilitating glucose uptake and/or reabsorbing glucose through the glomerulus (Gerich, 2010). The kidneys of healthy individuals filter approximately 180 litres of plasma each day. With a mean day-long plasma glucose concentration ranging between 5-5.5 mmol/L, this equates to roughly 162 g of glucose being filtered by the kidney each day, with virtually all of the glucose being reabsorbed along the proximal tubules and returned to the circulation. The net result is that no glucose is present in the urine. Hyperglycaemia, on the other hand, enhances the amount of glucose filtered and increases the reabsorption of glucose up to the renal transport maximum (Tm). It is important to note that the Tm in healthy human subjects is less than 375 mg/min, with the Tm increasing
by approximately 20% in patients with poorly controlled T2D (Farber, Berger, & Earle, 1951). Once this renal glucose threshold is exceeded, all of the glucose in excess of the $T_m$ is no longer reabsorbed and traces of glucose begin to appear in the urine. The presence of glycosuria was originally thought to be a marker of ill health in early BC. Indeed, our thought process to glycosuria has evolved significantly as our understanding of renal glucose handling has improved. This has led to the view that the kidneys “spill” the excess glucose into the urine in an attempt to mitigate the adverse effects associated with high glucose levels. Unfortunately, if the increasing glycaemia is not curbed by the implementation of lifestyle measures and/or pharmacological interventions, the concomitant increase in $T_m$ occurs and the process of renal glucose handling becomes defective. This means that glycosuria does not occur until the plasma glucose concentration is substantially higher than normal (often $>11\text{ mmol/L}$) and will eventually lead to microvascular and macrovascular problems associated with glucotoxicity. There are two types of SGLT transporters, SGLT1 and SGLT2, with both of these responsible for the reabsorption of filtered glucose by using the electrochemical sodium gradient as the source of energy generated by $\text{Na}^+/\text{K}^+$ ATPase transporters. The increase in transporter expression of SGLT2 at the mRNA and protein level is thought to be responsible for the maintenance of hyperglycaemia, and has been shown in a study comparing SGLT2 expression in human exfoliated proximal tubular epithelial cells isolated from individuals with NGT and T2D (Rahmoune et al., 2005). Additional studies in diabetic rodent models have reported similar findings (Vallon et al., 2014; Vallon et al., 2013; Vallon & Thomson, 2012). To date, the mechanisms involved in the regulation of SGLT2 are still relatively unclear. Some potential candidates include insulin, protein kinase C and protein kinase A. All three have been shown to increase $\text{Na}^+$-glucose currents in human embryonic kidney 293T cells expressing human SGLT2 (Ghezzi & Wright, 2012). Insulin is thought to act as an agonist and stimulate SGLT2-mediated glucose entry through oxidative stress (Nakamura, Matsui, Ishibashi, & Yamagishi, 2015), whilst protein kinases C and A directly phosphorylate SGLT2 (Ghezzi & Wright, 2012), unlike the indirect regulation of SGLT1 by protein kinases (Wright, Hirsch, Loo, & Zampighi, 1997). In addition to these positive regulators, there are other candidates that have been shown to prevent SGLT2 induction. These include angiotensin II receptors which were identified in a
study using the antagonist drug Losartan in hypertensive and diabetic rats (Osorio et al., 2009), and hepatocyte nuclear factor-1α (HNF-1α) (Pontoglio et al., 2000; Yamagata et al., 1996). HNF-1α is of particular clinical significance as dominant mutations in humans (Menzel et al., 1998) or its complete deletion in mice (Pontoglio et al., 2000) have been shown to reduce SGLT2 gene expression and subsequently lead to defects in renal glucose reabsorption. Furthermore, as HNF-1α is implicated in the differentiation of β-cells, a mutation or deletion of this factor causes progressive β-cell dysfunction and a particular form of T2D called maturity-onset diabetes of the young type 3 (Pontoglio et al., 1998; Yamagata et al., 1996). All of this occurs in the absence of hyperglycaemia due to excessive urinary glucose loss.

Genetic and acquired defects in SGLT1 and SGLT2 can occur and cause metabolic abnormalities. These consist of congenital small intestine glucose-galactose malabsorption (for SGLT1) (Turk, Martin, & Wright, 1994) and familial renal glycosuria (SGLT2) (Santer et al., 2003). SGLT1, a high affinity low capacity transporter, mediates glucose transport in the S2 and S3 segments, and filters a small proportion of glucose (up to 10%). In addition to its presence in the kidney, SGLT1 is also expressed in intestinal K- and L-cells where it is principally responsible for the uptake of sugars in the gut lumen. Mutations in SGLT1 are associated with mild glycosuria but cause glucose-galactose malabsorption which leads to impaired intestinal hexose transport and death, unless glucose and galactose are promptly removed from the diet (Turk et al., 1994). SGLT2, a low affinity high capacity transporter, mediates glucose transport in the S1 segment of the proximal tubule and is responsible for up to 90% of filtered glucose. Although individuals with mutations in SGLT2 have no intestinal phenotype, they do show persistent renal glycosuria in the presence of normal blood glucose levels, often in the range of 20-200 g/day (Santer et al., 2003). Genetic analysis of families with this disorder have shown at least 21 different mutations in the SGLT2 gene with the level of renal glycosuria dependent on the individual’s mutation and whether it is present on one or both alleles (Santer et al., 2003). Consistent with the phenotype described in humans, studies in mice without SGLT1 or SGLT2 clearly demonstrate that SGLT2 accounts for the majority (approximately 97 % in rodents) of renal glucose reabsorption with SGLT1 responsible for the remainder under normoglycaemic conditions (Powell et al., 2013). A schematic overview depicting the
localisation of SGLT1 and SGLT2 in the kidneys is shown in Figure 1.5. In summary, the discovery that glucose is transported across the proximal tubule membrane by SGLT2 and that a naturally occurring polymorphism of the gene results in renal glycosuria, has effectively paved the way for the development of SGLT2 inhibitors. The next section will touch on the developmental history of these inhibitors from conception to where we are currently now, as well as describe relevant pre-clinical and clinical data.

**Figure 1.5: The localisation of SGLT1 and SGLT2 in the kidneys.**

**Pharmacological Inhibitors of Renal Glucose Reabsorption – the Transition from SGLT1 to SGLT2 inhibitors**

**Phlorizin:** The concept of normalising blood glucose concentrations by inhibiting glucose reabsorption and inducing renal glycosuria was first observed more than 100 years ago when phlorizin in doses greater than 1 g caused glycosuria in man (von Mering, 1886). Phlorizin is comprised of two main moieties, a glucose ring connected via an oxygen atom (O-glucoside) to two phenol rings. This compound is found in the bark of apple trees and competitively inhibits both SGLT2 and SGLT1 in the proximal tubule, with a ten-fold higher affinity for SGLT1 compared to SGLT2 (Chasis, Jolliffe, & Smith, 1933). Despite the encouraging results of phlorizin in lowering glucose levels
in diabetic rat models, e.g., streptozotocin (STZ)-induced (Tsujihara et al., 1996) and partial pancreatectomised rats (Rossetti, Shulman, Zawalich, & DeFronzo, 1987; Rossetti, Smith, et al., 1987), phlorizin was deemed unsuitable for clinical development for several reasons. Firstly, phlorizin is readily broken down in the gastrointestinal tract and therefore must be administered intravenously; secondly, the active metabolite phloretin, is a potent inhibitor of insulin-stimulated glucose uptake into muscle (Battaglia, Manchester, & Randle, 1960) and adipose tissue (Frerichs & Ball, 1964); and thirdly, phlorizin is associated with frequent gastrointestinal adverse events such as diarrhoea. This last point most likely stems from the abundant expression of SGLT1 in the brush border of the small intestine, as humans with mutations in SGLT1 and mice lacking SGLT1 develop glucose-galactose malabsorption that leads to profuse diarrhoea if glucose and galactose are not removed from the diet (Gorboulev et al., 2012; Martin, Turk, Lostao, Kerner, & Wright, 1996). Further, unlike the expression of human SGLT2 which is localised to the kidney only, the SGLT1 gene is also expressed in the human heart, skeletal muscle and the trachea (J. Chen et al., 2010). At the translational level, a recently published study using novel affinity-purified antibodies against human SGLT1 identified that this protein is also expressed in the liver (biliary duct cells), the lung (alveolar epithelial type 2 and bronchial clara cells) and the heart (heart capillaries) (Vrhovac et al., 2015). These newly identified locations of SGLT1 implicate several extra-renal and -intestinal functions of this transporter, such as fluid absorption in the lung, energy supply to clara cells and the release of glucose from the heart capillaries. These functions may be blocked by reversible SGLT1 inhibitors like phlorizin and could have potentially led to compromises in the safety of individuals taking this drug if it was developed as a therapeutic tool for the management of T2D.

**T-1095:** T-1095 is a synthetic phlorizin derivative developed by Japanese investigators via the addition of a methyl-carbonate group in order to prevent its degradation by glucosidase in the gut and to improve its oral bioavailability. After its oral administration, T-1095 is metabolised by the liver to its active form T-1095A, where it then acts on the proximal tubule to inhibit SGLT2 to acutely increase urinary glucose excretion and lower blood glucose levels (Oku et al., 2000). Using mouse and rat models of T1D and T2D, these investigators showed that chronic treatment (28 days duration) with a high dose (0.1 % wt/wt) of T-1095 could significantly decrease both
blood glucose and HbA1c levels in STZ-induced diabetic rats, as well as prevent the appearance of hyperglycaemia, hyperinsulinaemia, hypertriglyceridaemia and obesity in diabetic yellow KK mice with advancing age (Oku et al., 1999). In a number of subsequent papers, the same investigators also described that T-1095 provides renoprotective effects such that the appearance of microalbuminuria is delayed, and increases in renal GLUT2 levels and kidney weight are prevented (Adachi et al., 2000; Oku et al., 1999). All of these can contribute to the improvement of diabetic renal complications. Like its predecessor phlorizin, T-1095 also has the potential to inhibit the SGLT1 transporter located in the gut, albeit with lesser affinity. In vitro studies using Chinese hamster ovary cells stably transfected with human SGLT genes have demonstrated that T-1095 has a 30-fold selectivity for SGLT2 compared to SGLT1 (Oku et al., 2000), which is in stark contrast to the pharmacokinetics of phlorizin described earlier. Despite T-1095’s improved specificity for SGLT2 versus SGLT1 when compared to phlorizin, along with the promising pre-clinical studies in various models of type 1 and 2 diabetic rodent models, this drug was discontinued soon after phase II clinical trials due to its co-inhibition of SGLT1 and potential safety concerns. As a result, there are no clinical data to report for this agent.

**SGLT2 inhibitors:** The lessons learned from the earlier SGLT2 inhibitors have resulted in pharmaceutical companies pursuing derivatives that possess increased bioavailability. This has led to the substitution of the O-link with a C-link conferred by a C-glycoside linkage between the glucose and phenol moieties in order to provide greater resistance against intestinal, hepatic and renal glycosidases that can rapidly hydrolyse O-glycoside bonds. The formation of C-glycoside analogs has therefore resulted in the development of longer-acting SGLT2 inhibitors that are appropriate for single daily dosing. Three SGLT2 inhibitors are currently available for use in Australia. They include dapagliflozin (label name Forxiga; developed by Bristol-Myers Squibb and AstraZeneca), canagliflozin (Invokana; Janssen) and empagliflozin (Jardiance; Boehringer Ingelheim and Eli Lilly and Company). The order that these agents have been listed coincides with the order in which they were approved by the Therapeutics Goods Administration (TGA) in Australia; a regulation authority that is parallel to the Food and Drug Administration (FDA) for the United States of America and the European Medicines Agency (EMA) for countries in the European Union. Due to the
previously mentioned structural modifications, these agents possess superior potency and selectivity for SGLT2 than do phlorizin or T-1095; with dapagliflozin being approximately 1200-, canagliflozin 160- and empagliflozin > 2500-times more selective for SGLT2 than for SGLT1 (Tahrani, Barnett, & Bailey, 2013). All of these SGLT2 inhibitors effectively reduce renal glucose reabsorption and induce a similar sustained urinary glucose loss in the vicinity of 40-80 g/day. This loss accounts for approximately one-third of the filtered glucose load and is strictly dependent on which inhibitor is used, given each one show different binding affinities and binding retention times at the SGLT2 channel transporter. A schematic diagram illustrating the mechanistic differences between normal and SGLT2 inhibition is shown in Figure 1.6.
Figure 1.6: SGLT2 inhibition reduces renal glucose reabsorption and increases urinary glucose excretion. Under normal circumstances (top panel), the renal tubule reabsorbs almost all of the glucose present in the glomerular filtrate. When the amount of glucose in the glomerular filtrate exceeds the capacity of the renal tubule to reabsorb it, urinary glucose excretion occurs. Individuals with T2D have an increased renal glucose reabsorption capacity. The administration of a SGLT2 inhibitor (bottom panel) increases urinary glucose excretion by inhibiting the reabsorption of glucose in the proximal tubule.
Since dapagliflozin is exclusively used in Chapters 4 and 5 of this thesis, the subject of the next section will focus on discussing published pre-clinical and clinical studies using this agent to date.

Dapagliflozin: Dapagliflozin was the first of the SGLT2 inhibitors to be TGA approved despite its several shortcomings when applying for FDA approval; dapagliflozin was initially rejected due to safety concerns made by the Advisory Panel surrounding the potential increases in the risk of bladder and breast cancers (Burki, 2012). These concerns were eventually addressed when new safety data were provided from other on-going clinical studies (Nauck et al., 2011; Rosenstock, Vico, Wei, Salsali, & List, 2012; Wilding et al., 2012). In defence of dapagliflozin, the nine patients who had bladder cancer were all aged over 60 years. Also, there was a current or past smoking history in six patients, and five had microscopic haematuria (blood in the urine) which is considered to be a potential predictor for bladder cancer. In the nine patients who developed breast cancer, all were aged over 50 years old and diagnoses were made within the first year of exposure; a time-frame considered too short to fully attribute dapagliflozin with these cancers. The imbalance in incidence of bladder and breast cancers with dapagliflozin exposure compared to canagliflozin or empagliflozin (Hinnen, 2015) emphasises the unlikelihood of a causal relationship between cancer development and SGLT2 inhibitor use. Moreover, long-term outcomes in excess of 30 years in patients with familial renal glycosuria show very little clinical consequences (Scholl-Burgi, Santer, & Ehrich, 2004). Soon after regulatory approval, an in-depth toxicological study of dapagliflozin was conducted in various animal models. Using a multitude of different approaches and at high multiples of human exposures, e.g., 100-3,000 times the human clinical exposure for up to 2 years in mice, rats and dogs, the results provided substantial evidence to suggest that dapagliflozin does not initiate, promote nor progress tumours even when used chronically and at supraphysiological levels (Reilly et al., 2014). However, despite the safety data in favour of dapagliflozin, it would be irresponsible for clinicians to not be wary of the incidence of cancer risk among patients taking this drug, or any SGLT2 inhibitor for that matter, over the long-term. Despite its regulatory history, dapagliflozin is the most clinically advanced SGLT2 inhibitor with 146 studies either currently underway or having been completed when the keyword “dapagliflozin” is searched on the Clinical Trial Registry (see
ClinicalTrials.gov, accessed on 3/11/2016). In comparison, using the keywords “canagliflozin” or “empagliflozin” only returned 107 and 109 studies, respectively.

**Clinical Trials with Dapagliflozin**

Both single and multiple ascending dose studies over the dose range of 2.5-50 mg/day in healthy and type 2 diabetic subjects have revealed that peak plasma concentrations of dapagliflozin is reached within the first hour of administration and that the mean-half-life remains in the circulation for approximately 8.1 to 12.2 hours; making it an effective therapeutic agent for once-daily dosing. A dose-dependent increase in the urinary glucose excretion rate of glucose was also observed, such that doses of 2.5, 10 and 20 mg and placebo treatment produced urine glucose of 37.9, 68.4, 76.7 and 9.4 g over a 24 hour period. These values were comparable when the same dosages were taken once-daily over a 14 day period (Kasichayanula et al., 2011). At the time of writing this literature review, dapagliflozin has been studied as monotherapy in only five clinical trials with a number of other studies currently in the recruiting process. These trials have been summarised in Table 1.1, where baseline and change from baseline at the conclusion of the clinical trial for FPG and HbA1c are shown. It is important to note that a number of these trials also included groups receiving 1, 2.5, 20 and 50 mg of dapagliflozin, but these have not been included as they are not the currently recommended doses for the use of dapagliflozin in Australia.
### Table 1.1: Clinical efficacy of dapagliflozin monotherapy in patients with type 2 diabetes.

<table>
<thead>
<tr>
<th>Study</th>
<th>Intervention</th>
<th>n</th>
<th>Age (years)</th>
<th>T2DM duration (years)</th>
<th>Baseline FPG (mmol/L)</th>
<th>ΔFPG from baseline (mmol/L)</th>
<th>Baseline HbA1c (%)</th>
<th>ΔHbA1c from baseline (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E. Ferrannini, Ramos, Salsali, Tang, &amp; List, 2010)</td>
<td>Placebo</td>
<td>75</td>
<td>52.7 ± 10.3</td>
<td>0.50</td>
<td>8.88 ± 2.34</td>
<td>-0.23 (-0.66, 0.20)</td>
<td>7.84 ± 0.87</td>
<td>-0.23 (-0.43, -0.03)</td>
</tr>
<tr>
<td></td>
<td>Dapagliflozin 5 mg (A.M.)</td>
<td>64</td>
<td>52.6 ± 10.9</td>
<td>0.25</td>
<td>9.01 ± 2.50</td>
<td>-1.34 (-1.81, -0.87)</td>
<td>7.86 ± 0.94</td>
<td>-0.77 (-0.99, -0.55)</td>
</tr>
<tr>
<td></td>
<td>Dapagliflozin 10 mg (A.M.)</td>
<td>70</td>
<td>50.6 ± 9.97</td>
<td>0.45</td>
<td>9.26 ± 2.31</td>
<td>-1.6 (-2.03, -1.17)</td>
<td>8.01 ± 0.96</td>
<td>-0.89 (-1.11, -0.67)</td>
</tr>
<tr>
<td></td>
<td>Dapagliflozin 5 mg (P.M.)</td>
<td>68</td>
<td>54.5 ± 11.0</td>
<td>0.50</td>
<td>8.72 ± 2.83</td>
<td>-1.52 (-1.97, -1.07)</td>
<td>7.82 ± 0.91</td>
<td>-0.79 (-1.01, -0.57)</td>
</tr>
<tr>
<td></td>
<td>Dapagliflozin 10 mg (P.M.)</td>
<td>76</td>
<td>50.7 ± 9.7</td>
<td>0.40</td>
<td>9.34 ± 3.22</td>
<td>-1.64 (-2.07, -1.21)</td>
<td>7.99 ± 1.05</td>
<td>-0.79 (-1.01, -0.57)</td>
</tr>
<tr>
<td>(Bailey, Iqbal, T’Joen, &amp; List, 2012)</td>
<td>Placebo</td>
<td>68</td>
<td>53.5 ± 11.08</td>
<td>1.1</td>
<td>8.97 ± 3.19</td>
<td>0.23 (-0.23, 0.69)</td>
<td>7.80 ± 1.12</td>
<td>0.02 (-0.22, 0.25)</td>
</tr>
<tr>
<td></td>
<td>Dapagliflozin 5 mg</td>
<td>68</td>
<td>51.3 ± 11.51</td>
<td>1.4</td>
<td>8.72 ± 2.31</td>
<td>-1.58 (-2.04, -1.12)</td>
<td>7.92 ± 1.03</td>
<td>-0.82 (-1.06, -0.58)</td>
</tr>
<tr>
<td>(List, Woo, Morales, Tang, &amp; Fiedorek, 2009)</td>
<td>Placebo</td>
<td>54</td>
<td>53 ± 11</td>
<td>NR</td>
<td>8.33 ± 2.56</td>
<td>-0.33 (-0.37, -0.29)</td>
<td>7.9 ± 0.9</td>
<td>-0.18 (-0.21, -0.15)</td>
</tr>
<tr>
<td></td>
<td>Dapagliflozin 5 mg</td>
<td>58</td>
<td>55 ± 12</td>
<td>NR</td>
<td>8.50 ± 2.67</td>
<td>-1.06 (-1.10, -1.02)</td>
<td>8.0 ± 0.9</td>
<td>-0.72 (-0.74, -0.70)</td>
</tr>
<tr>
<td></td>
<td>Dapagliflozin 10 mg</td>
<td>47</td>
<td>54 ± 9</td>
<td>NR</td>
<td>8.22 ± 2.11</td>
<td>-1.17 (-1.23, -1.11)</td>
<td>8.0 ± 0.8</td>
<td>-0.85 (-0.88, -0.82)</td>
</tr>
<tr>
<td>(Henry et al., 2012)</td>
<td>Dapagliflozin 5 mg (study 1)</td>
<td>203</td>
<td>52.2 ± 10.2</td>
<td>1.6</td>
<td>10.59 ± 3.14</td>
<td>-2.33 (-2.63, -2.04)</td>
<td>9.14 ± 1.37</td>
<td>-1.19 (-1.36, -1.02)</td>
</tr>
<tr>
<td></td>
<td>Dapagliflozin 10 mg (study 2)</td>
<td>219</td>
<td>51.1 ± 11.5</td>
<td>2.1</td>
<td>10.99 ± 3.43</td>
<td>-2.58 (-2.85, -2.30)</td>
<td>9.03 ± 1.27</td>
<td>-1.45 (-1.59, -1.31)</td>
</tr>
<tr>
<td>(Kaku et al., 2013)</td>
<td>Placebo</td>
<td>54</td>
<td>58.4 ± 10.0</td>
<td>4.74</td>
<td>8.83 ± 1.73</td>
<td>0.62 (0.25, 0.99)</td>
<td>8.12 ± 0.71</td>
<td>0.37 (0.23, 0.51)</td>
</tr>
<tr>
<td></td>
<td>Dapagliflozin 5 mg</td>
<td>58</td>
<td>58.0 ± 9.0</td>
<td>5.34</td>
<td>9.14 ± 1.31</td>
<td>-1.31 (-1.68, -0.94)</td>
<td>8.05 ± 0.66</td>
<td>-0.37 (-0.51, -0.23)</td>
</tr>
<tr>
<td></td>
<td>Dapagliflozin 10 mg</td>
<td>52</td>
<td>56.5 ± 11.5</td>
<td>4.73</td>
<td>9.08 ± 1.65</td>
<td>-1.77 (-2.16, -1.38)</td>
<td>8.18 ± 0.69</td>
<td>-0.44 (-0.58, -0.30)</td>
</tr>
</tbody>
</table>
All five of the dapagliflozin monotherapy trials shown in Table 1.1 possess some difference/s in their study design, e.g., dapagliflozin added onto a two-week background of diet and exercise, dapagliflozin compared to metformin monotherapy, dapagliflozin used in treatment-naïve and treatment-experienced patients, and/or the duration of the study (12 versus 24 weeks). Regardless of these differences, all of the trials show similar results with reductions in plasma glucose and HbA1c levels being approximately 1.6 mmol/L and 0.8 %, respectively. Despite several studies demonstrating that dapagliflozin can improve β-cell function (Merovci et al., 2015; Salsali et al., 2011), one of the major reasons for the relatively modest reductions in glycaemic measures is that glycosuria produced by SGLT2 inhibition stimulates an increase in EGP (E. Ferrannini et al., 2014; Merovci et al., 2014). Quantitatively, this paradoxical increase in EGP offsets about 50 % of the amount of glucose excreted in the urine and thus, significantly blunts the decrease in FPG concentration and HbA1c. As noted before, EGP is confined to the liver and the kidney only. The contribution of the kidney can vary from undetectable levels of EGP to as high as 23 % (Meyer & Gerich, 2002). Renal glucose uptake is roughly 0.2 mg/kg/min and in most cases equals renal EGP, which means that the kidney very seldom contributes to net total body glucose homeostasis (Cherrington, 1999). Therefore, the liver is responsible for up to 80 % of the EGP, making the regulation of HGP pivotal in understanding the effect of SGLT2 inhibitors on increased glucose production. The primary regulators of EGP in the basal state are insulin and glucagon, with plasma insulin strongly suppressing and plasma glucagon strongly stimulating glucose production. However, unlike the liver, renal EGP is not stimulated by glucagon (Gustavson et al., 2004; Stumvoll, Meyer, Kreider, Perriello, & Gerich, 1998). An important study has recently shown an association between the increase in EGP and a temporary rise in circulating glucagon after SGLT2 inhibition (Merovci et al., 2014). Since renal EGP is unresponsive to glucagon, the increase in EGP in response to glycosuria suggests the presence of a novel “reno-hepatic” interaction, whereby glycosuria activates a mechanism that increases HGP in order to prevent hypoglycaemia from occurring. Preventing this compensatory increase in HGP by SGLT2 inhibition with a drug that exerts a direct effect on the liver to suppress HGP, e.g., thiazolidinediones, metformin, a GLP-1 analogue or insulin, would be an attractive option as it would be expected to amplify the decrease in FPG and
HbA1c. Clinical trials using some of these drugs in conjunction with dapagliflozin will be discussed later in this section.

Further to the glycaemic benefits provided by SGLT2 inhibition in the five listed monotherapy trials, dapagliflozin also causes modest weight loss in the range of 2-3 kg (Nauck, 2014). This effect is consistent with the caloric loss of glucose through the urine which results in a negative energy balance. Interestingly, in the majority of clinical and rodent studies, weight loss during SGLT2 inhibition is much lower than what is initially expected given the amount of energy lost through excessive glycosuria (Bailey, Gross, Pieters, Bastien, & List, 2010; Devenny et al., 2012; E. Ferrannini et al., 2010). The control of food intake by the brain is a reasonable mechanism to help explain the increase in food intake with studies utilising SGLT2 inhibitors. However, evidence surrounding the presence of SGLT2 in the brain remains controversial, with one study showing extremely low levels (Zhou et al., 2003) and another showing none (J. Chen et al., 2010). Therefore, the currently accepted view with regards to this phenomenon is that chronic glycosuria causes an adaptive increase in energy intake rather than SGLT2 inhibition having a direct effect on food intake (Devenny et al., 2012).

In addition to monotherapy, dapagliflozin has also been studied as adjunct therapy to several oral and injectable agents. As mentioned earlier, implementing insulin sensitisers such as metformin, GLP-1 analogues or insulin, may help prevent the compensatory increase in HGP that occurs with excessive SGLT2 inhibitor-induced glycosuria and perhaps even offset the increase in plasma glucagon concentration associated with the drop in glucose levels. Reducing both would therefore lead to the further lowering of plasma glucose and HbA1c levels. Indeed, pairing dapagliflozin with metformin in T2D patients with inadequate glycaemic control provides additional HbA1c lowering benefits of 0.4-0.55 % and larger reductions in FPG concentrations of 0.98-1.3 mmol/L when compared with metformin alone (Bailey et al., 2010). In an extension of the same study, 71.2 % of the original patient cohort was followed for an additional 78 weeks, culminating a total of 102 weeks. At the conclusion of the trial, the combination of metformin plus dapagliflozin lowered both HbA1c by a further 0.78 % and FPG concentrations by 1.36 mmol/L. In comparison, the metformin alone group saw no changes in HbA1c and a slight improvement in FPG levels (-0.58 mmol/L) (Bailey et al., 2013).
The combination of a GLP-1 analogue with dapagliflozin has not been well studied in clinical trials for T2D. To the best of our knowledge, we are only aware of one study that was recently presented as an abstract at the Society for Endocrinology BES 2015 meeting held in Edinburgh, United Kingdom. The authors concluded that combination dapagliflozin and GLP-1 agonist therapy can effectively reduce HbA1c by approximately 0.9% over 12 months in patients with T2D of varied stages (Hayden, Huang, McConnell, Sainsbury, & Jones, 2015). Just recently, the Canagliflozin Cardiovascular Assessment Study (CANVAS) published an 18-week follow-up report on the glycaemic benefits of combining the SGLT2 inhibitor canagliflozin with a DPP-4 inhibitor or a GLP-1RA. The findings show that the addition of either agent to canagliflozin 100 or 300 mg significantly lowers HbA1c and FPG levels relative to placebo. At 18 weeks, placebo-subtracted changes in HbA1c for patients with canagliflozin 100 and 300 mg with a DPP-4 inhibitor were -0.56 and -0.75%, and FPG -1.1 and -1.5 mmol/L, respectively. For the canagliflozin 100 and 300 mg plus GLP-1RA subset, HbA1c levels were reduced in the order of 1.0 and 1.06%, and FPG 1.8 and 2.5 mmol/L, respectively (Fulcher et al., 2016). These results are quite interesting when one considers that a DPP-4 inhibitor is supposed to sensitise the liver and reduce glucagon secretion, both of which should greatly reduce glycosuria-induced HGP. Although the CANVAS study did not measure HGP, the dismal improvements in the parameters measured would suggest that a DPP-4 inhibitor may lose its ability to suppress HGP when co-administered with a SGLT2 inhibitor. Because GLP-1RAs are much more potent inhibitors of glucagon secretion and also stimulators of insulin secretion, and that they inhibit HGP independent of changes in insulin and glucagon concentrations (Hare et al., 2010), it is not surprising that adding a GLP-1RA to a SGLT2 inhibitor produces synergistic effects on glycaemia.

Dapagliflozin has also been studied in patients receiving concurrent treatment with insulin. In an adaptive trial, patients with T2D who were receiving at least 30 units of daily insulin, with or without up to two oral anti-hyperglycaemic agents and with inadequately controlled glycaemia were studied. These patients were randomised to receive placebo, dapagliflozin 2.5, 5 or 10 mg. Insulin doses were maintained unless there was a clinically indicated reason to up- or down-titrate dose. After 48 weeks, dapagliflozin 5 mg were increased to 10 mg. At 104 weeks, patients receiving dapagliflozin saw an HbA1c change of -0.6-0.8% while the group not receiving dapagliflozin had a change of -0.4%. Of particular interest to clinicians, the mean daily insulin dose for the placebo group increased by 18.3
units per day and this was associated with a 1.8 kg weight increase, whereas in the dapagliflozin groups, insulin dose was stable and weight decreased by 0.9-1.4 kg (Wilding et al., 2014). In addition to the study performed by Wilding et al., two other trials evaluating the efficacy of dapagliflozin in patients receiving insulin therapy with or without concurrent oral therapy have also shown superior glucose-lowering and weight loss effects (Wilding et al., 2009; L. Zhang, Feng, List, Kashchayanula, & Pfister, 2010). It is important to highlight that the patients involved in these trials had longer durations of T2D than in other dapagliflozin trials, further highlighting the efficacy of dapagliflozin (and SGLT2 inhibitors in general) across a range of patients who are in different stages of T2D.

Pre-Clinical Studies with Dapagliflozin

The earliest known reports to examine dapagliflozin as a potential treatment for T2D was a proof-of-concept study conducted in normal and experimentally diabetic rats. In two different rat models of diabetes: STZ and ZDF rats, hyperglycaemia was significantly reduced after a single oral dose of dapagliflozin within the first 6 hours of dosing (S. Han et al., 2008; Meng et al., 2008). Normal rats also exhibited improved glucose tolerance through excess urinary glucose loss, albeit less than the diabetic group, and this was associated with reductions in glucose excursions following oral glucose tolerance testing (S. Han et al., 2008). Interestingly, the experiment using the ZDF rat as a model of T2D showed that these rats excreted up to seven times more glucose on a gram-per-animal basis than non-diabetic rats, thus demonstrating that the glycosuric effect of dapagliflozin is proportional to the level of glucose in the blood (S. Han et al., 2008). Similarly, in C57BL/Ks-db/db mice, chronic treatment with dapagliflozin resulted in the normalisation of ambient fasting glucose levels and reductions in HbA1c in a dose-dependent manner (Terami et al., 2014).

As mentioned earlier, β-cell dysfunction and insulin resistance both contribute to the pathophysiology of T2D. SGLT2 inhibition is expected to circumvent both of these core defects through excessive glucose loss and partial weight loss. There are a number of studies that have examined whether dapagliflozin treatment can improve these parameters. Specifically, pre-diabetic ZDF rats treated with dapagliflozin for 15 days show enhanced glucose utilisation specifically in the liver and a reduction in HGP when a hyperinsulinaemic-euglycaemic clamp was performed (S. Han et al., 2008). These effects have also been observed in other ZDF rat studies; one with the treatment of dapagliflozin for 33 days at the initiation of a high-fat diet (F. R. Macdonald et al., 2010) and the other after treatment for 5 weeks (Zinker et al., 2011). However, the finding of reduced HGP in these rodent studies
goes against what is currently known and expected with SGLT2 inhibition, both in the clinical sense where plasma glucagon is increased in the presence of lowered insulin levels (Merovci et al., 2014) and in pre-clinical studies where SGLT2 inhibition can directly trigger glucagon secretion from the pancreatic α-cell (Bonner et al., 2015). An explanation for the reduction in HGP in the two studies has not been forthcoming. Preserving β-cell function and mass is the number one priority when treating T2D. The use of dapagliflozin in the ZDF rat has shown that both β-cell function and mass can be adequately preserved when dapagliflozin is given before or soon after moderate hyperglycaemia has been established (F. R. Macdonald et al., 2010). Likewise, long-term treatment (< 12 weeks) with dapagliflozin in C57BL/Ks-db/db mice significantly prevents the decrease in β-cell mass (Terami et al., 2014). There are currently no studies to date that have utilised dapagliflozin to precisely elucidate the mechanism/s by which glucose-lowering can preserve β-cell function and mass; however, the use of other SGLT2 inhibitors such as BI-38335 and luseogliflozin under diabetic conditions have shown that removing glucotoxicity increases islet cell proliferation as assessed by the number of Ki67-immunopositive cells and decreases islet cell apoptosis by suppressing genes associated with oxidative stress, ER stress and inflammation (L. Chen, Klein, & Leung, 2012; Okauchi et al., 2016). As neither SGLT2 nor SGLT1 (some SGLT2 inhibitors possess a low propensity to inhibit SGLT1, e.g., canagliflozin) are expressed in the β-cell (Bonner et al., 2015), the beneficial effects towards β-cell function and mass appear to be related to increased insulin sensitivity only.

**PEPCK Transgenic Rat**

The PEPCK rat is a transgenic model that was created by our laboratory to investigate the contribution of increased EGP in the pathogenesis of T2D (Rosella et al., 1995; Rosella, Zajac, Kaczmarczyk, Andrikopoulos, & Proietto, 1993). This was achieved by over-expressing the cytosolic form of the non-insulin responsive gluconeogenic enzyme, PEPCK, in the liver and kidney under the control of a metallothionein promoter with an iron responsive element (Rosella et al., 1993). Previous work using clones of the rat hepatoma cell line H4IIE-C3 have showed that transcription and translation of the PEPCK transgene could be controlled by manipulating the level of zinc and iron in the media (Rosella et al., 1993). Experiments in our laboratory have shown that this genetic modification results in a 2-3 fold induction of PEPCK in the liver and kidney (see Appendix II for expression of PEPCK in other tissues), which leads to the impaired suppression of EGP (Mangiafico et al., 2011). In addition, we have shown that this rodent model acquires several metabolic defects
that closely resemble those found in the pre-diabetic human state. These include the development of mild obesity, profound peripheral insulin resistance, glucose intolerance and high triglyceride levels (Lamont et al., 2003; Mangiafico et al., 2011; Rosella et al., 1995; Thorburn et al., 1999). Recent evidence from our laboratory has shown that these rats have NGT, insulin sensitivity and GSIS at 4-5 weeks of age, but progress to develop glucose intolerance, insulin resistance and impaired GSIS by 12-14 weeks of age (Mangiafico et al., 2011). Therefore, this rodent model provides a unique opportunity to study and dissect the relative contribution of obesity and chronic glucose excess in the progression of NGT to IGT and the consequences that this has on β-cell function.

**Study Rationale**

T2D is a complex disease that results in the compromise of insulin action on the liver and peripheral tissues, and insulin secretion from the β-cell. Besides the influence that genetic predispositions have on these defects, obesity is considered to be the major driver in bringing to head hyperglycaemia in type 2 diabetic subjects. From the literature review, it is clear that obesity can increase the output of glucose from the liver and impair insulin-stimulated glucose transport in skeletal muscle and adipose tissue. All of these defects place additional stress on the β-cell to hypersecrete insulin in order to compensate for the prevailing circulating glucose and over time, β-cell compensation fails and T2D ensues. Studies focusing on dietary and pharmacological interventions in humans and animal models of IGT have shown that implementing either intervention can slow the progression of T2D by preserving β-cell function through enhanced insulin sensitivity. However, it is currently not clear as to the level of contribution that obesity and glucose per se play in the progression from IGT to T2D, as obesity clearly induces hyperglycaemia, thus making attempts to dissociate one from the other difficult. The overall scope of this thesis was to utilise the PEPCK transgenic rat as a model of mild obesity and chronic glucose excess, and to use dietary and pharmacological interventions to determine the contribution of obesity and glucose per se in the development of IGT, hyperglycaemia and β-cell dysfunction.

**Overall Hypothesis**

The hypothesis of this thesis is that both obesity and chronic glucose excess contributes to the development of glucose intolerance, hyperglycaemia, insulin resistance and β-cell dysfunction in the PEPCK transgenic rat.
Overall Aim

The overall aim of this thesis is to investigate the contribution of obesity and a primary defect in glucose overproduction in the progression from NGT to IGT and β-cell dysfunction in the PEPCK transgenic rat. The specific aims are to:

1. **Characterise the PEPCK transgenic rat as a suitable model of mild obesity, glucose intolerance, hyperglycaemia and β-cell dysfunction:** PEPCK transgenic rats were studied at three different ages; 5, 14 and 20 weeks of age in order to adequately document the progression of mild obesity, glucose intolerance, hyperglycaemia and its effect on β-cell function (Chapter 3).

2. **Evaluate whether or not dapagliflozin treatment in PEPCK transgenic rats with established defects can normalise glucose intolerance, hyperglycaemia, insulin resistance and β-cell dysfunction:** PEPCK transgenic rats aged 14 weeks-old were given dapagliflozin for 6 weeks in order to determine whether pharmacologically removing excess glucose could improve glucose tolerance, hyperglycaemia, insulin resistance and β-cell function when these defects are already firmly established. A subset of PEPCK transgenic rats were calorie-restricted and used to control for any insulin sensitising benefits seen with associative weight loss from dapagliflozin treatment (Chapter 4).

3. **Evaluate whether or not early treatment with calorie-restriction or dapagliflozin can prevent the development of glucose intolerance, hyperglycaemia and β-cell dysfunction in the PEPCK transgenic rat:** PEPCK transgenic rats aged 5 weeks-old were either calorie-restricted or given dapagliflozin for 10 weeks in order to determine the relative contribution of obesity and chronic excess glucose on glucose tolerance, hyperglycaemia and β-cell function (Chapter 5).
Chapter 2 General Materials and Methods

Materials

Molecular Biology

Chemicals and Reagents

All chemicals and reagents used were of molecular biology grade. Ethanol absolute and glacial acetic acid were purchased from BDH Chemicals (Kilsyth, VIC, Australia). Isopropanol was purchased from Merck (Kilsyth, VIC, Australia). D-glucose, L-arginine monohydrochloride, tolbutamide, methanol, 10 % Neutral Buffered Formalin, chloroform, diethylpyrocarbonate (DEPC), Phosphate-buffered saline (PBS) tablets, Tris Base, Tris-hydrochloride and DPX mounting media were purchased from Sigma-Aldrich (St. Louise, MO, USA). Analytical and low melting point agarose powder was purchased from Amresco (Solon, OH, USA). Gel Red Nucleic Acid was purchased from Jomar Biosciences (Kensington, SA, Australia). TRIZol was purchased from Invitrogen, Life Technologies (Carlsbad, CA, USA). Glycogen (20 mg/mL) was purchased from Roche Applied Science (Penzberg, Germany).

Reverse Transcription Systems

DNA-free DNase Treatment Kit [Recombinant DNase I (rDNase I 2 U/µL), 10x DNase I Buffer], Nuclease-free Water and SUPERase·In RNase Inhibitor (20 U/µL) were purchased from Ambion, Life Technologies (Carlsbad, CA, USA). AMV Reverse Transcriptase (20 U/µL), Recombinant RNasin Ribonuclease Inhibitor, Random Hexamer Primers, dNTP Mixture, Reverse Transcription 10x Buffer and 25 mmol/L magnesium chloride were purchased from Promega (Madison, WI, USA).

Real-Time PCR Reagents

TaqMan Universal PCR Master Mix and Pre-Developed rat TaqMan Gene Expression Assays’ 18S (Hs99999901_s1), Glut2 (Rn00563565_m1), Gck (Rn00561265_m1), Sur1 (Rn01476317_m1), Kir6.2 (Rn01764077_s1), Pck1 (Rn01529014_m1), G6pc
(Rn00689876_m1) and *Fbp1* (Rn00561189_m1) were purchased from Applied Biosystems (Foster City, CA, USA).

**Western Blot Reagents**

Mouse monoclonal anti-GLUT4 IgG (100 µg/mL, ab48547) and mouse monoclonal anti-HSP90 (100 µg/mL, AC88) were purchased from Abcam (Cambridge, MA, USA). Polyclonal rabbit anti-mouse IgG-HRP and guinea pig polyclonal anti-porcine insulin antibodies were purchased from DAKO Corporation (Denmark). Bradford protein assay dye reagent, Dye Reagent Concentrate, bovine serum albumin (BSA, 2 mg/mL), 30 % acrylamide: 0.8 % bis solution and protein molecular weight standard were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Ethylenediaminetetraacetic acid (EDTA), ammonium persulphate (APS) and sodium dodecyl sulphate (SDS) were purchased from Merck (Kilsyth, VIC, Australia). Dithiothreitol (DTT) was purchased from ICN Biomedicals (OH, USA). Immobilon-P Transfer Polyvinylidene Difluoride Membrane (PVDF) was purchased from Millipore (MA, USA). Enhanced chemiluminescence reagents were manufactured by PerkinElmer Life Sciences (MA, USA). Complete 25x protease inhibitor cocktail tablets were purchased from Roche Applied Science (Penzberg, Germany). Tween-20 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Tetramethylethylenediamine (TEMED, electrophoresis grade) was purchased from MP Biomedicals (OH, USA).

**Cell Biology**

**Islet Isolation Reagents**

RPMI media 1640 (with L-glutamine, without sodium bicarbonate) manufactured by GIBCO BRL was purchased from Invitrogen, Life Technologies (Carlsbad, CA, USA). Hank’s Balanced Salt Solution (HBSS) was from Lonza Australia Pty Ltd (Mt Waverley, VIC, Australia). Calcium chloride was from BDH Chemicals (Kilsyth, VIC, Australia). Salts used to make Krebs-Ringer Bicarbonate Buffer (KRBB) were from Merck (Kilsyth, VIC, Australia) for potassium chloride and magnesium sulphate heptahydrate; Sigma-Aldrich (St Louis, MO, USA) for sodium chloride, sodium bicarbonate, sodium pyruvate and hydroxyethyl piperazineethanesulfonic (HEPES);
APS Ajax Finechem (Seven Hills, NSW, Australia) for calcium chloride dihydrate and potassium dihydrogen phosphate. Histopaque-1077, BSA (cell culture grade), PenStrep (penicillin 100 U/mL, streptomycin 100 µg/mL) and Collagenase XI were purchased from Sigma-Aldrich (St Louis, MO, USA).

**Animal Physiology**

Sodium pentobarbitone (Lethabarb) was used to anaesthetise rats for surgical procedures and was purchased from Rhone Merieux (QLD, Australia). Heparin was purchased from David Bull Laboratories (Mulgrave, VIC, Australia). Normal saline (0.9 %) and glucose intravenous injection (50 %) were purchased from Astra Pharmaceuticals (North Ryde, NSW, Australia). Insulin (Actrapid) was purchased from Novo Nordisk (Bagsværd, Denmark). D-[6-³H] Glucose [1 mCi; specific activity 25-50 Ci (925 GBq-1.85 TBq)/mmol] was purchased from PerkinElmer Life Sciences (MA, USA). The SGLT2 inhibitor, dapagliflozin (Forxiga) 10 mg, was manufactured by Bristol-Myers Squibb (Mulgrave, VIC, Australia) and AstraZeneca (North Ryde, NSW, Australia) and purchased from Sigma Pharmaceuticals Limited (Rowville, VIC, Australia).

**Equipment**

The MyCycler Personal Thermal Cycler was utilised and was manufactured by BIO-RAD Laboratories (Hercules, CA, USA). ABI PRISM 7500 Sequence Detection System was manufactured by Applied Biosystems (Foster City, CA, USA).

Gel electrophoresis apparatus was purchased from Plaztek Scientific (Beaconsfield, VIC, Australia). Proteins were resolved using a Mini-PROTEAN Tetra Cell and transferred using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell manufactured by BIO-RAD Laboratories (Hercules, CA, USA). For RNA, gel electrophoresis images were acquired using a BIO-RAD Molecular Imager Gel Doc XR System and analysed using Quantity One software. For protein, immunoreactive bands were visualised by exposure to a Fuji film Image Reader LAS-3000 and densitometry quantified using Fuji film Multi Gauge software.

Eppendorf tubes were centrifuged at room temperature in the Pico Biofuge (13000 rpm, 16060 x g, rcf) manufactured by Heraeus (Germany) or in an Eppendorf
Microcentrifuge 541D (13200 rpm, 16100 x g, rcf) manufactured by Eppendorf (Hauppauge, NY, USA) when temperature adjustment was required. Falcon tubes were centrifuged in a Sorvall RT 6000D Refrigerated Centrifuge and conical base StackPack tubes in a Sorvall RC 3B Plus manufactured by Dupont Instruments, Sorvall Biomedical Division (Newton, CT, USA).

Rats and food were weighed using Sartorius TE4101 digital scales (to 0.1 g) manufactured by Sartorius AG (Germany). Fat pads, kidneys and pancreata were weighed on TR-403 fine scales (to 0.001 g) manufactured by Denver Instrument Company (Arvada, CO, USA). KDS100 Syringe Pumps were used to deliver specified volumes of various solutions to rats and were manufactured by KD Scientific (Holliston, MA, USA).

Tissue samples were homogenised using a Polytron PT 3100 homogeniser from Kinematica (Switzerland). Sonication of samples was performed using a MICROSON XL 2000 Ultrasonic Liquid Processor manufactured by MISONIX (Farmingdale, NYC, USA). Colourimetric absorbance based assays were performed on a Sunrise plate reader and analysed using XFluor4 Software manufactured by TECAN Group Ltd (Switzerland). RNA quantification was performed using a Nanodrop Lite Spectrophotometer manufactured by Thermo Scientific Fisher (Waltham, MA, USA).

Hand-picking of islets was performed using a Leica MZ6 modular stereomicroscope (Leica Microsystems, Germany). Islet culture plastic wares were purchased from Becton Dickinson Biosciences (North Ryde, NSW, Australia).

Whole blood glucose measurements were conducted using an Accu-Chek Performa glucometer and glucose test strips manufactured by Roche Diagnostics (NSW, Australia). Plasma glucose was measured using a GM7 Micro-Stat glucose analyser, glucose reagent, and glucose standard at 8 mmol/L, manufactured by Analox Instruments Ltd (Hammersmith, London, UK). The presence of glucose in the urine was determined by using Diastix glucose test strips manufactured by Bayer (Leverkusen, Germany). The radioimmunoassay for insulin was purchased from Linco Research (St Charles, MO, USA) and performed using a Cobra II Auto Gamma Counter manufactured by Packard (Meriden, CT, USA). The enzyme-linked immunosorbent
assay (ELISA) for insulin manufactured by ALPCO (USA) was purchased from R-Biopharm/Laboratory Diagnostics (Caringbah, NSW, Australia). Whole blood β-ketone measurements were conducted using a Medisense Optium glucometer and β-ketone test strips manufactured by Medisense Precision Q.I.D (MA, USA). Plasma triglycerides were measured using a triglyceride determination kit purchased from Sigma-Aldrich (St. Louise, MO, USA). The Tri-Carb 2810 TR Liquid Scintillation Analyser was used for both [6-3H]-glucose and 2-deoxy-D-[1-14C]-glucose (2DG) measurements and was manufactured by PerkinElmer Life Sciences (MA, USA).

**Animal Source & Maintenance**

**Breeding and Maintenance**

Piebald Virology Glaxo control (PVG/c) rats were generated at the Animal Resources Centre (Perth, Western Australia, Australia) and served as the control strain in experiments. PEPCK transgenic rats expressing the PEPCK transgene in the liver and kidney under the control of the mouse metallothionein promoter were produced on the PVG/c background as previously described (Rosella et al., 1995). From 2009 to the completion of 2011, both colonies were housed and inbred in the University of Melbourne, Department of Medicine (Austin/Northern Health) Animal Facility (Austin Repatriation Hospital, Heidelberg West, Victoria, Australia). At the commencement of 2012, both colonies were housed and inbred in the Austin Health BioResources Facility (Austin Hospital, Heidelberg, Victoria, Australia). In both facilities, lightning was artificial and timer controlled with a 12 hour light/dark cycle (lights on at 0700 hr) at a room temperature of 19-22°C. Rats were housed in pairs with the exception of the calorie-restricted groups, and fed a standard laboratory chow diet manufactured by Barastoc (Pakenham, VIC, Australia) comprising, on a weight basis, 74 % carbohydrate, 20 % protein, 3 % fat and 3 % fibre (Energy = 16.8 MJ/kg), and water *ad-libitum* unless indicated. All procedures were performed in male rats only and were in accordance with the Animal Ethics Committee Guidelines and approved by the Austin Health (approval numbers A2006/02624 and A2010/04167).
General Protocols

Isolation of Pancreatic Islets
Islets were isolated by Collagenase digestion of the pancreas using a modification of the method of Lacy and Kostianovsky (Lacy & Kostianovsky, 1967) and Gotoh (Gotoh, Maki, Kiyoizumi, Satomi, & Monaco, 1985). Rats were weighed and anaesthetised with 60 mg/kg body weight of sodium pentobarbitone by intraperitoneal injection. The abdomen was opened and the bile duct clamped at its entrance to the duodenum. The pancreas was perfused by intraductal injection of 10 mL of Collagenase IX (0.4 mg/mL) in Complete HBSS (HBSS, 1 M HEPES pH 7.4, 2 mmol/L calcium chloride) and excised. An additional 10 mL of pre-warmed Complete HBSS was added and pancreata incubated at 37°C for 7 minutes. After incubation, the pancreata were transferred into 10 mL of RPMI (For 1 litre: 10.4 g RPMI-1640, 0.9 g sodium chloride, 2 g sodium bicarbonate, 0.11 g sodium pyruvate, 10 mL 2M HEPES pH 7.4) supplemented with 0.1 % BSA and PenStrep, and containing 11.1 mmol/L D-glucose, then disrupted by hand-shaking and passed through a 500 µm mesh. Islets were purified using histopaque-1077 (a solution of polysucrose and sodium diatrizoate adjusted to a density of 1.077 ± 0.001 g/mL) with RPMI layered on top. Upon centrifugation at 2,000 rpm for 15 minutes, islets accumulated at the histopaque-RPMI interphase. Islets were collected from the interface into 40 mL RPMI, pelleted by centrifugation at 1,000 rpm for 2 minutes, and transferred into a petri dish in a small volume of the supernatant. Islets were then hand-picked under a stereoscopic microscope into a petri dish containing fresh pre-warmed RPMI supplemented with 10 % (vol/vol) heat-inactivated fetal calf serum and PenStrep, and cultured overnight in a 37°C humidified incubator of 95 % air: 5 % CO₂.

Quantitative Real-Time PCR

RNA Extraction from Islets
Isolated islets (at least 80) were picked in RPMI into eppendorf tubes and subjected to centrifugation at 2,000 rpm for 5 minutes at room temperature. The supernatant was aspirated and the islet pellet washed twice in 1x PBS and centrifuged at 2,000 rpm for 5 minutes. After aspirating the supernatant, the islet pellet was resuspended in 300 µL of TRIzol reagent and the RNA immediately released from cells by repeatedly pipetting up and down. The samples were incubated at room temperature for 5 minutes to permit the
complete dissociation of nucleoprotein complexes. Next, 100 µL of chloroform was added, mixed by vigorous shaking for 15 seconds and incubated at room temperature for 3 minutes. Following centrifugation at 10,000 rpm for 15 minutes at 4°C, the aqueous phase was transferred to a new eppendorf tube and RNA precipitated by mixing with 2 µL of glycogen and 200 µL of isopropanol and incubated overnight at -80°C. The next day, samples were incubated at room temperature for 10 minutes before being centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was decanted and the RNA pellet washed once with 300 µL of 75 % ethanol in DEPC water and centrifuged at 6,000 rpm for 5 minutes at 4°C. The RNA pellets were briefly air dried and redissolved in 20 µL DEPC water.

**RNA Extraction from Whole Tissue**

Upon excision, whole tissues were frozen in liquid nitrogen and stored at -80°C. For RNA extraction, approximately 50-100 mg of the frozen tissue was cut and homogenised in 1 mL of TRIzol using a Polytron PT 3100 homogeniser for 20-30 seconds at 10,000 rpm. The homogenised samples were incubated at room temperature for 5 minutes to permit the complete dissociation of nucleoprotein complexes. Next, 200 µL chloroform was added, mixed by vigorous shaking for 15 seconds and incubated at room temperature for 3 minutes. Following centrifugation at 10,000 rpm for 15 minutes at 4°C, the aqueous phase was transferred to a new eppendorf tube and RNA precipitated by mixing with 500 µL of isopropanol and incubated at room temperature for 10 minutes. To pellet the RNA, centrifugation was performed at 10,000 rpm for 10 minutes at 4°C. The supernatant was decanted and the RNA pellet washed once with 1 mL of 75 % ethanol in DEPC water and centrifuged at 6,000 rpm for 5 minutes at 4°C. The RNA pellets were briefly air dried and dissolved in 30-50 µL DEPC water depending on pellet size.

**Determination of RNA Quality and Quantity**

Agarose gel electrophoresis was used to visualise RNA. Gels were prepared by weighing out molecular grade agarose powder in 1x TAE buffer at the desired concentration, dissolved by heating, and then adding 3 µL Gel Red Nucleic Acid to enable the visualisation of RNA under UV light. The hot agarose solution was immediately poured into a gel tray set with combs to create wells. The gel was then
placed into an electrophoresis tank containing 1x TAE buffer. Samples were prepared for electrophoresis with 2x RNA loading dye. The RNA was separated by electrophoresis at 110 volts for 20 minutes. The presence of 28S, 18S and 5S rRNA bands were visualised under UV light using a BIO-RAD Molecular Imager Gel Doc XR System and images taken with the Quantity One software. RNA was deemed good quality if the 28S and 18S rRNA bands were distinct.

RNA quality and concentration was determined by measuring the absorbance at 260 nm (A260) and 280 nm (A280) using a Nanodrop Lite Spectrophotometer. Samples with an A260/A280 ratio of greater than 1.8 were DNase treated and reverse transcribed.

**DNase Treatment of RNA**
DNA-free DNase Treatment Kit (Ambion) was used to remove residual DNA from RNA preparations. A 25 µL reaction was set up to contain 20 µL RNA (up to 0.25-0.5 µg/µL), 2.5 µL 10x DNA-free DNase I Buffer, 1.5 µL (30 U) of SUPERase-In RNase Inhibitor and 1 µL (2 U) DNA-free recombinant DNase 1. The reaction was incubated at 37°C for 30 minutes. The DNase was inactivated by incubating samples at 70°C for 10 minutes followed by chilling on ice for 5 minutes. Samples were either used as a template for cDNA synthesis or stored at -80°C for later use.

**Promega Reverse Transcription System**
The Promega Reverse Transcription System was used to synthesis cDNA from RNA derived from all tissues. Each reverse transcription reaction was 20 µL in volume containing nuclease-free water (Ambion), 1 µL Random Hexamer Primers (0.5 µg/µL), 1 µL recombinant RNasin Ribonuclease Inhibitor, total RNA (4 µg for islets and 1 µg for whole tissue), 10x Reverse Transcription Buffer, 25 mmol/L magnesium chloride, 10 mmol/L dNTP and 0.75 µL AMV Reverse Transcriptase. The nuclease-free water, Random Hexamer Primers and RNA were first combined and incubated at 70°C for 5 minutes and then chilled on ice for 5 minutes followed by the addition of the other reagents. Reverse transcription was then initiated in a MyCycler Personal Thermal Cycler with the following cycling conditions: room temperature for 10 minutes, 42°C for 15 minutes, 95°C for 5 minutes and 4°C for 5 minutes. The resulting cDNA samples were then diluted in PCR water and stored at -20°C or used immediately for
Real-Time PCR. A reaction without the addition of AMV Reverse Transcriptase was performed to control for genomic DNA contamination (no RT control).

**TaqMan Real-Time PCR**

Relative expression of mRNA transcripts was quantitated by Real-Time PCR using TaqMan chemistry. The reaction was performed in duplicate in a 20 µL volume containing 10 µL TaqMan Universal PCR Master Mix, 1 µL TaqMan Gene Expression Assay Mix, 6 µL PCR water and 3 µL cDNA (4 µg/µL for islets and 1 µg/µL for whole tissue). A no RT control sample was also run to detect any residual genomic DNA potentially leftover after DNase treatment. Reactions were loaded into a 96-well plate, sealed with optical adhesive film and briefly centrifuged. Real-Time PCR was then conducted in an ABI PRISM 7500 Sequence Detection System using the following cycling conditions for amplification: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 continuous cycles of 95°C for 15 seconds and 60°C for 1 minute.

**Real-Time PCR Analysis**

Data from Real-Time PCR runs were analysed using ABI Sequence Detection software. The comparative Ct (ΔΔCt) method was employed to assess the expression level of the target gene relative to the housekeeping gene 18S.

**Western Blot: Detecting total GLUT4 on Protein Gels**

**Extraction of Proteins from Whole Tissue**

A frozen piece of approximately 50-100 mg tissue was weighed and transferred into 0.5-1.5 mL of Homogenisation Buffer (20 mM HEPES pH 7.5, 2 mM EDTA pH 8, 50 mM sodium fluoride, 5 mM sodium orthovanadate, 1 % NP40 and 1 mM DTT) containing 1x Complete protease inhibitor cocktail, on ice. The tissue was homogenised using a Polytron PT 3100 homogeniser for 20-30 seconds at 10,000 rpm and the homogenate centrifuged for 15 minutes at 13,200 x rpm at 4°C. After centrifugation, the supernatant was retrieved for protein determination. Homogenates were stored at -80 °C for Western blotting.

**BIO-RAD Protein Assay**

Protein concentration was measured using a BIO-RAD protein assay based on the Bradford-dye binding method (Bradford, 1976). BSA standards in the range of 0.05
mg/mL to 0.5 mg/mL were prepared by a 1:10 serial dilution with distilled water, allowing for the creation of a five-point standard curve. Any dilutions of test samples were also prepared in distilled water. The dye reagent was prepared by diluting 1 part Dye Reagent Concentrate with 4 parts distilled water and filtered through a Whatman #1 filter to remove particulates. Next, 10 µL of each standard and test sample was pipetted into individual microtiter plate wells in duplicate followed by the addition of 200 µL of diluted dye reagent and incubated at room temperature for 5 minutes. The absorbance was then measured using a Sunrise microplate reader and XFluor4 Software at 570 nm.

**SDS-PAGE, Transfer and Probing**

Western blotting was performed on proteins extracted from the whole homogenate of red gastrocnemius muscle (50 µg protein) and epididymal fat (20 µg protein). In order to probe for GLUT4’s predicted band size of 54 kDa, extracted proteins were separated on a 10 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) resolving gel using reducing conditions. First, the protein extracts were mixed with 2x loading buffer (0.125 M Tris-HCl pH 6.8, 20 % glycerol, 4 % SDS, 10 % 2-mercaptoethanol, 0.05 % bromophenol blue) at a 1:1 ratio, and heated at 70°C for 5 minutes. Samples were immediately placed on ice for 5 minutes to ensure complete denaturing of proteins. An equal amount of protein (µg) from each sample, as determined by the BIO-RAD protein assay, was loaded onto a 10 % SDS-PAGE resolving gel (10 % acrylamide, 0.375 M Tris-HCl pH 8.8, 0.1 % SDS, 0.075 % APS, 0.05 % TEMED) with a 5 % stacking gel (5 % acrylamide, 0.125 M Tris-HCl pH 6.8, 0.1 % SDS, 0.05 % APS, 0.1 % TEMED) submerged in 1x running buffer (3 g Tris-HCl, 14.4 g of glycine and 1 g of SDS in 100 mL of water at pH 8.3). Electrophoresis was then conducted at 110 volts and 20 mA for approximately 2 hours.

Once the proteins had been separated, they were transferred onto a 0.45 µM PVDF membrane using a semi-dry method. This involved soaking two pieces of thick blot paper in transfer buffer (25 mM Tris, 192 mM glycine and 20 % methanol), pre-soaking a PVDF membrane in methanol and equilibrating for 5 minutes in transfer buffer. The SDS-PAGE gel/PVDF membrane sandwich was assembled from bottom to top: thick
blot paper, PVDF membrane, SDS-PAGE gel and thick blot paper. Electrophoresis was then conducted at 15 V for 35 minutes.

To process the blot, the membrane was cut in half to generate sections containing either GLUT4 or the loading control, HSP90. The membrane sections were blocked at room temperature in 5 % skim milk (0.5 g skim milk powder in 10 mL TBS containing 0.1 % Tween-20 [0.1 % TBS-t]) for a minimum of 1 hour with gentle agitation. A 0.1 % TBS-t dump wash was then performed and the membrane incubated overnight at 4°C in the same blocking solution with the requisite primary antibody (1:2500 dilution of mouse monoclonal anti-GLUT4 IgG or 1:2500 dilution of mouse monoclonal anti-HSP90 IgG1) with gentle agitation. After washing three times in 0.2 % TBS-t, the membrane sections were incubated for 1 hour at room temperature in 5 % skim milk containing 1:2000 dilution of the polyclonal goat anti-mouse IgG-HRP with gentle agitation. Membrane sections were then washed three times with 0.2 % TBS-t and immunoreactive bands detected by enhanced chemiluminescence (ECL) as per the manufacturer’s instructions.

Physiological Studies

Calorie-restriction

PEPCK transgenic rats were calorie-restricted from 5 weeks of age for 9 weeks (intervention), and from 14 weeks of age for 6 weeks duration (treatment). Body weights for the intervention group were assessed every week to ensure that the rats were gaining similar weights to that of the age-matched lean control with the amount of food given adjusted accordingly. Due to the significant differences in starting body weights at 14 weeks of age, rats from the treatment group could not be matched to the lean control due to ethical reasons. We therefore chose to prevent weight gain throughout the treatment period by adjusting the amount of food given accordingly so that final body weights were as close to the age-matched control. Since calorie-restricted PEPCK transgenic rats were fed at 0900 hr and ate their allocated food within a couple of hours, for experiments requiring a 6 hour (0600 – 1200 hr) or overnight fast, calorie-restricted PEPCK transgenic rats were allowed to eat ad-libitum before their fast to ensure comparable fasting times with rats from other experimental groups.
Dapagliflozin Administration
Six 10 mg dapagliflozin tablets were crushed into fine powder using a mortar and pestle, and dissolved in 1 litre of autoclaved water to make up a final concentration of 60 mg/L. This concentration was chosen following a 3 week preliminary study using a subset of female PEPCK transgenic rats and measuring fluid intake (see Appendix I for details). The drug solution was administered to PEPCK transgenic rats as drinking water from 5 weeks of age for 9 weeks, and from 14 weeks of age for 6 weeks duration. The drug solution was changed at least three times per week and freshly prepared each time. Body weight, food intake and fluid intake were assessed every week for the intervention/treatment periods. Random urinary glucose checks were performed to ensure that glycosuria was present. A wash-out period was not performed prior to experimentation.

Characterisation of PEPCK transgenic rats

General Surgical Procedures
Rats were weighed and anaesthetised with 60 mg/kg body weight of sodium pentobarbitone by intraperitoneal injection. The depth of anaesthesia was confirmed prior to surgery and throughout the one-step hyperglycaemic clamp, basal turnover and hyperinsulinaemic-euglycaemic clamp studies by the lack-of-reflex in response to a toe pinch. For the one-step hyperglycaemic clamp, basal turnover and hyperinsulinaemic-euglycaemic clamp studies, a silastic catheter filled with heparinised saline was inserted into the left carotid artery and the right jugular vein. The carotid catheter was used for blood sampling and the jugular catheter for infusion via KDS100 Syringe Pumps. A tracheostomy was performed to aid in breathing. In all experiments, rats were kept warm with the aid of heat mats and allowed to rest for approximately 20 minutes following the implantation of catheters. All blood samples taken were immediately centrifuged at top speed, the plasma collected, and the red blood cells resuspended in an equal volume of heparinised saline and reinfused into the rat via the carotid artery to avoid anaemic shock. The plasma samples were stored at -20°C for glucose, insulin and radioactivity measurements.
Phenotypic Analysis

Body weight & Food intake Measurements
Body weight and food intake were measured weekly during the light cycle. Food intake was measured by subtracting the weight of remaining chow from the weight of total chow provided and is shown as grams consumed per week. Feed efficiency was calculated as follows:

\[
\text{Feed efficiency (\%)} = \frac{\text{weight gained (grams per day)}}{\text{food intake (grams per day)}} \times 100
\]

Intraperitoneal Glucose Tolerance Test
The conscious intraperitoneal glucose tolerance test (IPGTT) was performed as previously described (Andrikopoulos, Blair, Deluca, Fam, & Proietto, 2008) but modified for rats. Following an overnight fast (approximately 16 hours), rats were weighed and a scalpel blade used to nick the tip of the tail to collect a droplet of blood for basal blood glucose measurement (0 minute time-point). A glucose bolus (1 g/kg body weight) was then injected into the intraperitoneal cavity and blood glucose measured at 15, 30, 45, 60 and 120 minute time-points. Insulin was not measured in these studies. At the conclusion of the conscious IPGTT, all rats were given standard laboratory chow ad-libitum with the exception of the calorie-restricted PEPCK transgenic rats, which were given a pre-weighed amount.

One-step Hyperglycaemic Clamp
The one-step hyperglycaemic clamp followed by an arginine bolus was performed as previously described (Lamontagne et al., 2013) but with some modifications. Following a 6 hour fast (0600 – 1200 hr), a 200 µL blood sample was collected to measure basal blood glucose and plasma insulin concentrations before glucose infusion at -20 and 0 minute time-points. A glucose bolus (375 mg/kg body weight) was then injected into a jugular catheter connected to a Y-junction to initially raise blood glucose levels. At 2 minutes, a pump containing various concentrations of glucose solution ranging from 30 % for PVG/c rats, 10-20 % for PEPCK transgenic rats, 20 % for calorie-restricted PEPCK transgenic rats and 30 % for PEPCK transgenic rats treated with dapagliflozin were infused to maintain blood glycaemia between 15-17 mmol/L for 120 minutes. Blood glucose was checked every 5 minutes throughout the clamp.
experiment and the speed of the glucose pump adjusted accordingly. Blood samples were collected every 20 minutes from 20 to 120 minutes. At the completion of the one-step hyperglycaemic clamp, glucose infusion was stopped and a bolus of arginine (1 g/kg body weight) was injected at 121 minutes to assess β-cell secretory capacity and blood collected at 2, 5, 10, 15 and 30 minutes thereafter.

**Basal Turnover & Hyperinsulinaemic-euglycaemic Clamp**

Basal turnover and hyperinsulinaemic-euglycaemic clamps were performed as previously described (Lamont et al., 2003; Nolan & Proietto, 1994) but with a 6 hour fast (0600 – 1200 hr).

For basal turnover, rats were infused from a single pump with an initial 2 minute priming dose of radiolabelled glucose tracer [6-3H]-glucose at a rate of 100 µBq.min⁻¹ in 0.9 % saline, followed by a constant infusion of tracer at a rate of 5.5 µBq.min⁻¹ in 0.9 % saline for the duration of the experiment. Blood glucose was monitored every 10 minutes using a hand-held glucometer. Blood samples were collected at baseline (0 minute time-point) and during steady-state at 90, 100 and 110 minute time-points.

For the hyperinsulinaemic-euglycaemic clamp, the jugular catheter was connected to a Y-junction and received solutions from two separate pumps. From the first pump, tracer and insulin were co-administered with an initial 2 minute priming dose at a rate of 100 µBq.min⁻¹ followed by a constant infusion rate at either 6 mU/kg body weight in PVG/c rats, 7 mU/kg body weight in PVG/c rats treated with dapagliflozin, 4 mU/kg body weight in PEPCK transgenic rats, 5 mU/kg body weight in calorie-restricted PEPCK transgenic rats and 6 mU/kg body weight in PEPCK transgenic rats treated with dapagliflozin to produce similar final plasma insulin concentrations. From the second pump, various concentrations of glucose solution ranging from 25 % for PVG/c rats, 35-40 % for PVG/c rats treated with dapagliflozin, 3.5-12.5 % for PEPCK transgenic rats, 15 % for calorie-restricted PEPCK transgenic rats and 25-40 % for PEPCK transgenic rats treated with dapagliflozin were infused to maintain euglycaemia. Blood samples were collected during steady-state conditions at 90, 100 and 110 minute time-points. At the completion of the hyperinsulinaemic-euglycaemic clamp, three samples of the infusate solution were collected in scintillation vials for 5 minutes each in order to accurately measure the tracer infusion rate (dpm/mL). An additional three samples of
the infusate solution were also collected in pre-weighed eppendorf tubes for 5 minutes each so that the rate of solution infused per minute could be accurately measured.

**Determination of Radioactivity in Plasma and Infusates**

Plasma samples collected during steady-state conditions in both the basal turnover and hyperinsulinaemic-euglycaemic clamp studies were treated so that the level of [6-³H]-glucose radioactivity could be measured as previously described (Lamont et al., 2003; Nolan & Proietto, 1994). To determine [6-³H]-glucose radioactivity, 25 µL of plasma was de-proteinised by combining with equal volumes of 0.3 M barium hydroxide and 0.3 M zinc sulphate, and centrifuged at 15,000 rpm for 5 minutes. The supernatant was then passed through an anion exchange column to remove radioactively labelled lactate and pyruvate and eluted with 4 mL distilled water into scintillation vials. The eluent was oven-dried at 60°C overnight to remove radioactively labelled water. To the treated plasma samples and infusates, 4 mL distilled water and 10 mL scintillation cocktail were added, shaken vigorously and the radioactivity measured as disintegrations per minute (dpm) on a Tri-Carb Liquid Scintillation Analyser. A scintillation vial containing only 4 mL distilled water and 10 mL scintillation cocktail was used as a blank control.

**Calculation of Whole-Body Glucose Turnover**

Whole-body glucose turnover was calculated based on the Two-Compartment Model which assumes that under steady-state, the ratio of [6-³H]-glucose is the same in all compartments measured. Given this assumption, the rate of glucose appearance (Ra) at steady-state under basal conditions can be calculated using the following equation:

\[
Ra = \frac{F}{SA}
\]

where \( F \) = the rate of [6-³H]-glucose infused into the animal (dpm/min) and is calculated by dividing the radioactivity of the [6-³H]-glucose infusate (dpm/mL) by the rate of the solution infused into the animal (mL/min), \( SA \) = the specific activity of glucose (dpm/µmol) and is calculated by dividing the radioactivity of the plasma [6-³H]-glucose measured from the scintillation counter (dpm/mL) by the plasma glucose concentration (µmol/mL).
**Insulin-stimulated 2DG Uptake in Peripheral Tissues**

Following the collection of blood for the last time-point in the hyperinsulinaemic-euglycaemic clamp, a bolus of 2DG (370 µBq) was infused into the carotid artery and approximately 200 µL of blood samples collected at 2, 5, 10, 15, 30 and 45 minutes thereafter. Immediately following the collection of the last blood sample at 45 minutes, the rats were sacrificed by a lethal dose of sodium pentobarbitone and tissues including the liver, kidney, white quadriceps, red gastrocnemius and epididymal white adipose tissue (WAT) were rapidly removed, frozen in liquid nitrogen and stored at -80°C. The time course of 2DG disappearance from plasma and the degree of phosphorylated 2DG accumulation in individual tissues were determined. Glucose uptake into white quadriceps, red gastrocnemius and epididymal fat were calculated using the method described by (Kraegen, James, Jenkins, & Chisholm, 1985).

**Fat Pad, Kidney and Pancreata Mass**

At the termination of experimental procedures, a number of PVG/c, PEPCK transgenic, calorie-restricted PEPCK transgenic and PEPCK transgenic rats treated with dapagliflozin were sacrificed and fat pads, kidneys and pancreata removed for subsequent weighing. The subcutaneous and epididymal fat pads were collected from the right side of the animal, weighed and doubled as an estimation of total subcutaneous and epididymal fat mass. The infrarenal fat pad was collected around the kidney on the left hand side of the animal, weighed and doubled as an estimation of total infrarenal fat mass. The total fat pad mass was calculated by adding up the total subcutaneous, infrarenal and epididymal fat mass together. Both kidneys were collected and the kidney capsule removed before being weighed. Kidneys belonging to calorie-restricted PEPCK transgenic rats were not weighed. Pancreata were dissected from the spleen, duodenal loop and the small and large intestine. Any visible fat was then removed and the pancreas weighed.

**Glucose Measurement**

**Blood Glucose**

A glucose strip (Accu-Chek Performa) was inserted into a calibrated hand-held glucometer (Accu-Chek Performa). Upon indication by the glucometer to apply blood, a droplet of tail blood was drawn onto the strip to measure the glucose concentration.
Plasma Glucose
Plasma glucose concentrations were measured using a GM7 Micro-Stat Glucose Analyser (Analox Instruments Ltd). This system employs a glucose oxidation method to determine the maximum rate of oxygen uptake which is directly proportional to the amount of glucose in the sample.

Urinary Glucose
The presence and concentration of glucose in the urine was determined by using Diastix glucose test strips. Briefly, a Diastix glucose test strip was dipped into fresh urine and the change in colour observed after 30 seconds and compared to the glucose colour chart located on the bottle.

Insulin Measurement

Radioimmunoassay
Insulin concentrations of samples from the one-step hyperglycaemic clamp and the islet insulin secretion/content studies were determined using a rat specific insulin radioimmunoassay kit manufactured by LINCO Research (St Charles, MO, USA). The assay uses the $^{125}$I-labelled insulin and rat insulin antiserum to determine the level of insulin by the double antibody technique. Where required, samples were diluted with the assay buffer provided in the kit and measured in a Packard Cobra II Auto Gamma Counter using the automated data reduction procedure.

Enzyme-linked Immunosorbent Assay
Insulin concentrations of samples from the basal turnover and hyperinsulinaemic-euglycaemic clamp studies were determined using a rat specific insulin enzyme-linked immunosorbent assay manufactured by ALPCO (USA). The assay uses a “sandwich” technique whereby two monoclonal antibodies are directed against specific antigens on the insulin sample. The wells are coated with the first antibody and after the sample is added, a second antibody layer is applied to bind to the insulin antigen. This second antibody contains an enzyme which reacts when the 3,3’,5,5’-Tetramethylbenzidine substrate is added. This reaction produces a detectable colour change used to determine the level of insulin. Where required, samples were diluted with the zero standard
provided in the kit and measured in a Sunrise plate reader (Switzerland) at 450 nm with a reference range of 620 nm.

**Blood β-ketone Measurement**
A β-ketone test strip was inserted into a calibrated hand-held glucometer (Medisense Optium). Upon indication by the glucometer to apply blood, a droplet of tail blood was drawn onto the strip to measure the β-hydroxybutyrate concentration.

**Triglyceride Measurement**
Triglyceride concentrations of plasma were determined using a triglyceride determination kit. The assay procedure involves enzymatic hydrolysis by lipase of the triglycerides to glycerol and free fatty acids. The glycerol produced is then measured by coupled enzyme reactions. Where required, samples were diluted with 0.9 % saline and measured in a Sunrise plate reader at 540 nm.

**Histology**
Epididymal adipose tissue was stored in 10 % Neutral Buffered Formalin at room temperature for up to a week and transferred to 70 % ethanol until processed. Fixed adipose tissue were sent to the Monash Histology Platform, Department of Anatomy and Developmental Biology, Monash University (Clayton, Victoria, Australia) to be paraffin embedded, sectioned at 4 µm and stained with haematoxylin and eosin.

**Immunohistochemistry**
Pancreata were stored in 10 % Neutral Buffered Formalin at room temperature for up to a week and transferred to 70 % ethanol until processed. Fixed pancreata were sent to Anatomical Pathology, Department of Medicine, University of Melbourne (Parkville, Victoria, Australia) to be paraffin embedded, sectioned at 5 µm, stained for insulin using a guinea pig polyclonal anti-porcine insulin antibody diluted 1:1000 and counterstained with haematoxylin. Three serial sections were taken to derive the entire pancreas.

**Digitising Images**
Digital images of microscopic sections were obtained through the Austin Health, Victorian Cancer Biobank Slide Scanning service (Heidelberg, Victoria, Australia). Using standard protocols, whole slide sections were line scanned using an Aperio
ScanScope XT manufactured by Aperio Technologies (Vista, CA, USA) at 40x magnification at a resolution of 0.5 µm/pixel. Digital images were analysed using Aperio image software, ImageScope version 12.0. The field of view used to analyse tissue sections were 8x magnification for pancreas and 11x for epidydymal adipose tissue.

β-cell mass was calculated using the following equation:

\[
\text{β-cell mass (mg)} = \frac{\text{total area of insulin stained β-cells (μm}^2\text{)}}{\text{total pancreas area (μm}^2\text{)}} \times \text{pancreas weight (mg)}
\]

In Vitro Islet Studies

All incubations were at 37°C in a humidified atmosphere of 95 % air: 5 % CO₂.

Islet Insulin Secretion

Following the overnight recovery of isolated islets as described in Section 2.4.1, islets were hand-picked into pre-warmed KRBB (129 mM sodium chloride, 4.8 mM potassium chloride, 2.5 mM calcium chloride, 1.2 mM potassium dihydrogen phosphate, 1.2 mM magnesium sulphate, 5 mM sodium bicarbonate, 10 mM HEPES pH 7.4) supplemented with 0.1 % BSA and containing 2.8 mmol/L D-glucose for 60 minutes. Triplicate batches of five islets of comparable size were then transferred to conical tubes containing pre-warmed 1 ml KRBB supplemented with 2.8 mmol/L D-glucose, 20 mmol/L D-glucose, 20 mmol/L D-glucose plus 10 mmol/L L-arginine or 20 mmol/L D-glucose plus 275 µM tolbutamide and incubated for 60 minutes. At the conclusion of the incubation time, the conical tubes containing the islets were centrifuged at 2,000 rpm for 5 minutes to pellet the islets. The 0.5 ml supernatant was removed for insulin secretion and the remaining 0.5 ml containing the pelleted islets was immediately pulse sonicated on ice very briefly (maximum of 10 seconds) to disrupt the individual cells. Both samples were stored at -20°C for insulin secretion and content measurements.

Islet mRNA Analysis: Glut2, Gck, Sur1 and Kir6.2

Following the overnight recovery of isolated islets as described in Section 2.4.1, total RNA was isolated from at least 80 islets per rat using the same method as previously described (Section 2.4.2.1). Once RNA quality and quantity was verified (Section
2.4.2.3), total RNA (4 µg) from each islet sample was reverse transcribed using the Promega Reverse Transcription System (Promega) as described in Section 2.4.2.5 in order to synthesis cDNA. TaqMan PCR was used for Real-Time quantitative PCR of Glut2, Gck, Sur1 and Kir6.2 mRNA expression. The relative mRNA transcript level of target genes was calculated as $\Delta\Delta C_t$ and normalised to the mRNA transcript level of 18S and reported as fold change.

**Statistical Analysis**

All data are presented as mean ± standard error of the mean (SEM) from repeated experiments. Statistical significance was assessed in two populations using the two-tailed, unpaired Student’s t-test with equal variances. In three or more populations, statistical significance was assessed using a one-way ANOVA with Tukey’s multiple comparison post hoc test. Incremental area under the curve was calculated using the trapezoid rule. A $P$-value < 0.05 was considered statistically significant. GraphPad Prism 6 (CA, USA) was used to plot graphs and perform statistical analyses.
Chapter 3 Characterisation of the PEPCK transgenic rat as a suitable model of progressive β-cell dysfunction

Introduction
T2D is a progressive disease that is characterised by elevated blood glucose levels which arise from a combination of defects in both insulin action and secretion (S. E. Kahn, 2003). Due to its increasing prevalence and high cost of treatment, T2D places an enormous economic burden on Australia with approximately $14.6 billion from the nation’s health care budget allocated to treatment per year (C. M. Lee et al., 2013). As a result, identifying preventative measures has become crucial, necessitating the need to use and develop pre-clinical models of T2D to evaluate potential strategies for treatment and prevention of the disease and its associated complications.

Some of the most commonly used rodent models of T2D include the high-fat fed rodent, the ZDF rat, the C57BL/Ks-db/db mouse and the NZO mouse, all of which exhibit obesity-induced insulin resistance that can lead to β-cell dysfunction and T2D. While these rodent models have contributed significantly towards our understanding of the mechanisms involved in the progression of T2D, they possess their own limitations that can make it difficult to accurately extrapolate findings into the clinical setting.

High-fat feeding in rodents has been extensively used as an easy and reliable way to investigate the progression of IGT and insulin resistance (C. Y. Wang & Liao, 2012). This diet can also be used to investigate the development of β-cell dysfunction and T2D; however, observing a β-cell phenotype is highly variable and is dependent upon a number of factors such as the genetic predisposition and sex of the background strain used, the macronutrient and micronutrient composition of the diet, and the timing in which diet-induced obesity is established. A comparative analysis between six separate studies using the same mouse strain, the C57BL/6J, clearly illustrates this disparity with the onset and degree of hyperglycaemia and hyperinsulinaemia occurring
at different stages of high-fat feeding (Burcelin, Crivelli, Dacosta, Roy-Tirelli, & Thorens, 2002; Gallou-Kabani et al., 2007; Reimer & Ahren, 2002; Sone & Kagawa, 2005; Winzell & Ahren, 2004; L. Wu et al., 2006). Indeed, one may argue that these variabilities should not be considered an issue since in human diabetes, patients often display a wide range of glucose intolerance, insulin resistance and loss of β-cell function depending on the stage of the disease. However, in reality, the discrepancies in phenotype seen in the C57BL/6J strain (and other strains not mentioned here) cannot be explained by differences in genetics because each C57BL/6J mouse has the same genetic background. Therefore, the major limitation with this model lies in the lack of reproducible results amongst researchers. For this reason, inducing T2D in rodents using a high-fat diet appears to have limited translatable benefit for understanding and treating human T2D.

The main cause of obesity and insulin resistance in the ZDF rat and the C57BL/Ks-db/db mouse is a monogenic mutation in the leptin receptor that results in deficient leptin signalling that is not commonly seen in the human population with T2D (H. Chen et al., 1996; Phillips et al., 1996). This primary genetic alteration results in the severe-onset of obesity due to hyperphagia and reduced energy expenditure, which then contributes to insulin resistance and hyperglycaemia at a very early age (Srinivasan & Ramarao, 2007). These phenotypes are in stark contrast to what naturally occurs in human obesity and insulin resistance that result in T2D, with both being less severe and present during adolescence or adulthood. Moreover, leptin has been shown to oppose the effects of insulin resistance via the activation of AMPK causing reduced lipid accumulation in insulin sensitive tissues (B. B. Kahn, Alquier, Carling, & Hardie, 2005). The lack of leptin signalling in both of these models may therefore play a role in the development of T2D involving mechanisms that do not commonly occur in human diabetes. These substantial differences between leptin receptor-deficient rodent models and human T2D greatly limit their appropriateness in translational and diabetes prevention studies.

Since T2D is considered a polygenic disease, it would follow on that polygenic rodent models such as the NZO mouse are more suitable to define and characterise human T2D. In attempts to identify genes contributing to T2D, a number of studies
utilising this rodent model have led to the identification of multiple susceptible loci. For example, our laboratory has performed genome-wide scans in conjunction with phenotypic experiments in congenic strains crossed on the NZO background to show that Sur1 contributes to early β-cell dysfunction (Andrikopoulos et al., 2016). Besides being a well-established model of obesity and glucose intolerance, it also exhibits all of the cardinal defects of human T2D including fasting hyperglycaemia, hyperinsulinaemia and hepatic and peripheral insulin resistance (Veroni et al., 1991). However, due to the contribution of many genes that are thought to be linked to a variety of defects that contribute to the overall diabetic phenotype seen in the NZO mouse, this model is not suitable to investigate the research question of whether a primary defect in hepatic insulin resistance can cause glucose intolerance and hyperglycaemia, and whether these perturbations can lead to β-cell dysfunction.

In order to address this question, our laboratory generated a rodent model genetically geared towards glucose overproduction that results in a primary defect in hepatic insulin resistance (Rosella et al., 1995). This rodent model was achieved by over-expressing the gluconeogenic enzyme, PEPCK, in the liver and kidney under the control of a non-insulin responsive promoter. The use of this promoter was particularly important to the success of the glucose overproduction phenotype observed in our transgenic rodent model as it ensured that this rate-limiting step of gluconeogenesis remains non-responsive to the suppressive effects of insulin. The overall aim of this chapter was to phenotypically characterise the PEPCK transgenic rat as a suitable model of mild obesity, glucose intolerance, hyperglycaemia and β-cell dysfunction. To this end, we examined the progression of normal to impaired GSIS in the PEPCK transgenic rat and utilised in vivo and in vitro techniques to elucidate the mechanisms by which a defect in GSIS occurs. Our results clearly show that the PEPCK transgenic rat progressively develops defective GSIS in parallel with the worsening of glucose tolerance and that this is primarily due to the reduction in key genes necessary for β-cell function. This model provides several advantages over some of the currently available models discussed herein. The first advantage is the development of mild obesity that is not precipitated by defective leptin signalling, an uncommon feature in human T2D, whilst the second advantage is that our model has a later age onset of β-cell dysfunction that gradually worsens with time, as observed in the majority of the population with
human T2D. This last point is of significant importance to this thesis as it allows scope to successfully conduct further experiments involving treatment and preventative measures using our rodent model.

Chapter Aims
The aims of this chapter are as follows:

1. To determine the mechanism/s involved in defective GSIS in the PEPCK transgenic rat using *in vivo* and *in vitro* experimental techniques.
2. To evaluate how the progression of IGT affects β-cell mass/β-cell area and pancreatic islet size in the PEPCK transgenic rat.

Results

**PEPCK transgenic rats become obese due to over-eating.**

Growth parameters and measures of adiposity in 5, 14 and 20 week-old male control and PEPCK transgenic rats are shown in Table 3.1. At 5 weeks of age, there were no differences in body weight, food intake, feeding efficiency or total white adiposity mass between control and PEPCK transgenic rats. As the rats aged, PEPCK transgenic rats became significantly heavier than their control counterparts. This increase in body mass at both 14 and 20 weeks of age was associated with significant increases in food intake and total white adiposity mass, in particular, infrarenal and epididymal fat depots. When fat pad weights were normalised to body weight, PEPCK transgenic rats still had significantly more fat at 14 and 20 weeks of age. Although there were no differences in feeding efficiency at 14 weeks of age despite the increased weight gain, feeding efficiency was significantly higher in 20 week-old PEPCK transgenic rats indicating that consumed calories were still being used for body growth.
Table 3.1: Body weight, food intake and white adiposity mass in 5, 14 and 20 week-old control and PEPCK transgenic rats.

<table>
<thead>
<tr>
<th></th>
<th>5 weeks old</th>
<th></th>
<th>14 weeks old</th>
<th></th>
<th>20 weeks old</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PEPCK transgenic</td>
<td>Control</td>
<td>PEPCK transgenic</td>
<td>Control</td>
<td>PEPCK transgenic</td>
</tr>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>108.6 ± 6.4 (6)</td>
<td>121.4 ± 4.0 (6)</td>
<td>277.3 ± 6.7 (6)</td>
<td>356.2 ± 5.9*** (6)</td>
<td>315.7 ± 8.9 (6)</td>
<td>419.1 ± 7.4*** (6)</td>
</tr>
<tr>
<td><strong>Weight gain from 5 weeks old (g)</strong></td>
<td>–</td>
<td>–</td>
<td>168.7 ± 3.1 (3)</td>
<td>234.7 ± 2.1*** (6)</td>
<td>207.2 ± 4.3 (3)</td>
<td>297.7 ± 7.4*** (6)</td>
</tr>
<tr>
<td><strong>Average food intake (g/day)</strong></td>
<td>14.8 ± 1.0 (6)</td>
<td>14.9 ± 1.3 (6)</td>
<td>19.4 ± 0.5 (7)</td>
<td>22.4 ± 1.0* (8)</td>
<td>22.5 ± 0.4 (4)</td>
<td>26.0 ± 0.8** (4)</td>
</tr>
<tr>
<td><strong>Feeding efficiency (%)</strong></td>
<td>33.4 ± 2.4 (6)</td>
<td>27.6 ± 2.7 (6)</td>
<td>10.4 ± 1.3 (7)</td>
<td>9.4 ± 2.6 (4)</td>
<td>2.5 ± 0.7 (7)</td>
<td>6.8 ± 1.2** (4)</td>
</tr>
<tr>
<td><strong>WAT mass (g)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>0.55 ± 0.09 (3)</td>
<td>0.58 ± 0.03 (3)</td>
<td>4.63 ± 0.43 (11)</td>
<td>5.00 ± 0.81 (9)</td>
<td>4.88 ± 0.45 (13)</td>
<td>5.41 ± 0.34 (10)</td>
</tr>
<tr>
<td>Infrarenal</td>
<td>0.10 ± 0.02 (3)</td>
<td>0.11 ± 0.01 (3)</td>
<td>2.15 ± 0.33 (11)</td>
<td>4.85 ± 0.70** (9)</td>
<td>1.27 ± 0.11 (13)</td>
<td>5.83 ± 0.37*** (10)</td>
</tr>
<tr>
<td>Epididymal</td>
<td>0.32 ± 0.02 (3)</td>
<td>0.23 ± 0.02* (3)</td>
<td>2.50 ± 0.24 (11)</td>
<td>4.16 ± 0.50** (9)</td>
<td>2.10 ± 0.09 (13)</td>
<td>5.98 ± 0.30*** (10)</td>
</tr>
<tr>
<td>Total WAT</td>
<td>0.97 ± 0.12 (3)</td>
<td>0.92 ± 0.05 (3)</td>
<td>9.28 ± 0.91 (11)</td>
<td>14.00 ± 1.82* (9)</td>
<td>8.26 ± 0.48 (13)</td>
<td>17.21 ± 0.79*** (10)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. The number of animals used for each strain is shown in parenthesis. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. age-matched control. Total WAT mass was calculated by adding subcutaneous, infrarenal and epididymal fat depots.
PEPCK transgenic rats develop fasting hyperglycaemia and hyperinsulinaemia.

Overnight fasted plasma glucose and insulin concentrations were measured and compared between 5, 14 and 20 week-old control and PEPCK transgenic rats to determine the effects of the PEPCK transgene on glycaemia and insulinaemia with age (Table 3.2). In addition, blood ketones and plasma triglycerides were measured in order to determine the effects of glucose overproduction on energy metabolism after an overnight fast in 14 and 20 week-old animals (Table 3.2). Six hour fasted blood glucose and plasma insulin were also measured, but only in 14 and 20 week-old rats since hyperglycaemic clamps were not performed at 5 weeks of age (Table 3.2). Despite expressing the PEPCK transgene in the liver and kidney, younger PEPCK transgenic rats showed no significant differences in overnight fasting parameters when compared to control. However, as both groups aged, transgenic rats developed mild hyperglycaemia following a 6 hour and overnight fast, which was accompanied by overnight fasting hyperinsulinaemia. Prolonged fasting caused a sustained decrease in the circulating blood ketone levels in PEPCK transgenic rats compared to the control group at both 14 and 20 weeks of age. The presence of increased fasting triglycerides in 14 and 20 week-old PEPCK transgenic rats confirm an associative relationship between increased weight gain, as shown in Table 3.1, and dyslipidaemia.
Table 3.2: Change in fasting glucose and insulin levels in control and PEPCK transgenic rats with age.

<table>
<thead>
<tr>
<th></th>
<th>5 weeks old</th>
<th>14 weeks old</th>
<th>20 weeks old</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PEPCK transgenic</td>
<td>Control</td>
</tr>
<tr>
<td><strong>6 hour fast</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood glucose (mmol/L)</td>
<td>Not measured</td>
<td>5.9 ± 0.1 (6)</td>
<td>6.6 ± 0.1** (6)</td>
</tr>
<tr>
<td>Plasma insulin (ng/mL)</td>
<td>Not measured</td>
<td>2.0 ± 0.3 (6)</td>
<td>2.0 ± 0.1 (6)</td>
</tr>
<tr>
<td><strong>Overnight fast</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma glucose (mmol/L)</td>
<td>5.2 ± 0.2 (15)</td>
<td>5.1 ± 0.2 (14)</td>
<td>5.9 ± 0.2 (8)</td>
</tr>
<tr>
<td>Plasma insulin (ng/mL)</td>
<td>0.15 ± 0.03 (15)</td>
<td>0.11 ± 0.02 (14)</td>
<td>0.71 ± 0.21 (8)</td>
</tr>
<tr>
<td>Blood ketones (mmol/L)</td>
<td>Not measured</td>
<td>1.83 ± 0.09 (4)</td>
<td>1.13 ± 0.05 (7)**</td>
</tr>
<tr>
<td>Plasma triglycerides (mmol/L)</td>
<td>Not measured</td>
<td>0.412 ± 0.036 (11)</td>
<td>1.067 ± 0.004*** (6)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. The number of animals used for each strain is shown in parenthesis. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. age-matched control.
PEPCK transgenic rats develop glucose intolerance with age.
To determine the progression of glucose intolerance in the PEPCK transgenic rat, 5, 14 and 20 week-old animals were subjected to an IPGTT (Figure 3.1A). The IPGTT revealed that PEPCK transgenic rats have NGT at 5 weeks of age but progress to develop glucose intolerance at 14 and 20 weeks as indicated by the significantly elevated incremental area under the glucose curve when compared to the control (Figure 3.1B).
Figure 3.1: Intraperitoneal glucose tolerance tests in 5, 14 and 20 week-old control and PEPCK transgenic rats. (A) Blood glucose and (B) incremental area under the glucose curve in 5, 14 and 20 week-old control and PEPCK transgenic rats. Data are presented as mean ± SEM. Number of animals included: 5 weeks-old, n = 3 for control and n = 4 for PEPCK transgenic, 14 weeks-old, n = 6 for each group, and 20 weeks-old, n = 3 for control and n = 5 for PEPCK transgenic. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. control.
**PEPCK transgenic rats have impaired glucose-stimulated insulin secretion.**

We have previously shown that 12-14 week-old PEPCK transgenic rats develop an acquired impairment in GSIS when challenged with an acute intravascular glucose tolerance test (IVGTT) (Mangiafico et al., 2011). Although the IVGTT is regularly used as a tool to assess β-cell function in rodent models of T2D, the test itself is artificial in that the glucose bolus given can raise blood glucose levels in excess of 30 mmol/L, a concentration that is at least three times higher than what is naturally seen in the PEPCK transgenic rat regardless of age (Blair, A. R.; personal communication, 2011). Achieving such high glucose levels therefore makes the insulin response entirely non-physiological and introduces the possibility of “washing out” any real effects. Finally, the IVGTT does not take into consideration the relationship between β-cell function and insulin sensitivity. On account of these reasons, we performed a one-step hyperglycaemic clamp which is a technique considered to be the “gold standard” for accurately determining both phases of insulin secretion in relation to insulin sensitivity. As we have previously shown no discernible differences in insulin sensitivity or secretion at 5 weeks of age (Mangiafico et al., 2011), control and PEPCK transgenic rats were not studied at this particular age. The glucose infusion rate (GIR) required to maintain glycaemia between 15-17 mmol/L during the experiment were significantly decreased by 40-47 % in PEPCK transgenic rats at both ages, indicative of profound insulin resistance (Figure 3.2A and E for 14 weeks-old and Figure 3.2B and F for 20 weeks-old). Hyperglycaemia induced robust insulin secretory responses in control rats at both 14 and 20 weeks of age, but not in PEPCK transgenic rats over the 120 minutes time-course (Figure 3.2C and D). The incremental area under the insulin curve for the duration of the one-step hyperglycaemic clamp experiment clearly shows impaired GSIS at 14 and 20 weeks of age (Figure 3.2C and D panel inserts).

To assess the total secretory capacity of the β-cell, glucose infusion was stopped and a bolus of arginine was injected at 121 minutes. In both ages tested, arginine stimulation significantly prolonged the rise in blood glucose in PEPCK transgenic rats, with this observation being clearly more prominent in the 14 week-old rats (Figure 3.2A and B dotted areas). In addition, the effect of arginine on the potentiation of insulin secretion was significantly higher at the 2 minute time-point in 14 week-old PEPCK transgenic rats, but was reversed at 20 weeks of age (Figure 3.2C and D dotted areas).
However, when this was expressed as incremental area under the insulin curve over the
30 minute testing period, no differences were observed between strains at either age
(Figure 3.2E and F right panels). These findings confirm that the PEPCK transgenic rat
has a defect that is selective for glucose, while having the same capacity to secrete
comparable levels of insulin as the control when stimulated with the non-glucose
secretagogue, arginine.
Chapter 3  Characterisation of the PEPCK transgenic rat

A) 14 weeks-old
   - Blood glucose (mmol/L)
   - Time (min)
   - Control vs. PEPCK

B) 20 weeks-old
   - Blood glucose (mmol/L)
   - Time (min)
   - Control vs. PEPCK

C) GSIS (0-120 min)
   - Plasma insulin (ng/mL)
   - Control vs. PEPCK

D) GSIS (0-120 min)
   - Plasma insulin (ng/mL)
   - Control vs. PEPCK

E) Glucose infusion rate
   - Arginine (0-30 min)
   - Control vs. PEPCK

F) Arginine (0-30 min)
   - IACU insulin (ng/mL x min)
   - Control vs. PEPCK
Figure 3.2: One-step hyperglycaemic clamp in 14 and 20 week-old control and PEPCK transgenic rats. (A) and (B) blood glucose, (C) and (D) plasma insulin levels, (E) and (F) glucose infusion rate during the course of the one-step hyperglycaemic clamp and incremental area under the insulin curve following a bolus of arginine (right panels) at the conclusion of the one-step hyperglycaemic clamp in 14 and 20 week-old control and PEPCK transgenic rats. Incremental area under the insulin curve for GSIS during the one-step hyperglycaemic clamp is illustrated as corresponding panel inserts. An arginine bolus (1 g/kg) was administered one minute after last sampling (121 minute time-point) and is represented by dotted areas. Data are presented as mean ± SEM. Number of animals included: 14 weeks-old, n = 6 for each group, and 20 weeks-old, n = 4 for control and n = 5 for PEPCK transgenic. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. control.
To determine the site of defective GSIS in the PEPCK transgenic rat, static incubations with glucose and other secretagogues were performed in isolated islets from 14 week-old control and PEPCK transgenic rats (Figure 3.3A). Insulin secretion in response to glucose was also measured in 20 week-old control and PEPCK transgenic rats (Figure 3.3B) to determine whether this parameter deteriorates with age. Non-glucose secretagogues were not used at this older age to avoid redundancy as they were performed at 14 weeks of age when the defect in GSIS first appears. Insulin content from extracted pancreata was also measured and compared at both ages (Figure 3.3C and D). During the isolation process, islet yield was much greater for control than PEPCK transgenic rats at both ages so islets of similar size were chosen for comparison. Both groups at 14 weeks of age had similar basal glucose insulin secretion. Consistent with our *in vivo* findings (Figure 3.2C and panel insert), islets from 14 week-old PEPCK transgenic rats displayed a defect in insulin secretion when challenged with 20 mmol/L glucose. Next, we assessed the responses to the non-glucose secretagogues, arginine (depolarises membrane and activates voltage-dependent calcium channels) and tolbutamide (binds to SUR1 and closes K\(_{ATP}\) channels), to determine whether the insulin secretory defect observed in the PEPCK transgenic rat was unique to the glucose-mediated insulin secretory pathway (Figure 3.3A). Similar to the findings in the hyperglycaemic clamp study performed in 14 week-old rats (Figure 3.2C dotted areas and E right panel), stimulation with glucose plus arginine showed no differences in the insulin secretory response between transgenic and control rats. In contrast, when challenged with glucose plus tolbutamide, a significant suppression of insulin secretion was observed in the pancreatic islets from PEPCK transgenic rats. Consistent with the worsening of GSIS with age, 20 week-old PEPCK transgenic rat islets did not respond to stimulatory levels of glucose (Figure 3.3B). These findings at both ages are particularly striking considering that PEPCK transgenic rats contain 1.2- and 2.4-fold more islet insulin content at 14 and 20 weeks of age, which may have been expected to lead to higher insulin secretion. These *in vitro* findings together with the *in vivo* GSIS work strongly suggest that the insulin secretory defect in the PEPCK transgenic rat is functional in nature and not driven by a lack of insulin production and/or storage as indicated by the increase in islet insulin content.
Figure 3.3: Glucose and secretagogue-stimulated insulin secretion in isolated pancreatic islets from 14 and 20 week-old control and PEPCK transgenic rats. Insulin release from (A) 14 week-old control and PEPCK transgenic rat pancreatic islets stimulated with 2.8 mmol/L glucose, 20 mmol/L glucose, 20 mmol/L glucose plus 10 mmol/L arginine or 20 mmol/L glucose plus 275 μM tolbutamide; (B) 20 week-old control or PEPCK transgenic rat pancreatic islets stimulated with 2.8 mmol/L glucose or 20 mmol/L glucose, and insulin content in islets isolated from (C) 14 week-old
and (D) 20 week-old control and PEPCK transgenic rats. Data are presented as mean ± SEM. Number of animals included: 14 week-old, $n = 3-10$ for each group, and 20 week-old, $n = 3-5$. For 14 week-old glucose and secretagogue-stimulated insulin secretion: *$P < 0.05$ vs. control islets stimulated with 20 mmol/L glucose, **$P < 0.01$ vs. control islets with 20 mmol/L glucose plus tolbutamide; *$P < 0.01$ vs. 2.8 mmol/L glucose control; †$P < 0.05$ vs. 2.8 mmol/L glucose PEPCK transgenic. For 14 week-old islet insulin content: *$P < 0.05$ vs. control. For 20 week-old glucose-stimulated insulin secretion: **$P < 0.01$ vs. control islets with 20 mmol/L glucose; *$P < 0.01$ vs. 2.8 mmol/L glucose control. For 20 week-old islet insulin content: ***$P < 0.001$ vs. control. Abbreviations: N.S., not significant.
The down-regulation of Glut2 may contribute to the initial impairment in glucose-stimulated insulin secretion in the PEPCK transgenic rat.

To determine which genes may contribute to the defect in GSIS in PEPCK transgenic rats at 14 and 20 weeks of age, the mRNA levels of the glucose sensors, Glut2 and Gck, and the subunits that make up the ATP-sensitive $K^+$ channel, Sur1 and Kir6.2, were measured in isolated pancreatic islets (Figure 3.4B and C). These genes were also measured in 5 week-old PEPCK transgenic rats (Figure 3.4A). There were no differences in gene expression at 5 weeks; however, Glut2 was found to be significantly down-regulated by 34 % and 76 % in 14 and 20 week-old PEPCK transgenic rat islets, respectively. In addition, Gck gene expression was normal at 14 weeks of age and slightly reduced at 20 weeks of age in PEPCK transgenic rats, although this was not statistically significant ($P = 0.1$). In light of the compelling in vivo and in vitro data shown in Figure 3.2 and 3.3, there were no differences at the mRNA level of the constituents that make up the $K^{+}_{ATP}$ channel in islets taken from 14 week-old PEPCK transgenic rats (Figure 3.4B). On the other hand, Sur1 and Kir6.2 were significantly reduced in 20 week-old PEPCK transgenic islets (Figure 3.4C). Together, these results suggest that the initiating defect in GSIS at 14 weeks of age may be due to a reduction in Glut2 gene expression. GSIS in the PEPCK transgenic rat deteriorates further with the concomitant reduction in Glut2, Gck, Sur1 and Kir6.2 gene expression, as seen in the 20 week-old PEPCK transgenic rat.
Figure 3.4: Gene expression analysis of Glut2, Gck, Sur1 and Kir6.2 in isolated pancreatic islets from 5, 14 and 20 week-old control and PEPCK transgenic rats. Relative mRNA expression of the glucose sensing genes, Glut2 and Gck, and the subunits that make up the K⁺ATP channel, Sur1 and Kir6.2, in pancreatic islets isolated from (A) 5 week-old, (B) 14 week-old and (C) 20 week-old control and PEPCK transgenic rats. Data are presented as mean ± SEM. Number of animals included: 5 weeks-old, n = 4-6 for each group, 14 weeks-old, n = 3-6, and 20 weeks-old, n = 3-5. *P < 0.05 vs. control.
PEPCK transgenic rats have larger but fewer pancreatic islets.

To determine whether the observed defects in GSIS resulted from progressive alterations in islet morphology and/or reduced β-cell mass, morphometric and immunohistological analyses on pancreatic islets from 5, 14 and 20 week-old control and PEPCK transgenic rats were performed (Table 3.3). Representative pancreatic sections are 8x magnification (scale bar, 300 µm) and show insulin immunohistochemical staining (Figure 3.5). The pancreas from 14 and 20 week-old control and PEPCK transgenic rats were weighed and used as an approximate measure of pancreatic growth. There were no differences in pancreas weight at 14 weeks of age however, pancreata of 20 week-old PEPCK transgenic rats were significantly heavier than those from age-matched controls. However, when pancreas weight was corrected for body weight, no differences between groups at either age were observed. Our findings show that young transgenic rats begin with 43% fewer islets than their control counterparts and this continues to significantly decline with age. Moreover, 5 week-old PEPCK transgenic rats possess comparable sized islets as the control, with these islets becoming substantially larger at 14 weeks of age when profound insulin resistance is present (Lamont et al., 2003). The observation of increased islet size is consistent with the PEPCK transgenic rat having 14.9% of distributed islet size skewed toward islets larger than 20,001 µm² versus the 5.8% in the control. This suggests that the islets are compensating for the insulin resistance by either increasing β-cell number or β-cell size as is evident by the significant increase in insulin positive staining and a trend for increased β-cell mass ($P = 0.09$) compared to age-matched controls. Consistent with the worsening of both in vivo and in vitro GSIS and the reduction in key genes involved in the GSIS pathway, the islets of 20 week-old PEPCK transgenic rats become smaller and decline in number when compared with 14 week-old PEPCK transgenic rats. These changes coincide with a significant reduction in β-cell mass ($P = 0.01$) compared to 14 week-old PEPCK transgenic rats even though insulin positive staining remains the same and significantly higher than the age-matched control. Since we did not weigh pancreata at 5 weeks of age, we used β-cell area as an approximate measure for β-cell mass. We found that 5 week-old PEPCK transgenic rats possess significantly lower total β-cell area than control, but this is restored at 14 weeks of age. Whether the initial reduction in total β-cell area (and perhaps β-cell mass) and islet number in 5 week-old
PEPCK transgenic rats contribute to the defect in GSIS at 14 weeks of age is not currently known. Taken together, these data suggest that a functional defect in the β-cells of the PEPCK transgenic rat is present before a decline in β-cell mass is observed.
Figure 3.5: Insulin immunohistochemical staining of the pancreas of 5, 14 and 20 week-old control and PEPCK transgenic rats. Representative images of insulin immunohistochemical staining of pancreatic sections in 5 week-old (A) control and (B) PEPCK transgenic, 14 week-old (C) control and (D) PEPCK transgenic, and 20 week-old (E) control and (F) PEPCK transgenic rats. The insulin-containing β-cells stain brown and the vascular endothelial cells and exocrine portion of the pancreas appear blue. 8x magnification; scale bar, 300 µm.
Table 3.3: Quantitative insulin immunohistochemical staining of pancreatic sections of 5, 14 and 20 week-old control and PEPCK transgenic rats.

<table>
<thead>
<tr>
<th></th>
<th>5 weeks-old</th>
<th>14 weeks-old</th>
<th>20 weeks-old</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PEPCK</td>
<td>Control</td>
</tr>
<tr>
<td>Pancreas weight (g)</td>
<td>Not measured</td>
<td>1.3 ± 0.1 (6)</td>
<td>1.5 ± 0.1 (4)</td>
</tr>
<tr>
<td>Average islet size (µm²)</td>
<td>2258 ± 154 (6)</td>
<td>2148 ± 369 (6)</td>
<td>4807 ± 526 (9)</td>
</tr>
<tr>
<td>Islet number (per mm² pancreas area)</td>
<td>2.75 ± 0.13 (6)</td>
<td>1.57 ± 0.22*** (6)</td>
<td>1.61 ± 0.14 (9)</td>
</tr>
<tr>
<td>Islet size distribution (%)</td>
<td></td>
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<tr>
<td>&lt;1,000 µm²</td>
<td>58.8 ± 1.7</td>
<td>63.4 ± 4.4</td>
<td>33.3 ± 3.6</td>
</tr>
<tr>
<td>1,001 – 3,000 µm²</td>
<td>22.9 ± 1.2</td>
<td>12.7 ± 2.2*</td>
<td>31.3 ± 2.2</td>
</tr>
<tr>
<td>3,001 – 5,000 µm²</td>
<td>6.7 ± 1.0</td>
<td>11.0 ± 2.9</td>
<td>12.6 ± 1.7</td>
</tr>
<tr>
<td>5,001 – 10,000 µm²</td>
<td>7.3 ± 0.8</td>
<td>8.6 ± 0.8</td>
<td>10.0 ± 1.0</td>
</tr>
<tr>
<td>10,001 – 20,000 µm²</td>
<td>2.7 ± 0.3</td>
<td>3.8 ± 1.8</td>
<td>7.0 ± 1.1</td>
</tr>
<tr>
<td>&gt;20,001 µm²</td>
<td>1.6 ± 0.4</td>
<td>0.4 ± 0.4</td>
<td>5.8 ± 1.4</td>
</tr>
<tr>
<td>Insulin+ area (% islet area)</td>
<td>47.7 ± 2.4 (6)</td>
<td>51.2 ± 3.8 (6)</td>
<td>60.0 ± 2.8 (9)</td>
</tr>
<tr>
<td>β-cell area (% pancreas area)</td>
<td>0.24 ± 0.02 (6)</td>
<td>0.15 ± 0.03* (6)</td>
<td>0.45 ± 0.07 (9)</td>
</tr>
<tr>
<td>β-cell mass (mg)</td>
<td>Not measured</td>
<td>5.2 ± 0.6 (6)</td>
<td>6.7 ± 0.2 (4)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. The number of animals used for each strain is shown in parenthesis. *P < 0.05 and **P < 0.01 vs. age-matched control. ImageScope software was used for quantification of pancreatic images.
Discussion

In order for a rodent model to be translatable to human disease, it must effectively recapitulate the natural history, pathophysiology and the associated complications in a manner similar to what is observed in humans. This is very seldom the case with the vast majority of diabetic rodent models developing obesity, insulin resistance and β-cell dysfunction in a very short period of time, or in an inconsistent manner. In contrast, the progression of T2D in the human population results from a mixture of genetic and environmental factors that leads to disease development and which can take years to decades to fully develop. In this regard, it is important to acknowledge that rodent models of T2D have their limitations; however, they can be used effectively to answer specific research questions with respect to various stages in the pathogenesis of human T2D, i.e., IGT, β-cell dysfunction and β-cell failure. Data generated from rodent models that progress to T2D or are made to have severe hyperglycaemia have shown marked β-cell dysfunction and impaired GSIS (Bonner-Weir, Trent, & Weir, 1983; Clark, Palmer, & Shaw, 1983; Leahy, Cooper, Deal, & Weir, 1986; Portha et al., 2001; Rossetti, Shulman, et al., 1987; Sinha, Baxter, Larson, & Vanderlaan, 1979; Veroni et al., 1991; Weir, Clore, Zmachinski, & Bonner-Weir, 1981). These studies support the concept that chronic hyperglycaemia can cause structural and functional damage in β-cells. In this chapter, our aim was to characterise the progression of defective GSIS in the PEPCK transgenic rat, a model of primary chronic low-grade hyperglycaemia, using in vivo and in vitro experimental techniques.

Obesity is considered to be the number one driver in the development of T2D with a large majority of patients being either overweight or obese. One of the major flaws with many of the commonly used type 2 diabetic rodent models is that they possess defects in leptin signalling or are leptin receptor-deficient and as a consequence, become severely obese. The C57BL/Ks-db/db mouse is one such example of a rodent model with defective leptin signalling. Using data derived from the Jackson Laboratory, 14- and 20 week-old C57BL/Ks-db/db mice are on average 78 % heavier than the most commonly used control strain for diabetes research, the C57BL/6J. This large difference in body weight is not a fair representation of the current human population with T2D. Therefore, an important part of characterising the PEPCK transgenic rat as an appropriate model of β-cell dysfunction with translatable benefits to
the clinical setting was to confirm its mild obese phenotype. Consistent with observations in our laboratory, PEPCK transgenic rats gained significantly more body weight at both 14 and 20 weeks of age, which were accompanied by increases in infrarenal, epididymal and total fat pad mass (Lamont et al., 2003; Mangiafico et al., 2011; Rosella et al., 1995; Thorburn et al., 1999). Of note, the difference in body weight percentage between both strains at 14 and 20 weeks old was only a modest 28% and 33%, respectively. Since over-eating and reduced physical activity contribute greatly to the obesity epidemic (McAllister et al., 2009), we examined whether an increase in food intake was associated with the PEPCK transgenic rats’ mild obesity phenotype. We found that food consumption was higher at 14 and 20 weeks of age when compared to age-matched controls. We also calculated how efficient both groups were in converting calories consumed into weight gained (feeding efficiency) across the three ages tested. As expected, feeding efficiency was at its highest in 5 week-old rats, indicative of their rapid growth during weaning. As the rats aged, the amount of calories consumed that were translated into weight gain steadily decreased. Interestingly, 20 week-old PEPCK transgenic rats were still utilising consumed calories for body growth partly explaining their persistent weight gain at this older age compared to controls. As mentioned before, reduced physical activity can also contribute to obesity. Although physical activity was not measured in the current study, previous work have shown comparable physical activity levels in both 4-5 and 12-14 week-old PEPCK transgenic rats when compared to their age-matched control (Mangiafico et al., 2011). A contentious point that needs to be addressed is the possibility of ectopic expression of the PEPCK transgene into other organs implicated in increased food intake and obesity. It is well documented that the hypothalamus is the most critical tissue in the regulation of food intake and body weight, with it being able to sense and respond to changes in nutritional status of the animal (Schwartz, Woods, Porte, Seeley, & Baskin, 2000). We have previously shown that there is no PEPCK expression in the brain (Rosella et al., 1995) while unpublished results from our laboratory have shown undetectable levels of Pck1 mRNA in the hypothalamus of fed 4 week-old control and PEPCK transgenic rats (Appendix II). In addition, there is also some evidence to suggest that WAT may play a role in the development of obesity. This has been shown by Franckhauser et al. in which the over-expression of PEPCK in
WAT in mice fed a high-fat diet led to obesity via decreased energy expenditure, impaired adipose tissue lipid buffering, i.e., suppression of FFAs released and the increase in triglyceride clearance, and decreased thermogenesis (Franckhauser, Munoz, Elias, Ferre, & Bosch, 2006). This finding is particularly interesting considering that fed 4 week-old rats show significantly increased $Pck1$ mRNA expression in the subcutaneous WAT of PEPCK transgenic rats when compared to age-matched controls (Appendix II). Finally, the liver can act as a key regulator in appetite and body weight (Jambor de Sousa, Arnold, Langhans, Geary, & Leonhardt, 2006; Jambor de Sousa, Benthem, et al., 2006; Russek, 1963). Our laboratory has shown that the over-expression of FBPase in the livers of C57BL/6J mice consistently reduces body weight by 10-15% from weaning to 22 weeks of age compared to negative littermate controls. These findings were associated with reduced food intake but not voluntary physical activity levels or resting energy expenditure (Visinoni et al., 2012). With respect to the other two gluconeogenic enzymes, PEPCK and G6Pase, a lean phenotype has not been reported in liver-specific transgenic rodent models (Sun et al., 2002; Trinh, O'Doherty, Anderson, Lange, & Newgard, 1998). Based on these findings, we propose that the PEPCK transgenic rat becomes mildly obese due to hyperphagia and not from physical inactivity or from the PEPCK transgene being activated in the hypothalamus. Moreover, our finding of a ~2-fold induction over and above what was observed in the control strain could partly explain the mild obesity phenotype in the PEPCK transgenic rat. However, given that all of our experiments were conducted in rats given a standard laboratory diet devoid of high-fat and that mice over-expressing PEPCK specifically in adipose tissue only develop severe obesity when fed a high-fat diet and not a normal chow diet, we hypothesise that the additional $Pck1$ found in the subcutaneous adipose of the PEPCK transgenic rat is unlikely to be due to leakage of the PEPCK transgene. Although the exact underlying cause/s for hyperphagia in this rat model is still elusive and is outside the scope of this thesis, it is interesting to note that the increased weight gain in our transgenic model coincides with the appearance of hyperinsulinaemia which has recently been shown to have a potential role in diet-induced obesity (Mehran et al., 2012). Whether the increased expression of $Pck1$ in subcutaneous adipose tissue and perhaps in other white adipose depots, hyperinsulinaemia or a combination of both, are
contributing factors to the increase in weight gain observed in the PEPCK transgenic rat clearly warrants further investigation.

Insulin action and GSIS are considered to be the major determinants of glucose tolerance (Maheux, Chen, Polonsky, & Reaven, 1997). Previous work in our laboratory has established that hyperglycaemia alone is not sufficient to induce glucose intolerance without a defect in GSIS (Mangiafico et al., 2011). The progression from NGT to IGT with age in the PEPCK transgenic rat has been well documented (Mangiafico et al., 2011; Rosella et al., 1995). However, the relative importance of insulin resistance in relation to β-cell dysfunction in the glucose intolerant state has not been properly addressed as the initial defect in GSIS was found using an IVGTT, a method that does not distinguish the hyperbolic relationship between insulin sensitivity and β-cell function (S. E. Kahn et al., 1993). We therefore employed a more physiological technique, the one-step hyperglycaemic clamp, which takes into consideration this complex hyperbolic relationship. This test is the “gold-standard” for measuring β-cell sensitivity in both early- and late-phase GSIS in vivo (DeFronzo, Tobin, & Andres, 1979). Since 4-5 week-old PEPCK transgenic rats have NGT due to preserved glucose disposal and GSIS (Mangiafico et al., 2011), we did not perform a one-step hyperglycaemic clamp at this age. During the one-step hyperglycaemic clamp in both 14 and 20 week-old PEPCK transgenic rats, the GIR needed to maintain hyperglycaemia was significantly lower than that of the control group. As GIR reflects whole-body insulin sensitivity; the sum of EGP suppression and the stimulation of glucose disposal, this finding is in accordance with the insulin resistant phenotype of the PEPCK transgenic rat (Lamont et al., 2003; Mangiafico et al., 2011). The hyperglycaemic challenge at 14 weeks of age revealed significant reductions in early- (0-20 minutes, \( P = 0.02 \)) and late-phase GSIS (\( P = 0.01 \)) when the trapezoidal rule was used to determine the incremental area under the curve, with the former finding not being observed in the IVGTT (Mangiafico et al., 2011). It is well accepted that the selective loss of early-phase GSIS is a sign of β-cell dysfunction contributing to the development of both IGT and T2D (Bruttomesso et al., 1999; Calles-Escandon & Robbins, 1987; Cerasi & Luft, 1967; Gerich, 1997; Mitrakou et al., 1992). However, the reduction in the late-phase of GSIS and its role in glucose intolerance has not been well established (Pimenta et al., 1995). Therefore, the initial finding of a selective
defect in late-phase GSIS in the PEPCK transgenic rat in our previously published work was unexpected. The discrepancy between our previously published findings and the current findings in this thesis is most likely due to the methodological differences in the tests used to measure GSIS. The IVGTT involves a transient glucose excursion that reaches non-physiological levels whereas in the one-step hyperglycaemic clamp, glycaemia can be clamped at a more relevant physiological level. In addition to this, we cannot discount the possibility that the reduced early-phase of GSIS during the one-step hyperglycaemic clamp was masked in the IVGTT due to the non-physiological level of plasma glucose (~ 30 mmol/L) reached following glucose injection. This increased level of glycaemia could potentially facilitate the increased transport of glucose into the β-cell and elicit the over-compensatory increase in GSIS (Luni, Marth, & Doyle, 2012). Similarly, hyperglycaemia failed to induce a robust insulin secretory response at 20 weeks of age and was accompanied by a greater deterioration in early-phase GSIS as well as a similar reduction in late-phase GSIS to that found at 14 weeks of age. It is a common misconception that the level of circulating plasma insulin is an accurate reflection of the amount of insulin that has been secreted from the β-cell. Instead, circulating insulin involves a balanced relationship between the insulin produced and secreted by the pancreatic β-cell, and the rate of which it is cleared from the plasma by the liver, kidney and muscle (Duckworth, Bennett, & Hamel, 1998). The production of insulin within the β-cell involves many intermediate steps that occur in the endoplasmic reticulum, with the final step being the packaging of mature insulin and C-peptide. This makes C-peptide a useful bio-marker for insulin secretion as it is secreted alongside insulin in equimolar amounts into the bloodstream and therefore can be measured by clinicians to approximate the amount of insulin that the β-cell is secreting. Unfortunately, we did not measure C-peptide in the current study and so were unable to estimate the metabolic clearance rate of insulin or how much circulating insulin was actually attributed to the release from the β-cell directly. However, insulin clearance rates for insulin in humans have been shown to decrease in the presence of glucose intolerance (Bonora, Zavaroni, Coscelli, & Butturini, 1983), obesity (Meistas, Margolis, & Kowarski, 1983), insulin resistance (Flier et al., 1982) and elevated free fatty acids (Lewis, Carpentier, Adeli, & Giacca, 2002); all of which are metabolic abnormalities found in the PEPCK transgenic rat (Lamont et al., 2003; Mangiafico et al., 2011;
Thorburn et al., 1999). Therefore, we would hypothesise that a large proportion of the secreted insulin would remain in the systemic circulation and not be cleared by the liver, kidney and muscle. This would result in lower plasma C-peptide levels over the course of the clamp experiment which would cause us to be underestimating the insulin secretory defects present in both phases of GSIS at both ages. It is important to make mention that although there are a number of genetically modified rodent models in which some of the key gluconeogenic enzymes have been overexpressed in the liver, studies involving these rodent models have only directly tested the effects of increased EGP on glucose intolerance and hyperglycaemia, and not the consequences that increased EGP may have on β-cell function (Sun et al., 2002; Trinh et al., 1998; Valera, Pujol, Pelegrin, & Bosch, 1994). However, since the reported phenotypes in the rodent models mentioned above closely resemble that of our PEPCK transgenic rat, we would assume that β-cell function would deteriorate in concert with glucose intolerance and insulin resistance with age.

We next examined the β-cell secretory capacity in vivo of control and PEPCK transgenic rats at both ages by infusing a bolus of arginine after the hyperglycaemic challenge. Previous work has suggested that arginine-induced insulin secretion bypasses both glucose metabolism and the closure of the $K^{+}_{ATP}$ channels (Blachier, Mourtada, Sener, & Malaisse, 1989; P. A. Smith et al., 1997), thereby making it an attractive tool to estimate functional β-cell reserve (Ward, Bolghino, McKnight, Halter, & Porte, 1984). We hypothesised that stimulating the β-cell with arginine after a hyperglycaemic challenge would help us determine whether the defect was confined to glucose only, or whether it was also due to a defect in the insulin reserve pool. Since the magnitude of insulin secretion in response to arginine is dependent upon the ambient glucose level (Larsson & Ahren, 1998; Palmer, Walter, & Ensink, 1975; van Haeften, Voetberg, Gerich, & van der Veen, 1989), we infused arginine at the conclusion of the one-step hyperglycaemic clamp when glucose levels were carefully matched. Stimulation with arginine resulted in increased blood glucose excursions in PEPCK transgenic rats, but not in the control, at both ages. As the PEPCK transgenic rat is genetically geared for glucose overproduction and arginine is known to be a substrate utilised by the liver for gluconeogenesis (Floyd, Fajans, Conn, Knopf, & Rull, 1966), the concomitant increase in blood glucose was, in hindsight, to be expected. The
infusion of arginine at both ages did not reveal any subsequent defects in insulin secretion. As arginine does not stimulate insulin biosynthesis due to its electrogenic-natured entry, our results indicate that PEPCK transgenic rats have adequate levels of insulin pre-docked on the plasma membrane ready for release. This finding closely resembles that observed in newly diagnosed and non-obese type 2 diabetics evaluated by a glucose-dependent arginine stimulation test where there is a selective loss of acute GSIS but a preserved response to arginine (Fasching et al., 1994; Sjostrand et al., 2014). We cannot rule out that the increase in blood glucose concentration following the arginine bolus may have potentiated the direct action of arginine in stimulating the β-cell to secrete insulin. In order to remove the glucose potentiating effect seen in our transgenic rat model \textit{in vivo}, it would be worth performing the same experiment with a gluconeogenic inhibitor such as 3-mercaptopicolinic acid on-board, or even with a non-glucose secretagogue that is not involved in gluconeogenesis, such as leucine, but not in the presence of high glucose, e.g., after a hyperglycaemic challenge, as its proposed mechanism to induce insulin secretion is inhibited (M. J. MacDonald et al., 1991).

Glucotoxicity as a result of persistently high and chronic plasma glucose levels can have deleterious effects on the β-cell. There is substantial evidence from diabetic animal models, isolated pancreatic islets and transformed β-cell lines to suggest that persistent elevated levels of glucose can induce oxidative (Piro et al., 2002; Tanaka et al., 1999) and endoplasmic reticulum stress (Seo et al., 2008; B. Song, Scheuner, Ron, Pennathur, & Kaufman, 2008) that contribute to a reduction in β-cell function and mass. Together, all of these can lead to impaired GSIS via the reduced expression of several important genes such as insulin (Nielsen et al., 1985; Pirot et al., 2007; Wicksteed, Alarcon, Briaud, Lingohr, & Rhodes, 2003) and those involved in the insulin secretory pathway (Gremlich et al., 1997; Laybutt et al., 2003; Marselli et al., 2010; H. Wang, Kouri, & Wollheim, 2005). Consistent with our \textit{in vivo} GSIS findings, islets from 14 week-old PEPCK transgenic rats showed significantly reduced GSIS when stimulated with glucose. Furthermore, pancreatic islets that have been exposed to an unfavourable metabolic milieu, i.e., chronic mild hyperglycaemia and obesity for an additional six weeks, as seen in islets of the 20 week-old PEPCK transgenic rat, completely inhibits GSIS. There are a few reasons for these observations. Firstly, our findings from islets isolated from 14 week-old PEPCK transgenic rats can be explained, in part, by a 34 %
reduction in Glut2 gene expression. However, there are conflicting reports with respect to the level of Glut2 required to sustain normal GSIS. For example, a study using Glut2 null mice found that rescuing Glut2 expression to only 20% of the normal level of rat islets completely normalised GSIS (Thorens, Guillam, Beermann, Burcelin, & Jaquet, 2000). This raises the question on how much of a reduction of Glut2 expression is required to have an impact on islet metabolism considering that GCK is rate-controlling. Therefore, the use of a non-glycolytic metabolic stimulus such as glutamine or leucine may be an alternative method of determining whether GLUT2 and/or GCK are limiting in islets of the PEPCK transgenic rat.

Secondly, the level of hyperglycaemia “seen” by the islets of the PEPCK transgenic rat may also cause the internalisation of Glut2 and reduce the facilitated diffusion of glucose into the β-cell. This is most likely to be a consequence of the prevailing glycaemia, rather than the cause (Kluth et al., 2011). The lack of GSIS in the islets of 20 week-old PEPCK transgenic rats is the result of a combination of defects, with the most obvious being a further progressive loss in Glut2, significant reductions in Sur1 and Kir6.2 gene expression and an approximate 38% decline in β-cell mass. Interestingly, in light of defective GSIS, insulin content of size-matched islets and insulin positive staining of pancreata belonging to 14 and 20 week-old PEPCK transgenic rats were significantly increased when compared to their age-matched control. A plausible explanation for the preservation of pancreatic islet insulin in a rat model of chronic mild hyperglycaemia and mild obesity could be improved adaptations to oxidative and/or ER stress by mechanisms currently unknown to us, which in turn prevents the dysregulation of insulin, but not insulin secretion. This may occur via the increased storage of mature peptide hormone through the budding of new islets from existing ones in order to maintain optimal islet size (Seymour, Bennett, & Slack, 2004) and would explain the reduction in islet number observed in the PEPCK transgenic rat from weaning, as well as the subsequent enlargement of islet size as they become insulin resistant with age.

Lastly, there is strong evidence of metallothionein activity in the pancreatic islet (Laychock, Duzen, & Simpkins, 2000; Marselli et al., 2010). Since the PEPCK transgene is under the control of a metallothionein promoter, it is worth considering that
ectopic expression of PEPCK in the islet may have partly caused the reduction in islet number at 5 weeks of age when the impact of adiposity and glucose homeostasis on this parameter would have been minimal. However, unpublished data from our laboratory in control and PEPCK transgenic islets (data not shown) suggest that there are no significant differences in \textit{Pck1} gene expression. These data potentially exclude an islet phenotype in the PEPCK transgenic rat.

In order to determine the site of the insulin secretory defect in the PEPCK transgenic rat, we used an assortment of non-glucose secretagogues; arginine and tolbutamide, in the presence of stimulatory levels of glucose. While the mechanisms underlying GSIS has been well studied since at least the 1970’s (Cerasi, 1975), it is not completely understood. In brief, the major signaling pathway of GSIS consists of the closure of K\textsuperscript{+}\textsubscript{ATP} channels, membrane depolarisation, activation of voltage-dependent Ca\textsuperscript{2+} channels, Ca\textsuperscript{2+} influx and the elevation of cytosolic Ca\textsuperscript{2+} which helps facilitate the exocytosis of secretory granules containing insulin. Arginine directly depolarises the β-cell membrane and thereby elicits Ca\textsuperscript{2+}-dependent electrical activity, Ca\textsuperscript{2+} entry and insulin secretion, whereas tolbutamide directly binds to the SUR1 subunit of the K\textsuperscript{+}\textsubscript{ATP} channel complex. Our findings demonstrate that 14 week-old PEPCK transgenic rat islets are able to respond to arginine, but not to the level seen \textit{in vivo}, further reinforcing the notion that the gluconeogenic capacity of the PEPCK transgenic rat influences \textit{in vivo} arginine-induced insulin secretion. Conversely, pancreatic islets isolated from PEPCK transgenic rats were not able to respond to tolbutamide. The regulation of the K\textsuperscript{+}\textsubscript{ATP} subunits is important for normal β-cell function, particularly when the β-cell is exposed to unfavourable environmental conditions such as high levels of glucose. This is clearly evident when exposing isolated rat pancreatic islets to high (16.7 mM) glucose conditions for 24 hours with the result being decreased K\textsuperscript{+}\textsubscript{ATP} channel function as measured by \textsuperscript{86}Rb efflux (a tracer for K\textsuperscript{+}) and defective glucose-induced insulin release (Purrello et al., 1990). Another study utilising the INS-1 clonal rat β-cell line in high (25 mM) glucose conditions for 72 hours show \textit{Sur1} mRNA levels being reduced by 26% and \textit{Kir6.2} by 36% compared with those cells kept at 5 mM glucose (100% mRNA levels) (Moritz et al., 2001). Similar findings with respect to \textit{Kir6.2} gene expression have also been documented in pancreatectomised, highly hyperglycaemic rats, but not in rats that were made only mildly hyperglycaemic (Jonas et al., 1999). Since the
PEPCK transgenic rat is a model of mild hyperglycaemia, this last point corroborates the results in our study which shows that there are no significant differences relative to control between the genes that make up the K\(^{+}\)ATP channel complex, *Sur1* and *Kir6.2*. It therefore seems that this defect may affect one or more of the steps beyond expression that are required for proper K\(^{+}\)ATP channel function, i.e., channel assembly or trafficking. The use of patch-clamp techniques to measure membrane potential may aid in our understanding of β-cell K\(^{+}\)ATP channel function in the PEPCK transgenic rat.

**Summary**

In summary, the findings in this chapter provide evidence that mild chronic glucose excess due to a 2-3 fold over-expression in PEPCK in the liver and kidney can lead to impaired GSIS in the 14 week-old PEPCK transgenic rat. This predominantly arises due to a reduction in *Glut2* expression and the inability of the K\(^{+}\)ATP channel to function properly. Interestingly, defective GSIS in the PEPCK transgenic rat appears to be driven by the decline in β-cell function in the first instance, with β-cell mass remaining unchanged. The defect in GSIS progressively worsens with age due to the combination of β-cell dysregulation and declined β-cell mass. Therefore, the current study shows that the PEPCK transgenic rat is an excellent model to investigate the link between mild obesity and chronic glucose excess and β-cell dysfunction. The next chapter will investigate the potential of the selective glucose-lowering SGLT2 inhibitor, dapagliflozin, in preventing the progression of insulin resistance and β-cell dysfunction when both are already firmly established.
Chapter 4 Dapagliflozin reduces insulin resistance but does not improve the insulin secretory capacity of the β-cell in the PEPCK transgenic rat.

Introduction
Defective insulin secretion in the presence of insulin resistance is the principal cause of hyperglycaemia in T2D (S. E. Kahn, 2003). As the maintenance of near-normal glycaemia is important in reducing the risk of long-term complications, management of this disease is therefore targeted at resolving insulin resistance and insulin secretion. In the last 25 years, there has been a significant increase in the number of pharmacological options available for the treatment of T2D (S. E. Kahn, Cooper, & Del Prato, 2014). However, currently approved antidiabetic treatments only specifically address the underlying defects by either increasing insulin sensitivity or insulin secretion. At the time of clinical diagnosis, the majority of patients with T2D have already lost between 40-50 % of their β-cell function (UKPDS, 1998), putting into question the durability of drugs such as sulphonylureas and GLP-1 agonists which require adequate β-cell function in order to elicit their glycaemic benefits. Similarly, forcing the β-cell to secrete insulin at a time when it is struggling with the demands of obesity and insulin resistance can accelerate its demise (Aston-Mourney, Proietto, Morahan, & Andrikopoulos, 2008). Many blood glucose-lowering agents also cause hypoglycaemia, weight gain and gastrointestinal discomfort. Furthermore, there is much controversy surrounding the incretin-based therapies and whether or not they can negatively alter pancreatic morphology in diabetic human subjects (Butler et al., 2013; Lamont & Andrikopoulos, 2014). Consequently, the limitations and side effects of these drugs have paved the way for the development of a new class of agent that acts independent of insulin and glucose metabolism through the pharmacological inhibition of SGLT2.

The SGLT2 inhibitors reduce blood glucose levels by inhibiting the reabsorption of glucose in the S1 segment of the proximal tubule in the kidney, resulting in glucose
being excreted from the urine (Bailey et al., 2010; Henry et al., 2012; Nauck et al., 2011). As the mechanism of action of SGLT2 inhibitors is independent of insulin action and secretion, their efficacy is not expected to deteriorate with severe insulin resistance and β-cell dysfunction. More importantly, by removing the excess glucose from the circulation, SGLT2 inhibitors provide a promising strategy in preventing glucotoxicity. Glucotoxicity is a resultant of persistently high plasma glucose levels and is a mechanism that can induce insulin resistance in the liver, muscle and fat, as well as impair β-cell function. Experimental work performed in Chapter 3 of this thesis and by other members from our group has shown that the PEPCK transgenic rat is characterised by low-grade chronic hyperglycaemia, glucose intolerance, skeletal muscle and fat insulin resistance and defective GSIS (Lamont et al., 2003; Mangiafico et al., 2011; Rosella et al., 1995). These attributes make the PEPCK transgenic rat an excellent model of glucotoxicity-induced impairments in insulin action and secretion.

Given the deleterious effects of glucotoxicity, it may be hypothesised that treatment with a SGLT2 inhibitor would provide beneficial effects on insulin resistance and impaired insulin secretion. Dapagliflozin is a potent competitive SGLT2 inhibitor with high selectivity to SGLT2 over other SGLT isoforms. Clinical studies have suggested that short-term dapagliflozin treatment in patients with T2D can improve both insulin resistance and insulin secretion (Merovci et al., 2015; Merovci et al., 2014). Similarly, treatment with dapagliflozin in male ZDF rats with established moderate hyperglycaemia improves glucose tolerance and insulin sensitivity (S. Han et al., 2008), while adequately preserving β-cell function and mass (F. R. Macdonald et al., 2010). However, these studies have not conclusively shown that the SGLT2 inhibitors improve defects in both insulin resistance and insulin secretion concomitantly as each of these studies have opted to perform either a hyperinsulinaemic-euglycaemic or a hyperglycaemic clamp, and not both. As a result, it is not clear whether SGLT2 inhibitors directly improve β-cell function via a direct effect on the β-cell or whether it is because of increased insulin sensitivity. Hence, the purpose of this study was to use the “gold-standard” techniques for measuring insulin sensitivity and β-cell function, the hyperinsulinaemic-euglycaemic and hyperglycaemic clamp respectively, to determine whether dapagliflozin treatment can improve or reverse insulin resistance and β-cell dysfunction in the glucose intolerant PEPCK transgenic rat. Our data suggests that
Dapagliflozin treatment selectively improves peripheral insulin sensitivity which in turn may provide a β-cell sparing effect since the insulin secretory capacity of the β-cell was unchanged compared to untreated PEPCK transgenic rats.

**Chapter Aims**

The aims of this chapter are as follows:

1. To assess whether initiating dapagliflozin treatment in glucose intolerant PEPCK transgenic rats can improve or resolve insulin resistance.
2. To assess whether initiating dapagliflozin treatment in glucose intolerant PEPCK transgenic rats can prevent or reduce the β-cell’s decline in insulin secretory capacity and mass.

**Results**

**Calorie-restriction and dapagliflozin treatment reduces weight gain in PEPCK transgenic rats.**

Growth parameters and measures of adiposity in 20 week-old ad-libitum fed, calorie-restricted and dapagliflozin-treated PEPCK transgenic rats are shown in Table 4.1. There were no differences in the starting body weights between PEPCK transgenic rats randomised into *ad-libitum* fed, calorie-restriction or dapagliflozin-treated groups. Due to the significant body weight differences at the start of the treatment period and the ethical guidelines provided by the Austin Health Animal Ethics Committee for the care and use of animals for scientific purposes, we were unable to match the body weights of calorie-restricted PEPCK transgenic rats with the lean control from Chapter 3. We therefore chose to reduce the amount of allowable calories consumed so that body weight remained constant throughout the treatment period and as close to the final body weight of age-matched controls shown in Table 3.1. By experimental design, the amount of weight gained after the 6 week treatment period was significantly lower in the calorie-restricted PEPCK transgenic rats compared to the *ad-libitum* fed and dapagliflozin-treated groups. Of particular interest is that PEPCK transgenic rats treated with dapagliflozin ate significantly more food yet gained significantly less body weight over the treatment period when compared to the *ad-libitum* fed PEPCK transgenic group. This reduction in weight gain did not result in significantly different final body
weights since PEPCK transgenic rats randomised into dapagliflozin treatment started slightly heavier than the *ad-libitum* fed group. The reduction in weight gain in the dapagliflozin-treated PEPCK transgenic rat group did, however, result in significant decreases in all three of the fat depots measured when compared to *ad-libitum* fed PEPCK transgenic rats. WAT depots were not weighed in calorie-restricted PEPCK transgenic rats as only one cohort was maintained and tissues were immediately snap frozen at the conclusion of the hyperinsulinaemic-euglycaemic clamp for glucose uptake and immunoblotting studies. PEPCK transgenic rats treated with dapagliflozin consumed nearly double the amount of fluid per day compared to the PEPCK transgenic rats given water. This was expected since the SGLT2 inhibitor class of drugs promote glycosuria and have a mild diuretic effect. Based on the fluid intake over the 6 week period, PEPCK transgenic rats treated with dapagliflozin were administered approximately 3.8 mg of drug per day or 10 mg/kg body weight. Interestingly, dapagliflozin treatment significantly increased kidney wet weight compared to *ad-libitum* fed PEPCK transgenic rats.
Table 4.1: The effects of treatment on growth parameters and adiposity in PEPCK transgenic rats.

<table>
<thead>
<tr>
<th>PEPCK transgenic rats fed <em>ad-libitum</em>, calorie-restricted or treated with dapagliflozin from 14 to 20 weeks of age</th>
<th>Ad-libitum fed PEPCK transgenic</th>
<th>Calorie-restricted PEPCK transgenic</th>
<th>Dapagliflozin-treated PEPCK transgenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting body weight at 14 weeks of age (g)</td>
<td>343.8 ± 6.6 (9)</td>
<td>354.7 ± 6.6 (6)</td>
<td>362.7 ± 6.3 (9)</td>
</tr>
<tr>
<td>Weight gained over treatment period (g)</td>
<td>60.1 ± 5.5 (9)</td>
<td>-4.2 ± 4.6 *** (6)</td>
<td>31.4 ± 2.8 *** ††† (9)</td>
</tr>
<tr>
<td>Final body weight at 20 weeks of age (g)</td>
<td>403.9 ± 4.6 (9)</td>
<td>350.6 ± 3.9 *** (6)</td>
<td>394.1 ± 5.4 ††† (9)</td>
</tr>
<tr>
<td>Average food intake over treatment period (g/day)</td>
<td>24.1 ± 0.7 (4)</td>
<td>14.7 ± 0.0 *** (6)</td>
<td>27.8 ± 1.0 *** ††† (4)</td>
</tr>
<tr>
<td>Feeding efficiency over treatment period (%)</td>
<td>7.0 ± 0.8 (4)</td>
<td>-0.8 ± 0.9 *** (6)</td>
<td>2.5 ± 0.6 † (4)</td>
</tr>
<tr>
<td>Average fluid intake over treatment period (mL/day)</td>
<td>29.8 ± 1.3 (4)</td>
<td>Not measured</td>
<td>62.9 ± 2.8 *** (4)</td>
</tr>
<tr>
<td>WAT mass (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>5.4 ± 0.3 (10)</td>
<td>Not measured</td>
<td>3.1 ± 0.4 *** (11)</td>
</tr>
<tr>
<td>Infra-renal</td>
<td>5.8 ± 0.4 (10)</td>
<td>Not measured</td>
<td>4.2 ± 0.4 *** (11)</td>
</tr>
<tr>
<td>Epididymal</td>
<td>6.0 ± 0.3 (10)</td>
<td>Not measured</td>
<td>4.7 ± 0.4 † (11)</td>
</tr>
<tr>
<td>Total WAT</td>
<td>17.2 ± 0.8 (10)</td>
<td>Not measured</td>
<td>12.0 ± 0.9 *** (11)</td>
</tr>
<tr>
<td>Kidney wet weight (g)</td>
<td>5.9 ± 0.3 (10)</td>
<td>Not measured</td>
<td>7.0 ± 0.2 *** (10)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. The number of animals used for each strain is shown in parenthesis. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. *ad-libitum* fed PEPCK transgenic; †P < 0.05 and †††P < 0.001 vs. calorie-restricted PEPCK transgenic.
The effect of calorie-restriction and dapagliflozin treatment on fasting glucose and insulin levels in PEPCK transgenic rats.

Overnight and 6 hour fasting glucose and insulin levels were measured and compared between 20 week-old *ad-libitum* fed, calorie-restricted and dapagliflozin-treated PEPCK transgenic rats to determine whether a loss of adiposity and glucose-lowering could prevent fasting hyperglycaemia and hyperinsulinaemia (Table 4.2). Due to the increased incidence of ketoacidosis in patients exposed to dapagliflozin, we also examined blood ketones (as assessed by β-hydroxybutyrate) in all three groups following an overnight fast (Table 4.2). Calorie-restricting PEPCK transgenic rats at the onset of 14 weeks of age where obesity, glucose intolerance, insulin resistance and β-cell dysfunction are prevalent for 6 weeks duration did not improve fasting hyperglycaemia or hyperinsulinaemia. However, preventing weight gain during the 6 week treatment period significantly lowered blood ketone levels suggesting that calorie-restricted PEPCK transgenic rats are utilising glucose metabolism for energy during fasting conditions. The treatment with dapagliflozin for 6 weeks normalised both overnight and 6 hour fasting hyperglycaemia and hyperinsulinaemia back to levels similar to that seen in age-matched control rats (Chapter 3, Table 3.2). In addition, modest weight gain and SGLT2 inhibitor-induced glycosuria significantly raised blood ketone levels compared to *ad-libitum* fed and calorie-restricted PEPCK transgenic rats.
Table 4.2: The effects of treatment on fasting biomarkers in PEPCK transgenic rats.

<table>
<thead>
<tr>
<th>PEPCK transgenic rats fed ad-libitum, calorie-restricted or treated with dapagliflozin from 14 to 20 weeks of age</th>
<th>Ad-libitum fed PEPCK transgenic</th>
<th>Calorie-restricted PEPCK transgenic</th>
<th>Dapagliflozin-treated PEPCK transgenic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>6 hour fast</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood glucose (mmol/L)</td>
<td>6.3 ± 0.1 (5)</td>
<td>7.1 ± 0.2 (5)</td>
<td>4.8 ± 0.3*** ††† (5)</td>
</tr>
<tr>
<td>Plasma insulin (ng/mL)</td>
<td>1.9 ± 0.1 (5)</td>
<td>1.9 ± 0.6 (5)</td>
<td>0.6 ± 0.1* † (5)</td>
</tr>
<tr>
<td><strong>Overnight fast</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma glucose (mmol/L)</td>
<td>6.3 ± 0.3 (7)</td>
<td>6.8 ± 0.2 (6)</td>
<td>4.4 ± 0.2*** ††† (4)</td>
</tr>
<tr>
<td>Plasma insulin (ng/mL)</td>
<td>1.07 ± 0.25 (4)</td>
<td>Not measured</td>
<td>0.26 ± 0.05## (5)</td>
</tr>
<tr>
<td>Blood ketones (mmol/L)</td>
<td>0.79 ± 0.04 (7)</td>
<td>0.22 ± 0.02 (6)</td>
<td>3.65 ± 0.31*** ††† (8)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. The number of animals used for each strain is shown in parenthesis. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. ad-libitum fed PEPCK transgenic; †P < 0.05 and †††P < 0.001 vs. calorie-restricted PEPCK transgenic.
Calorie-restriction and dapagliflozin treatment improves glucose tolerance in PEPCK transgenic rats.

To determine whether ameliorating mild obesity and mild hyperglycaemia in the PEPCK transgenic rat would reverse glucose intolerance, we performed an IPGTT in overnight fasted 20 week-old *ad-libitum* fed, calorie-restricted and dapagliflozin-treated PEPCK transgenic rats. The glucose tolerance test revealed that preventing weight gain in the calorie-restricted PEPCK transgenic rat (Table 4.1) significantly improved the blood glucose excursion over the 120 minutes when compared to *ad-libitum* fed PEPCK transgenic rats (Figure 4.1A). Interestingly, the incremental area under the glucose curve shows that reducing adiposity and resolving hyperglycaemia through the treatment of dapagliflozin for 6 weeks only slightly, but not significantly, improves glucose tolerance when compared to the *ad-libitum* fed PEPCK transgenic group when a one-way ANOVA was used (Figure 4.1B).
Figure 4.1: Intraperitoneal glucose tolerance test in PEPCK transgenic rats fed *ad-libitum*, calorie-restricted or treated with dapagliflozin for 6 weeks starting from 14 weeks of age. (A) Blood glucose and (B) incremental area under the glucose curve in 20 week-old *ad-libitum* fed, calorie-restricted and dapagliflozin-treated PEPCK transgenic rats. Data are presented as mean ± SEM. Number of animals included: *n* = 5 for *ad-libitum* fed PEPCK transgenic, *n* = 6 for calorie-restricted PEPCK transgenic and *n* = 4 for dapagliflozin-treated PEPCK transgenic. *#P* < 0.05, ***P* < 0.01 and ####P* < 0.001 vs. *ad-libitum* fed PEPCK transgenic; †*P* < 0.05 and ††*P* < 0.01 vs. calorie-restricted PEPCK transgenic. Abbreviations: i.p., intraperitoneal; CR, calorie-restricted.
Dapagliflozin treatment does not restore glucose-stimulated insulin secretion in glucose intolerant PEPCK transgenic rats.

Based on our *in vivo* and *in vitro* GSIS findings from Chapter 3 (Figure 3.2 and 3.3), PEPCK transgenic rats develop signs of β-cell dysfunction at approximately 14 weeks of age. Since the use of SGLT2 inhibitors are becoming more prominent in the management of prevailing blood glucose levels in T2D, we wanted to determine whether starting dapagliflozin treatment when β-cell function is already compromised, as is generally the case at the time of clinical diagnosis, could preserve β-cell function. As we only maintained one cohort of calorie-restricted PEPCK transgenic rats throughout the treatment study, we did not perform a one-step hyperglycaemic clamp in these rats. Dapagliflozin treatment significantly increased the GIR required to maintain glycaemia between 15-17 mmol/L when compared to *ad-libitum* fed PEPCK transgenic rats throughout the clamp experiment (Figure 4.2A and C). This significant increase is most likely due to a combination of SGLT2 inhibitor-induced glycosuria and improvements in both insulin sensitivity and tissue glucose uptake (as indicated in Figure 4.4). The administration of dapagliflozin for 6 weeks duration did not improve the defect in GSIS in the PEPCK transgenic rat (Figure 4.2B and panel insert).

To assess whether dapagliflozin could improve the β-cell’s capacity to secrete insulin, glucose infusion was stopped and a bolus of arginine injected at 121 minutes. Arginine stimulated a robust insulin secretory response above that of glucose alone in both groups, with no discernible differences between *ad-libitum* fed or dapagliflozin-treated PEPCK transgenic rats (Figure 4.2B dotted areas and D). Blood glucose levels following an arginine bolus showed a similar pattern whether dapagliflozin was administered or not (Figure 4.2A dotted areas).
Chapter 4  Dapagliflozin reduces insulin resistance

A) Blood glucose (mmol/L) vs. Time (min)
- Arginine

B) GSIS (0-120 min)
- ad-lib fed PEPCK
- SGLT2i PEPCK

C) Glucose infusion rate (μmol/min/kg) vs. Time (min)
- #

D) Arginine (0-30 min)
- ad-lib fed PEPCK
- SGLT2i PEPCK
Figure 4.2: One-step hyperglycaemic clamp in PEPCK transgenic rats fed *ad-libitum* or treated with dapagliflozin for 6 weeks starting from 14 weeks of age. (A) Blood glucose, (B) plasma insulin levels and (C) glucose infusion rate during the course of the one-step hyperglycaemic clamp, and (D) incremental area under the insulin curve following a bolus of arginine at the conclusion of the one-step hyperglycaemic clamp in 20 week-old *ad-libitum* and dapagliflozin-treated PEPCK transgenic rats. Incremental area under the insulin curve for GSIS during the one-step hyperglycaemic clamp is illustrated as corresponding panel insert. An arginine bolus (1 g/kg) was administered one minute after last sampling (120 minute time-point) and is represented by dotted areas. Data are presented as mean ± SEM. Number of animals included: *n* = 5 for each group. **P < 0.01 vs. ad-libitum fed PEPCK transgenic.**
Dapagliflozin treatment does not alter islet size or islet number in glucose intolerant PEPCK transgenic rats.

Given that 14 week-old PEPCK transgenic rats possess significantly larger but fewer pancreatic islets than their control counterparts and that β-cell mass significantly declines with age, we wanted to determine if glucose-lowering with dapagliflozin treatment could resolve pancreatic islet size and number, and more importantly, preserve β-cell mass (Table 4.3). Representative images of the pancreas from PEPCK transgenic rats fed ad-libitum or treated with dapagliflozin are 8x magnification (scale bar, 300 µm) and show insulin immunohistochemical staining (Figure 4.3). Pancreas was weighed prior to formalin fixation with no differences being reported. Interestingly, the sustained reduction of mild hyperglycaemia, as shown in Table 4.2 with dapagliflozin administration over the 6 week treatment period, did not alter any of the islet morphological parameters analysed in Table 4.3. Therefore, these data show that dapagliflozin does not improve islet morphology and preserve β-cell mass when administered to PEPCK transgenic rats with firmly established profound insulin resistance and β-cell dysfunction.
Figure 4.3: Insulin immunohistochemical staining of the pancreas of PEPCK transgenic rats fed *ad-libitum* or treated with dapagliflozin for 6 weeks starting from 14 weeks of age. Representative images of insulin immunohistochemical staining of pancreatic sections from PEPCK transgenic rats fed either (A) *ad-libitum* or (B) treated with dapagliflozin for 6 weeks starting from 14 weeks of age. The insulin-containing β-cells stain brown and the vascular endothelial cells and exocrine portion of the pancreas appear blue. 8x magnification; scale bar, 300 µm.
Table 4.3: Quantitative insulin immunohistochemical staining of pancreatic sections from PEPCK transgenic rats fed *ad-libitum* or treated with dapagliflozin for 6 weeks starting from 14 weeks of age.

<table>
<thead>
<tr>
<th></th>
<th>Ad-libitum fed PEPCK transgenic</th>
<th>Dapagliflozin-treated PEPCK transgenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreas weight (g)</td>
<td>1.8 ± 0.1 (4)</td>
<td>1.7 ± 0.1 (3)</td>
</tr>
<tr>
<td>Average islet size (µm²)</td>
<td>7183 ± 808 (5)</td>
<td>8344 ± 530 (5)</td>
</tr>
<tr>
<td>Islet number (per mm² pancreas area)</td>
<td>0.57 ± 0.01 (5)</td>
<td>0.53 ± 0.06 (5)</td>
</tr>
<tr>
<td>Islet size distribution (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1,000 µm²</td>
<td>38.0 ± 1.6</td>
<td>38.1 ± 2.6</td>
</tr>
<tr>
<td>1,001 – 3,000 µm²</td>
<td>24.2 ± 1.5</td>
<td>24.1 ± 2.1</td>
</tr>
<tr>
<td>3,001 – 5,000 µm²</td>
<td>10.5 ± 1.3</td>
<td>8.7 ± 0.9</td>
</tr>
<tr>
<td>5,001 – 10,000 µm²</td>
<td>9.7 ± 0.9</td>
<td>9.3 ± 1.0</td>
</tr>
<tr>
<td>10,001 – 20,000 µm²</td>
<td>7.0 ± 0.2</td>
<td>6.7 ± 0.5</td>
</tr>
<tr>
<td>&gt;20,001 µm²</td>
<td>10.6 ± 1.4</td>
<td>13.1 ± 1.5</td>
</tr>
<tr>
<td>Insulin⁺ area (% islet area)</td>
<td>70.9 ± 0.5 (5)</td>
<td>66.5 ± 1.9 (5)</td>
</tr>
<tr>
<td>β-cell area (% pancreas area)</td>
<td>0.27 ± 0.04 (5)</td>
<td>0.26 ± 0.03 (5)</td>
</tr>
<tr>
<td>β-cell mass (mg)</td>
<td>4.1 ± 0.4 (4)</td>
<td>3.5 ± 0.1 (3)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. The number of animals used for each strain is shown in parenthesis. ImageScope software was used for quantification of pancreatic images.
Calorie-restriction and dapagliflozin treatment improves insulin sensitivity in glucose intolerant PEPCK transgenic rats.

To formally assess whether insulin resistance could be improved with calorie-restriction and dapagliflozin treatment in PEPCK transgenic rats, a hyperinsulinaemic-euglycaemic clamp was performed. In addition, since the effects of SGLT2 inhibition in normal glucose tolerant and insulin sensitive rodents have not been well characterised, we also assessed insulin sensitivity in a subset of control rats treated with dapagliflozin from 14 weeks of age for 6 weeks duration. As expected, treatment with dapagliflozin in control and PEPCK transgenic rats significantly reduced baseline blood glucose levels when compared to their relative vehicle-treated group (ad-libitum fed control; 6.38 mmol/L ± 0.03 vs. dapagliflozin-treated control; 4.86 ± 0.46, \( P = 0.02 \) and ad-libitum fed PEPCK transgenic; 6.68 ± 0.24 vs. dapagliflozin-treated PEPCK transgenic; 4.96 ± 0.15, \( P = 0.0001 \); Figure 4.4A-C and E). After insulin infusion, blood glucose levels were matched in control and PEPCK transgenic groups and clamped at 7 mmol/L (Figure 4.4A-E dotted areas). Fasting plasma insulin levels were not statistically different between control and dapagliflozin-treated control rats (3.1 ng/mL ± 0.1 vs. 2.1 ± 0.4, \( P = 0.08 \)) while dapagliflozin treatment reduced hyperinsulinaemia in PEPCK transgenic rats (3.0 ng/mL ± 0.3 vs. 1.7 ± 0.3, \( P = 0.02 \)) and are denoted by insulin ‘–’ in Figure 4.4F. Plasma insulin levels under insulin stimulation were maintained between 5-6 ng/mL and are denoted by insulin ‘+’ in Figure 4.4F. Under hyperinsulinaemic conditions, ad-libitum fed PEPCK transgenic rats showed profound insulin resistance as demonstrated by a 84 % reduction in the rate of glucose infusion required to maintain euglycaemia when compared to ad-libitum fed control rats (Figure 4.4G). Preventing weight gain through calorie-restriction over the treatment period provided a subtle improvement in insulin sensitivity as evident by a small increase in GIR, whereas treatment with dapagliflozin completely normalised insulin sensitivity back to the ad-libitum fed control group (Figure 4.4G and H). Furthermore, dapagliflozin treatment for 6 weeks in the healthy control group resulted in a trend for improved insulin sensitivity, but this did not reach statistical significance (\( P = 0.2 \)) (Figure 4.4G). The various levels of insulin sensitivity in the different strains and treatments tested were predominantly due to enhanced whole-body glucose disposal (Figure 4.4H). At basal (insulin ‘–’), EGP was slightly enhanced by dapagliflozin treatment in the control (ad-libitum fed...
Chapter 4  
Dapagliflozin reduces insulin resistance

control; 48.3 µmol/min/kg ± 1.6 vs. dapagliflozin-treated control; 60.2 ± 3.7, \( P = 0.03 \), with this effect being more obvious in the PEPCK transgenic group (ad-libitum fed PEPCK; 42.7µmol/min/kg ± 2.6 vs. dapagliflozin-treated PEPCK; 52.3 ± 2.2, \( P = 0.02 \)) (Figure 4.4I). Basal EGP was not examined in calorie-restricted PEPCK transgenic rats as this group was used to control for any weight-induced insulin-sensitising benefits seen in the dapagliflozin-treated PEPCK transgenic group. Furthermore, the level of insulin achieved during the clamp (insulin ‘+’) was adequate to suppress EGP in all groups except for ad-libitum fed PEPCK transgenic rats.

As insulin suppresses hepatic and renal EGP by negatively regulating the gluconeogenic enzymes, we next examined whether the dysregulation of the genes that encode these enzymes; \( Pck1 \), \( G6pc \) and \( Fbp1 \), could contribute to the impairment in EGP suppression seen in the PEPCK transgenic rat under insulin-stimulated conditions and whether preventing weight gain and sustained glucose-lowering through dapagliflozin treatment could alter their gene expression profile (Figure 4.4J and K). There were no statistical differences in gene expression of hepatic or renal \( Pck1 \) between ad-libitum fed control and PEPCK transgenic rats. Insulin-stimulation in both PEPCK transgenic treatment groups greatly reduced hepatic and renal \( Pck1 \) gene expression levels. Despite this reduction in both tissues, \( G6pc \) in calorie-restricted rats remained elevated to a level similar to that seen in the ad-libitum fed PEPCK transgenic group. In contrast, treatment with the glucose-lowering agent, dapagliflozin, significantly reduced \( G6pc \) in both tissues. Hepatic \( Fbp1 \) gene expression levels were no different between any of the groups whilst kidney \( Fbp1 \) was significantly reduced with either treatment versus the ad-libitum fed control, and a trend for a reduction when compared to the ad-libitum fed PEPCK transgenic group. Taken together, these data suggest that the effects that dapagliflozin provide, i.e., a small reduction in weight gain and glucose-lowering, are more beneficial to insulin sensitivity than preventing weight gain alone, in the insulin resistant PEPCK transgenic rat.
Figure 4.4: Insulin sensitivity in control and PEPCK transgenic rats fed ad-libitum, calorie-restricted or treated with dapagliflozin for 6 weeks starting from 14 weeks of age. (A)-(E) Blood glucose and (F) plasma insulin levels at basal (denoted by insulin ‘-’), (G) glucose infusion rate, (H) whole-body rate of glucose disappearance, (I) endogenous glucose production under basal (insulin ‘-’), and during hyperinsulinaemia clamp conditions (insulin ‘+’), and relative gluconeogenic enzyme mRNA in (J) the liver and (K) the kidneys at the conclusion of the hyperinsulinaemic-euglycaemic clamp. Data are presented as mean ± SEM. Number of animals included: $n = 4$ for ad-libitum fed control, $n = 5$ for dapagliflozin-treated control, $n = 8$ for ad-libitum fed PEPCK transgenic, $n = 5$ for calorie-restricted PEPCK transgenic and $n = 7$ for dapagliflozin-treated PEPCK transgenic. *$P < 0.05$ and **$P < 0.01$ vs. ad-libitum fed control; §§$P < 0.01$ and §§§$P < 0.001$ vs. dapagliflozin-treated control; #*$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$ vs. ad-libitum fed PEPCK transgenic; †$P < 0.05$ and ††$P < 0.01$ vs. calorie-restricted PEPCK transgenic.
Calorie-restriction and dapagliflozin treatment improves insulin sensitivity in skeletal muscle and adipose tissue in glucose intolerant PEPCK transgenic rats.

To determine if the prevention of weight gain or if a combination of glucose-lowering and a small reduction in weight gain could improve insulin resistance in peripheral tissues, glucose uptake in white quadriceps, red gastrocnemius and epididymal fat were measured using 2DG (Figure 4.5A). During the hyperinsulinaemic-euglycaemic clamp, the rate of glucose uptake into white quadriceps and epididymal fat was significantly lower in *ad-libitum* fed PEPCK transgenic compared to control rats. Both calorie-restriction and dapagliflozin treatment greatly improved insulin sensitivity in all three peripheral tissues tested, with glucose uptake returning to levels comparable to that of the control in red gastrocnemius and epididymal fat.

We next determined whether or not the improvements in insulin-stimulated glucose uptake in red gastrocnemius and epididymal fat with either treatment were due to an increase in total GLUT4 protein content. Representative immunoblots for GLUT4 and the loading control HSP90, with corresponding densitometry levels are shown for red gastrocnemius in Figure 4.5B and epididymal fat in Figure 4.5C. There was no significant differences in total GLUT4 protein content in red gastrocnemius between any of the groups irrespective of the level of glucose uptake achieved (Figure 4.5B). Interestingly, despite having similar rates of glucose uptake in epididymal fat, the total GLUT4 protein content of dapagliflozin-treated PEPCK transgenic rats was significantly increased when compared to the equally insulin sensitive calorie-restricted PEPCK transgenic rat (Figure 4.5C). Therefore, these data show that lowering glucose using dapagliflozin treatment significantly improves the defect in GLUT4 protein content that is present in the glucose intolerant PEPCK transgenic rat.
Chapter 4  Dapagliflozin reduces insulin resistance

A) 

- **Rg'** (umol/min/100g)
- **ad-lib fed Control**
- **ad-lib fed PEPCK**
- **CR PEPCK**
- **SGLT2i PEPCK**

B) 

- **Red gastrocnemius**

C) 

- **Epididymal WAT**
Figure 4.5: Peripheral insulin sensitivity in control and PEPCK transgenic rats fed *ad-libitum*, calorie-restricted or treated with dapagliflozin for 6 weeks starting from 14 weeks of age. (A) Rate of glucose uptake in white quadriceps, red gastrocnemius and epididymal adipose tissue, and representative immunoblots of total GLUT4 protein content and corresponding densitometry levels in (B) red gastrocnemius and (C) epididymal adipose tissue from 20 week-old *ad-libitum* fed control rats and *ad-libitum* fed, calorie-restricted and dapagliflozin-treated PEPCK transgenic rats. Data are presented as mean ± SEM. Number of animals included: skeletal muscle and white adipose tissue 2DG uptake, \( n = 4 \) for *ad-libitum* fed control, \( n = 8 \) for *ad-libitum* fed PEPCK transgenic, \( n = 5 \) for calorie-restricted PEPCK transgenic and \( n = 7 \) for dapagliflozin-treated PEPCK transgenic, and GLUT4 protein content in red gastrocnemius and epididymal adipose tissue, \( n = 4 \) for each group. \( *P < 0.05 \) and \( **P < 0.01 \) vs. *ad-libitum* fed control; \( ^{#}P < 0.05 \) and \( ^^P < 0.01 \) vs. *ad-libitum* fed PEPCK transgenic; \( \texttt{†††}P < 0.001 \) vs. calorie-restricted PEPCK transgenic.
Calorie-restriction and dapagliflozin treatment reduces adipocyte size in glucose intolerant PEPCK transgenic rats.

We next examined whether initiating calorie-restriction or dapagliflozin treatment at 14 weeks of age when PEPCK transgenic rats are mildly obese and have significantly larger fat pads could normalise adipocyte size and number back to the lean control. Representative histology images of epididymal adipose tissue sections from *ad-libitum* fed control and *ad-libitum* fed, calorie-restricted and dapagliflozin-treated PEPCK transgenic rats are 11x magnification (scale bar, 200 µm) and show H&E staining (Figure 4.6A-D). Results from image analysis of epididymal adipose tissue sections reveal that *ad-libitum* fed PEPCK transgenic rats have significantly larger and fewer adipocytes than their control counterparts (Figure 4.6E and F). Implementing either treatment protocol in PEPCK transgenic rats significantly decreased the average adipocyte area and increased the number of adipocytes per mm² of fat area back to levels seen in the lean control.
Chapter 4  Dapagliflozin reduces insulin resistance

A)  

B) 

C)  

D)  

200 μm
Figure 4.6: Histology of epididymal adipose tissue with H&E staining and morphological analyses in control and PEPCK transgenic rats fed *ad-libitum*, calorie-restricted or treated with dapagliflozin for 6 weeks starting from 14 weeks of age. Representative images of H&E staining of epididymal adipose tissue sections in *ad-libitum* fed (A) control and (B) PEPCK transgenic rats, (C) calorie-restricted PEPCK transgenic rats and (D) PEPCK transgenic rats treated with dapagliflozin for 6 weeks starting from 14 weeks of age, (E) average adipocyte area and (F) adipocyte number per mm² of fat area. The cytoplasm stain pink/red and the nuclei appear blue. 11x magnification; scale bar, 200 µm. Data are presented as mean ± SEM. Number of animals included: n = 4 for each group. *P < 0.05 and ***P < 0.001 vs. *ad-libitum* fed control; #P < 0.05 and ###P < 0.001 vs. *ad-libitum* fed PEPCK transgenic. ImageScope software was used for quantification of images.
Discussion

The SGLT2 inhibitor class of drugs are a relatively new treatment option for T2D that have the potential to improve glycaemic control, both in the fasting and postprandial state, through a mechanism independent of insulin action and secretion by lowering plasma glucose levels via the urine. Pre-clinical studies have suggested that the use of SGLT2 inhibitors initiated after the onset of hyperglycaemia can improve glucose tolerance, insulin sensitivity and β-cell function (S. Han et al., 2008; Hansen et al., 2014; Millar et al., 2016; Neschen et al., 2015; Tahara, Takasu, Yokono, Imamura, & Kurosaki, 2016). However, none of these studies have made a concerted attempt to show that SGLT2 inhibition can improve both insulin resistance and insulin secretion defects, or whether the effect on the β-cell is indirect by enhancing insulin sensitivity. Therefore, the primary goal of this study was to address this knowledge gap by using the “gold-standard” techniques; the hyperinsulinaemic-euglycaemic and hyperglycaemic clamp, to determine whether treatment with the SGLT2 inhibitor, dapagliflozin, could improve or reverse insulin resistance and/or β-cell dysfunction in a transgenic rat model overexpressing the gluconeogenic enzyme, PEPCK, in the liver and kidney (Rosella et al., 1993).

The pharmacological inhibition of SGLT2 has the potential to promote negative energy balance and in turn lead to weight loss. This has been shown in clinical trials where dapagliflozin can cause modest reductions in the range of 2-3 kg in body weight over 12-24 weeks of therapy (Nauck, 2014). These observations also hold true in pre-clinical studies where other SGLT2 inhibitors including dapagliflozin result in marked weight loss or reductions in weight gain (Devenny et al., 2012; Fujimori et al., 2008; Katsuno, Fujimori, Ishikawa-Takemura, & Isaji, 2009). Our results are consistent with these findings and are predominantly due to a decrease in fat mass via the loss of calories through glucose in the urine, lower insulin levels and potentially increased oxidative metabolism of fat, as indicated by elevated levels of blood β-hydroxybutyrate. One of the main points raised with the pharmacological inhibition of SGLT2 is that weight loss is much lower than what was initially expected given the amount of energy being lost through glycosuria (Bailey et al., 2010; E. Ferrannini et al., 2010; L. Zhang et al., 2010). For example, a recent study in overweight patients with T2D showed that SGLT2 inhibitor treatment resulted in a modest 3 kg weight loss rather than the
predicted 11 kg that was calculated through urinary glucose loss (G. Ferrannini et al., 2015). This study suggested an increase in energy intake as a possible mechanism for this difference in the predicted and actual weight loss (G. Ferrannini et al., 2015). As we did not consistently measure urinary glucose excretion, we were unable to determine the exact level of energy loss over the 6 week treatment period. In spite of this, our study clearly shows a paradoxical relationship between reduced weight gain and increased food intake which would make us hypothesise that chronic urinary glucose loss was present during the study. Interestingly, this compensatory hyperphagic response was not sufficient to prevent the continuous reduction in weight gain during the time period that these rats were studied. Our findings of reduced weight gain and adiposity, and higher food intake and β-hydroxybutyrate levels corroborate with another pre-clinical study which utilised diet-induced obese rats treated with dapagliflozin (Devenny et al., 2012). This study showed that food-restricting SGLT2 inhibitor-treated animals to the food intake of vehicle-treated counterparts improves weight loss significantly (Devenny et al., 2012), suggesting that any further potential weight loss with a SGLT2 inhibitor is attenuated by compensatory hyperphagia. The increased circulation of ketone bodies such as β-hydroxybutyrate has been associated with satiety in obese individuals (Chearskul, Delbridge, Shulkes, Proietto, & Kriketos, 2008; Sumithran et al., 2013) and in transgenic mice with a lean phenotype (Visinoni et al., 2012). Therefore, the finding of elevated fasting and fed (these were measured sporadically throughout the treatment period and so data are not shown) ketones in the presence of hyperphagia in PEPCK transgenic rats treated with dapagliflozin is counter-intuitive. However, since the level of hyperphagia was not sufficient to offset the loss of energy through the urine as indicated by the persistent reduction in weight gain, we postulate that fatty-acid oxidation is being used in order to maintain overall energy balance. It would be worth investigating whether mitigating hyperphagia associated with dapagliflozin treatment in the PEPCK transgenic rat by either adding an appetite suppressant such as phentermine, topiramate or a combination of both (Neoh et al., 2014), or implementing a stringent caloric deficit diet further improves weight loss which in turn could lead to even further improvements in both glycaemic and non-glycaemic benefits, e.g., blood pressure and lipid levels. Since the brain is considered to be the most critical tissue in the regulation of food intake and body weight (Schwartz
et al., 2000), and quantitative expression profiling studies have shown unappreciable amounts of SGLT1 and SGLT2 in the brain (J. Chen et al., 2010), our laboratory hypothesise that SGLT2 inhibitor-induced glycosuria may signal through a neural reflex to the central nervous system to alter appetite regulation.

In addition to their glycaemic and non-glycaemic benefits, SGLT2 inhibitors are also able to prevent the early proximal tubule from “seeing” the level of glucose present in the diabetic state. This is hypothesised to prevent the negative effects of glucotoxicity on renal structure and function, with both being linked to the development of diabetic nephropathy (Caramori et al., 2002). As the primary focus of the current study was to look at the potential effects of SGLT2 inhibition on insulin action and secretion in the PEPCK transgenic rat, we did not perform any experiments pertaining to renal structure or function. Therefore, we can only speculate on the possible reason/s why treatment with a SGLT2 inhibitor for 6 weeks resulted in significantly larger kidney weights. We postulate that SGLT2 inhibitor-induced glycosuria would promote increased water retention in the tubular lumen due to osmotic effects of unabsorbed glucose and/or increase the accumulation of glycogen granules in the distal tubules. This condition is called the Armanni-Ebstein lesion and is present in untreated type 2 diabetics with severe glycosuria (Holck & Rasch, 1993). A similar finding with regards to a modest increase in kidney weight has been shown in C57BL/Ks-db/db mice genetically lacking Sglt2 (Vallon et al., 2013). However, the significance of increased kidney weight due to SGLT2 deficiency has been challenged in two separate studies using different backgrounds of mice. Using mutagenised C57BL/6 and C3H/HeJ mice, Quaggin et al showed that there Sweet Pee mutants that carry a nonsense mutation in the Slc5a2 gene have significantly smaller kidneys while Jurczak et al. showed no differences between SGLT2 knockout and wild-type controls on the C57BL/6 strain (Jurczak et al., 2011; Ly et al., 2011). It is most likely that the different background strains used in both studies played a significant role in these discrepancies; the C57BL/Ks-db/db mouse is one of the most investigated models of diabetic kidney disease while the C57BL/6 are quite resistant to developing nephropathy (Brosius et al., 2009). Considering that mutations in the SLC5A2 gene that reduces the expression and activity of SGLT2 are found in persons with familial renal glycosuria and that long-term follow-up studies in excess of 30 years have shown this condition to be benign (Scholl-
Burgi et al., 2004), these data suggest that the long-term use of SGLT2 inhibitors represent a safe strategy for the management of patients with T2D.

At 10 mg/kg, dapagliflozin was well tolerated over the 6 week treatment period with very few signs of gastrointestinal side effects indicative of significant gut SGLT1 inhibition, e.g., diarrhoea and soft stools, as seen with studies involving phlorizin. This is most likely due to dapagliflozin’s greater selectivity for SGLT2 versus SGLT1 (~200-fold in rats) (S. Han et al., 2008). Furthermore, dapagliflozin induced significant urinary excretion of glucose (> 111 mmol/L) based on the Diastix colour chart which reduced the burden of glucose intolerance. Glucotoxicity as a result of persistent high glucose levels is a mechanism that can cause insulin resistance in liver, muscle and fat, as well as impair β-cell function. The glucotoxicity hypothesis on insulin action and secretion in rodents has been well established. One of the first proof-of-concept studies in support of this hypothesis was conducted by Rossetti et al., where they showed that a small persistent increase in glucose levels in partial pancreatectomised rats can have deleterious effects on insulin sensitivity and β-cell function (Rossetti, Shulman, et al., 1987). Both parameters were returned to normal by ameliorating hyperglycaemia through phlorizin treatment (Rossetti, Shulman, et al., 1987). In another study, when phlorizin was withdrawn, hyperglycaemia and insulin resistance returned (B. B. Kahn et al., 1991). Unlike pre-clinical studies, evidence in support of the glucotoxic effect of hyperglycaemia in humans have been somewhat lacking until recently (E. Ferrannini et al., 2014; Merovci et al., 2015; Mudaliar et al., 2014; Polidori, Mari, & Ferrannini, 2014). Studies in humans have shown that SGLT2 inhibitor treatment in patients with T2D improves both insulin resistance and insulin secretion as assessed by a meal tolerance test (E. Ferrannini et al., 2014). In fact, Ferrannini and colleagues performed β-cell function modelling to show that empagliflozin treatment improves β-cell glucose sensitivity, that is, for any given plasma glucose level, the patient on SGLT2-inhibitor treatment secreted more insulin than baseline (E. Ferrannini et al., 2014). Similarly, the use of dapagliflozin for 12 weeks in patients with T2D with inadequate glycaemic control showed a non-significant improvement in acute insulin secretion following an intravenous glucose tolerance test (Mudaliar et al., 2014). Based on these studies, we hypothesised that resolving mild hyperglycaemia in the 20 week-old PEPCK transgenic rat with 6 weeks of dapagliflozin treatment would protect the β-cell against further
glucotoxic-induced damage and thus, preserve the decline in β-cell function and mass when compared to PEPCK transgenic rats given no treatment. We initially measured β-cell function in vivo by using an IVGTT but found that dapagliflozin treatment led to significant glucose loss over the experimental period which in turn greatly blunted insulin secretion, particularly early-phase (0-5 minutes) GSIS (Appendix III). Due to the presence of SGLT2-induced glycosuria, we employed the one-step hyperglycaemic clamp to ensure that the glucose stimulus remained matched throughout the experiment so as to accurately compare the insulin secretory capacity of the β-cell between treated and untreated PEPCK transgenic rats. Surprisingly, initiating dapagliflozin had no beneficial effects on the β-cell’s ability to secrete insulin or β-cell mass. This was unexpected considering that treatments that improve hyperglycaemia have been shown to correct impairments in insulin secretion in patients with T2D (Aston-Mourney, Proietto, & Andrikopoulos, 2005). This is also evident in pre-clinical rodent models where the pharmacological inhibition of SGLT2 during firmly established hyperglycaemia can lead to improved β-cell function and mass. For example, treatment with the SGLT2 inhibitor, BI-38335, in C57BL/Ks-db/db mice resulted in improved glucose-mediated insulin secretion in isolated islets (L. Chen et al., 2012). Similarly, high-fat fed and STZ-induced diabetic mice treated with dapagliflozin or impragliflozin showed improved islet insulin secretion and significantly increased pancreatic insulin content (Millar et al., 2016; Tahara et al., 2016). Increased islet and β-cell mass were also reported in ZDF rats treated with empagliflozin after the onset of moderate hyperglycaemia (Hansen et al., 2014; L. Thomas et al., 2012) but interestingly, not when dapagliflozin was used (F. R. Macdonald et al., 2010). Lastly, improvements in β-cell function and mass have also been shown in SGLT2 whole-body knockout mice backcrossed onto the C57BL/Ks-db/db background (Jurczak et al., 2011). These particular mice show preserved pancreatic β-cell function as measured by a hyperglycaemic clamp, increased β-cell mass and improved islet yield during the isolation process (Jurczak et al., 2011). Therefore, our data shows that the sustained reversal of mild hyperglycaemia with dapagliflozin treatment in PEPCK transgenic rats after which insulin resistance and glucose intolerance have been present for at least 6 weeks duration, is not adequate to directly improve β-cell function or preserve β-cell mass. In the first instance, this data suggests that the functional and morphological
changes in the β-cell of PEPCK transgenic rats are irreversible when dapagliflozin treatment is started too late.

Unrestrained EGP is a consistent feature that contributes to fasting hyperglycaemia in T2D (Consoli, Nurjhan, Capani, & Gerich, 1989). Clinical studies in type 2 diabetic individuals have demonstrated that inducing glucosuria with a SGLT2 inhibitor is associated with a compensatory increase in basal EGP (E. Ferrannini et al., 2014; Merovci et al., 2014). Our results are in agreement with the clinical findings mentioned above and to the best of our knowledge, are the first in the pre-clinical arena to report this compensatory response in the basal state. The mechanism/s by which glucosuria causes this increase in basal EGP in both the control and PEPCK transgenic rats treated with dapagliflozin compared to their respective untreated groups is currently not clear. Studies have shown that the level of glycaemia dictates liver glucose output, such as when the plasma glucose level rises, glucose production by the liver decreases, and vice-versa. As treatment with dapagliflozin caused decreases in fasting blood glucose concentrations in both strains of rats, a possible explanation could be that this decrease leads to an increase in EGP due to the removal of the inhibitory effect of hyperglycaemia on EGP (Cherrington, 1999; DeFronzo, 1987). Additionally, evidence in human studies suggest that the increase in plasma glucagon concentration observed with dapagliflozin stimulates EGP, particularly in the presence of lowered plasma insulin levels which in turn increases the plasma glucagon/insulin ratio (Cherrington, 1999; DeFronzo & Ferrannini, 1987; Matsuda et al., 2002). Recent work in rodent islets have established that SGLT2 appears to be predominantly expressed and localised to pancreatic islet glucagon-producing α-cells (L. Chen et al., 2012) and that inhibition of either SLC5A2 via siRNA-induced gene silencing or SGLT2 by dapagliflozin triggers glucagon secretion in human islets (Bonner et al., 2015). These findings illustrate that this class of drug can mediate effects on the pancreatic islet and not just the kidney as originally thought. In the present study, we attempted to confirm the mechanistic link between glucagon and the compensatory increase in basal EGP by measuring plasma glucagon concentrations throughout the basal turnover studies. However, due to the lack of sensitivity of commercially available rat-specific ELISA kits available, we were unable to accurately determine circulating levels. On a side note, it would be of considerable clinical interest to prevent the compensatory increase in EGP in response
to glycosuria through combination therapy with compounds known to effectively lower basal EGP, e.g., metformin (Hundal et al., 2000), or compounds that inhibit glucagon production and stimulate insulin secretion, e.g., incretin mimetics (Lovshin & Drucker, 2009). Either of these add-on compounds would be expected to further augment the glucose-lowering ability of SGLT2 inhibitors. It is important to highlight that care needs to be taken when combination therapy of this kind is prescribed as not to increase the incidence of hypoglycaemic and ketoacidosis episodes in patients. Our decision not to measure basal EGP in the calorie-restricted PEPCK transgenic group makes it difficult for us to comment on the level of suppression of EGP in response to insulin during the clamp. In the hyperinsulinaemic-euglycaemic clamp studies, the calorie-restricted PEPCK transgenic rat was strictly used to account for any potential insulin-sensitising effects associated with reduced weight gain from SGLT2 inhibition. Studies investigating the major metabolic pathways involved in fuel usage in calorie-restricted rodents have reported increases in transcriptional and enzymatic activity pertinent to gluconeogenesis, and reductions in those involved in glycolysis (Dhahbi et al., 2001; Dhahbi et al., 1999; Hagopian, Ramsey, & Weindruch, 2003). These data, in conjunction with our slightly elevated 6 hour fasting glucose levels compared to PEPCK transgenic rats fed ad-libitum, provide some evidence to suggest that basal EGP in the calorie-restricted PEPCK transgenic rat should be slightly higher than that found in the ad-libitum fed PEPCK transgenic rat.

Given that β-cell function is one of two major determinants that contribute to glucose homeostasis and that there was no improvement with dapagliflozin treatment, we next examined whether the sustained reversal of mild hyperglycaemia could enhance insulin sensitivity and therefore, offer an explanation to the improvement in glucose tolerance. A subset of PEPCK transgenic rats were calorie-restricted with the intention of preventing body weight gain over the 6 week treatment period in order to account for the potential insulin-sensitising effects associated with reduced weight gain from SGLT2 inhibition. Under insulin-stimulation, the GIR required to maintain euglycaemia in rats treated with dapagliflozin were increased by 86 % in PEPCK transgenic and 40 % in normal healthy control rats when compared to their respective vehicle-treated groups, and 73 % in calorie-restricted PEPCK transgenic versus ad-libitum fed PEPCK transgenic rats. It is important to note that we did not implement a
wash-out period prior to experimentation and so SGLT2 inhibitor-induced glycosuria may have affected our interpretation of the GIR. We initially assumed that urine glucose loss during the basal turnover studies would be negligible considering that we were only infusing radiolabelled tracer made up in 0.9 % saline and that the differences in the blood glucose levels between PEPCK transgenic rats given vehicle and dapagliflozin at the beginning of the experiment were not elevated to levels that would be considered clinically inducive of excess glycosuria (Turk et al., 1994). As such, we proceeded to collect urine directly from the bladders of all rats tested at the conclusion of the insulin-stimulated 2DG uptake studies with the expectation that we would be able to correct GIR during insulin-stimulation with the urinary glucose excretion rate. Indeed, using this method to correct GIR proved difficult as our calculations gave inconsistent values, suggesting that there must have been considerable glucose loss during the basal glucose turnover studies which we did not account for. As a result, the GIR for the hyperinsulinaemic-euglycaemic clamp in the present study has been calculated using the infusion speed from the syringe pumps. Pre-clinical studies performing basal glucose turnover and hyperinsulinaemic-euglycaemic clamp studies in rodents given SGLT2 inhibitors have attempted to circumvent this problem by regularly collecting urine over several pre-determined time periods during the course of the experiment (S. Han et al., 2008; Neschen et al., 2015; Ueta et al., 2014). In theory, this method should allow both urinary glucose excretion rates and total urinary glucose loss to be calculated so that the GIR can be adjusted accordingly; however, the accuracy in determining glucose appearance and particularly glucose disappearance due to the constant infusion and loss of glucose has been challenging (Ueta et al., 2014). Despite the technical difficulties that we encountered, our results show that the increase in GIR with calorie-restriction and dapagliflozin treatment in the PEPCK transgenic rat all translated into improvements in whole-body glucose disposal. This data suggests that dapagliflozin treatment selectively improves insulin sensitivity in addition to glycaemia, which in turn would lessen the burden and provide a β-cell sparing effect. This would help explain why the SGLT2 inhibitor, dapagliflozin, did not improve the insulin secretory capacity of the β-cell when a hyperglycaemic clamp was performed. Furthermore, while insulin-stimulated EGP was not suppressed in the PEPCK transgenic compared with control rats, this was not significantly reduced with either
calorie-restriction or dapagliflozin treatment. This is contrary to studies performed in ageing rats that were calorie-restricted or ZDF rats treated with SGLT2 inhibitors, which all show suppression of EGP during a hyperinsulinaemic-euglycaemic clamp (Catalano, Bergman, & Ader, 2005; S. Han et al., 2008; L. Thomas et al., 2012). It is important to reiterate that the PEPCK transgene in our transgenic rodent model is driven by a promoter that does not respond to the suppressive effects of insulin (Rosella et al., 1995) and so we were not expecting that insulin would have any effect on EGP. The lack of significance for hepatic and renal Pck1 in the ad-libitum fed PEPCK transgenic rat may have been due to the fact that these rats are hyperinsulinaemic and this increase in insulin levels may well be inhibiting the expression of endogenous PEPCK (but not the PEPCK transgene) such that total PEPCK expression is not different. On the other hand, the irregular expression pattern of all of the gluconeogenic genes, in particular hepatic and renal Pck1 in calorie-restricted and dapagliflozin-treated PEPCK transgenic rats was unexpected given that the plasma insulin levels achieved during the hyperinsulinaemic clamp were not high enough to suppress EGP as we have previously shown (Lamont et al., 2003). Although PEPCK has been proposed to be an important regulatory enzyme of gluconeogenesis (Hanson & Reshef, 1997) with our laboratory showing data in support of this in both in vitro (Rosella et al., 1993) and in vivo systems (Mangiafico et al., 2011), its mechanism of action might not be as simple as increased levels of PEPCK mRNA and protein equals an increase in the rate of gluconeogenesis, and vice-versa. It is likely that PEPCK expression must coordinate with other mechanisms to regulate this metabolic pathway, as is the case in livers from mice lacking Pck1 which are still able to produce glucose at a rate 40% of that in control mice (Burgess et al., 2007). The authors suggest that PEPCK content alone only weakly influences gluconeogenesis and that hepatic energy metabolism through TCA cycle flux appear to be intimately linked (Burgess et al., 2007). This “disconnect” paradigm also holds true for the gluconeogenic enzyme gene Fbp1, where a 2-fold upregulation in the livers of the NZO mouse (Andrikopoulos et al., 1993) and in high-fat fed rats (S. Song et al., 2001) results in increased EGP, but not in a lean liver-specific transgenic mouse (Lamont et al., 2006). These studies therefore suggest that enzymatic activity may be more important than transcriptional regulation and that the nutrient state of an animal, e.g., hepatic energy production from fatty acids and/or excess of peripheral substrate
supply from amino acids and fatty acids, dictates whether the overall rate of gluconeogenesis and EGP is increased.

To determine the site/s responsible for the improvement in insulin sensitivity, we measured glucose uptake in skeletal muscle (white and red fibre types) and WAT using radioactively labelled 2DG. Our results of improved whole-body glucose disposal partially due to the increase in muscle and WAT-specific glucose uptake with dapagliflozin treatment are in contrast with other studies, namely those performed in ZDF rats treated with either empagliflozin (L. Thomas et al., 2012) or dapagliflozin (S. Han et al., 2008; Ueta et al., 2014). It is not clear why we were able to see an improvement in muscle and WAT insulin resistance in the dapagliflozin-treated PEPCK transgenic compared to the ZDF rat studies mentioned above. We would hypothesise that glucotoxicity may be the primary driver of insulin resistance in the PEPCK transgenic rat while in the ZDF rat there are other contributing factors such as lipotoxicity (De Feyter et al., 2008; A. C. Smith et al., 2007) and inflammation (Ndisang & Jadhav, 2013; Rodriguez-Calvo et al., 2008). The loss of fat mass has also been shown to sensitise muscle and fat to the effects of insulin in humans (Knowler et al., 2002; Tuomilehto et al., 2001) and pre-clinical rodent models (Gazdag et al., 1999; S. Y. Park et al., 2005; Sharma et al., 2011). Another possible reason that may have offset the improvement in glucose disposal in the ZDF rat is that dapagliflozin treatment did not reduce body weight (S. Han et al., 2008; Ueta et al., 2014) as was the case in our study. Interestingly, calorie-restriction also significantly enhanced peripheral insulin sensitivity despite the presence of mild hyperglycaemia. These findings do not support our original hypothesis of excess glucose being the primary cause of peripheral insulin resistance in the PEPCK transgenic rat. As there is already a link between fat-induced hepatic and peripheral insulin resistance in obese individuals that is partly mediated by oxidative stress (Pereira et al., 2015; Pereira et al., 2014), this evidence suggests that lipotoxicity may also play a part role in the insulin resistant phenotype observed in our transgenic rodent model. This would be an interesting prospect worth pursuing considering that it is difficult to dissociate between the contribution of obesity (and potentially lipotoxicity) and glucose per se in the pathogenesis of insulin resistance in currently studied diabetic rodent models.
The disposal of glucose into skeletal muscle and WAT under insulin-stimulated conditions is predominantly dependent on the action of GLUT4 (James, Brown, Navarro, & Pilch, 1988; B. B. Kahn, 1992). We have previously shown that insulin resistance in WAT, but not skeletal muscle, is associated with reduced total GLUT4 protein levels in the PEPCK transgenic rat (Lamont et al., 2003). This tissue-specific regulation of GLUT4 is consistent with reports in patients with T2D with a decrease in adipose tissue but normal skeletal muscle GLUT4 content (Garvey et al., 1992; Garvey et al., 1991; Pedersen et al., 1990). Likewise, tissue-specific regulation of GLUT4 has also been described in animals fed a high-fat diet (D. H. Han, Hansen, Host, & Holloszy, 1997; Ikemoto, Thompson, Itakura, Lane, & Ezaki, 1995). In the current study, we show a positive association between improved insulin sensitivity in WAT and total GLUT4 protein content with dapagliflozin treatment. However, this increase in GLUT4 protein content was not observed in calorie-restricted PEPCK transgenic rats, despite comparable improvements in white adipose glucose uptake and reductions in adipocyte size and increased number relative to fat area when compared to the dapagliflozin treatment group. These conflicting reports concerning the role of altered GLUT4 protein expression in the face of enhanced insulin sensitivity are interesting and corroborate with another study which found that weight loss did not alter total GLUT4 protein content but did increase the translocation of GLUT4 to the plasma membrane in calorie-restricted Wistar rats (Seraphim, Nunes, & Machado, 2001). Indeed, it is important to disclose that this difference was only found when the authors expressed GLUT4 protein content relative to grams of tissue used. In contrast, total GLUT4 protein content from red gastrocnemius was unchanged with either treatment. However, since we measured the protein in whole tissue homogenate, we cannot rule out that an increase in the translocation efficiency of GLUT4 from the cytoplasm to the plasma membrane surface that with our current technique we could not capture. Other mechanisms such as increased functional activity of GLUT4 or even the compensatory upregulation of other GLUT’s may also be responsible for the increase in glucose uptake. To be confident in our results, it would be beneficial to measure the different fractions of GLUT4, i.e., the membrane and cytosolic protein, as well as other GLUT’s in both skeletal muscle and white adipose in order to determine whether or not an increase in GLUT4 (or other GLUT’s) translocation can explain the improvement in...
insulin sensitivity seen in the calorie-restricted and dapagliflozin-treated PEPCK transgenic groups.

**Summary**

The current study sought to determine whether dapagliflozin treatment could improve or reverse the already established core defects in insulin resistance and β-cell function found in the PEPCK transgenic rat. Calorie-restriction was also used to account for any potential insulin-sensitising effects associated with reduced weight gain from dapagliflozin treatment. The findings in this chapter show that dapagliflozin treatment in the hyperglycaemic, hyperinsulinaemic and glucose intolerant PEPCK transgenic rat resulted in reduced glucose and insulin levels, and enhanced glucose tolerance which was associated with improvements in both skeletal muscle and WAT insulin resistance. Importantly, this treatment did not increase the capacity of insulin secretion or alter islet morphology, suggesting that the SGLT2 inhibitor, dapagliflozin, does not directly increase the insulin secretory capacity of the β-cell when treatment is started too late but rather provides a β-cell sparing effect. The next chapter will investigate the contribution of obesity and glucose *per se* in the progression of the metabolic disturbances associated with the PEPCK transgenic rat, namely hyperglycaemia, glucose intolerance and β-cell dysfunction by using calorie-restriction and dapagliflozin treatment.
Chapter 5 The effects of obesity and chronic mild excess glucose in the progression of β-cell dysfunction in the PEPCK transgenic rat

Introduction
Given that the majority of currently used rodent models of T2D are reliant on obesity-induced insulin resistance in order to place secretion pressure on the β-cell, it has become inherently difficult for researchers to dissociate the relative contribution of obesity and glucose per se in the development of β-cell dysfunction. Several pre-clinical studies utilising dietary interventions have shown interplay between obesity and T2D. For example, calorie-restricting C57BL/Ks-db/db mice for 3 months can significantly improve β-cell function, reduce β-cell dedifferentiation and restore the expression of genes that are important for β-cell function and maintenance (Sheng et al., 2016). Another study using obese and diabetic ZDF rats found that a similar intervention completely prevents the loss of GSIS, the reduction in β-cell Glut2 and the decline in β-cell volume (Ohneda et al., 1995). Notwithstanding the beneficial effects on β-cell function, whether or not ameliorating diabetes in these rodent models with calorie-restriction can be attributed to by obesity alone or a combination of obesity and hyperglycaemia is difficult to determine due to their reliance for one another. In contrast, it has been well documented that glucotoxicity in the absence of obesity can escalate the development of β-cell dysfunction. Evidence in support of this is shown in the pre-clinical model, the Goto-Kakizaki rat, which was created through the repetitive breeding of Wistar rats with the poorest glucose intolerance (Goto, Kakizaki, & Masaki, 1976). This leads to the development of a lean model of T2D characterised by defective GSIS due to insufficient β-cell function and mass (Ostenson & Efendic, 2007; Portha et al., 2001).

As shown in this thesis and in previous published reports made by our laboratory, the PEPCK transgenic rat concurrently develops mild obesity, hyperglycaemia and glucose intolerance, which together, leads to muscle and fat insulin
resistance as well as a defect in insulin secretion (Lamont et al., 2003; Mangiafico et al., 2011; Rosella et al., 1995). Unlike other commonly used rodent models of T2D discussed in this thesis, the PEPCK transgenic rat does not rely on the presence of obesity to drive hyperglycaemia and glucose intolerance. Instead, the presence of mild hyperglycaemia is a by-product of a primary defect in glucose overproduction due to the over-expression of PEPCK in the kidney and liver (Rosella et al., 1995). The PEPCK transgenic rat therefore provides us with the unique ability to delineate obesity and glucose per se.

The purpose of the present study was to determine the relative contribution of obesity and chronic hyperglycaemia in the progression of β-cell dysfunction in the PEPCK transgenic rat. This was achieved by preventing obesity and hyperglycaemia by starting calorie-restriction and dapagliflozin treatment prior to any defects in β-cell function. Our data shows that early intervention with calorie-restriction or dapagliflozin does not protect nor increase insulin secretory capacity or β-cell mass. These findings suggest that treatments that prevent obesity and hyperglycaemia in the PEPCK transgenic rat act to selectively improve insulin sensitivity, which may alleviate additional stress on the β-cell.

Chapter Aims
The aims of this chapter are as follows:

1. To determine the relative contribution of obesity and glucose per se in the development of β-cell dysfunction in the PEPCK transgenic rat.
2. To determine whether early intervention with dapagliflozin, i.e., at 5 weeks of age when no metabolic defects are observed, is necessary to improve or preserve the β-cell’s insulin secretory capacity and mass in the PEPCK transgenic rat.

Results

Early intervention with calorie-restriction or dapagliflozin treatment causes reduced weight gain in PEPCK transgenic rats.

Growth parameters and measures of adiposity in 14 week-old ad-libitum fed, calorie-restricted and dapagliflozin-treated PEPCK transgenic rats are shown in Table 5.1. There were no differences in the starting body weights between PEPCK transgenic rats
randomised to *ad-libitum* fed, calorie-restriction or dapagliflozin-treated groups. All calorie-restricted PEPCK transgenic rats were given the same amount of food each day regardless of their body weight. The amount of weight gained after the 9 week intervention period was significantly lower in the calorie-restricted PEPCK transgenic rats compared to the *ad-libitum* fed PEPCK transgenic rats. Consistent with the food intake and weight gain findings in Chapter 4, PEPCK transgenic rats treated with dapagliflozin ate significantly more food yet gained significantly less body weight over the intervention period when compared to the *ad-libitum* fed PEPCK transgenic group. This is further highlighted by the decrease in feeding efficiency and is most likely due to a loss of calories in the urine resulting in a net loss of energy. The reduction in weight gain in both calorie-restricted and dapagliflozin-treated PEPCK transgenic rat groups resulted in smaller infra-renal and epididymal adipose tissue depots but not subcutaneous fat. PEPCK transgenic rats treated with dapagliflozin consumed nearly double the amount of fluid per day compared to the PEPCK transgenic rats given water. This was expected since the SGLT2 inhibitor class of drugs promote glycosuria and have a mild diuretic effect. Based on the fluid intake over the 9 week intervention period, PEPCK transgenic rats treated with dapagliflozin were administered approximately 3.3 mg of drug per day or 10 mg/kg body weight. Similar to findings from Chapter 4, kidney wet weight was significantly increased following dapagliflozin treatment for 9 weeks (Table 4.1).
Table 5.1: The effects of preventative treatment on growth parameters and adiposity in PEPCK transgenic rats.

<table>
<thead>
<tr>
<th>PEPCK transgenic rats fed ad-libitum, calorie-restricted or treated with dapagliflozin from 5 to 14 weeks of age</th>
<th>Ad-libitum fed PEPCK transgenic</th>
<th>Calorie-restricted PEPCK transgenic</th>
<th>Dapagliflozin-treated PEPCK transgenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting body weight at 5 weeks of age (g)</td>
<td>109.4 ± 3.9 (6)</td>
<td>107.7 ± 4.9 (6)</td>
<td>121.0 ± 4.8 (6)</td>
</tr>
<tr>
<td>Weight gained over intervention period (g)</td>
<td>236.6 ± 5.5 (6)</td>
<td>172.1 ± 6.8 * (6)</td>
<td>202.6 ± 5.6 ** †† (6)</td>
</tr>
<tr>
<td>Final body weight at 14 weeks of age (g)</td>
<td>346.0 ± 8.2 (6)</td>
<td>279.7 ± 5.0 ** (6)</td>
<td>323.6 ± 6.9 †† (6)</td>
</tr>
<tr>
<td>Average food intake over intervention period (g/day)</td>
<td>23.0 ± 0.6 (6)</td>
<td>17.7 ± 0.0 ** (6)</td>
<td>26.6 ± 0.5 ** ††† (6)</td>
</tr>
<tr>
<td>Feeding efficiency over intervention period (%)</td>
<td>17.7 ± 0.2 (6)</td>
<td>17.4 ± 0.7 (6)</td>
<td>13.0 ± 0.3 ** ††† (6)</td>
</tr>
<tr>
<td>Average fluid intake over intervention period (mL/day)</td>
<td>27.5 ± 1.2 (6)</td>
<td>Not measured</td>
<td>54.6 ± 0.9 *** (6)</td>
</tr>
<tr>
<td><strong>WAT mass (g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>5.00 ± 0.81 (9)</td>
<td>4.32 ± 0.77 (5)</td>
<td>3.40 ± 0.41 (12)</td>
</tr>
<tr>
<td>Infra-renal</td>
<td>4.85 ± 0.70 (9)</td>
<td>2.31 ± 0.50 * (5)</td>
<td>2.58 ± 0.25 ** (12)</td>
</tr>
<tr>
<td>Epididymal</td>
<td>4.16 ± 0.50 (9)</td>
<td>1.88 ± 0.22 ** (5)</td>
<td>2.50 ± 0.23 ** (12)</td>
</tr>
<tr>
<td>Total WAT</td>
<td>14.00 ± 1.82 (9)</td>
<td>8.52 ± 1.49 * (5)</td>
<td>8.48 ± 0.69 ** (12)</td>
</tr>
<tr>
<td><strong>Kidney wet weight (g)</strong></td>
<td>2.8 ± 0.1 (9)</td>
<td>Not measured</td>
<td>3.1 ± 0.1 * (12)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. The number of animals used for each strain is shown in parenthesis. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. ad-libitum fed PEPCK transgenic; †P < 0.01 and ††P < 0.001 vs. calorie-restricted PEPCK.
The effect of early intervention with calorie-restriction or dapagliflozin treatment on fasting glucose and insulin levels in PEPCK transgenic rats.

To assess the contribution that obesity and glucose per se have on fasting glucose and insulin levels in the PEPCK transgenic rat, calorie-restriction and dapagliflozin-treatment were started at a time when no metabolic disturbances were present, i.e., just after weaning. Overnight and 6 hour fasted plasma glucose and insulin concentrations were measured and compared in 14 week-old ad-libitum fed, calorie-restricted and dapagliflozin-treated PEPCK transgenic rats (Table 5.2). Blood ketones and plasma triglycerides were also measured in order to determine the impact that early intervention in the presence of glucose overproduction has on energy metabolism after an overnight fast (Table 5.2). Despite being genetically identical, calorie-restricted PEPCK transgenic rats displayed significantly higher 6 hour fasting blood glucose and an increased trend for overnight fasted plasma glucose levels when compared to the ad-libitum fed PEPCK group. Six hour and overnight fasting hyperinsulinaemia did not improve with calorie-restriction. However, matching the body weight of PEPCK transgenic rats so that they were similar to that of age-matched control rats (Chapter 3, Table 3.1) resulted in significantly decreased fasting blood ketone and plasma triglyceride levels. As expected, dapagliflozin treatment for 9 weeks duration prevented both 6 hour and overnight fasting hyperglycaemia and hyperinsulinaemia. Consistent with our findings from Chapter 4, treatment with dapagliflozin also significantly raised blood ketone levels (Table 4.2) but did not improve overnight fasting plasma triglycerides compared to ad-libitum fed PEPCK transgenic rats.
Table 5.2: The effect of early intervention on fasting biomarkers in PEPCK transgenic rats.

<table>
<thead>
<tr>
<th>PEPCK transgenic rats fed <em>ad-libitum</em>, calorie-restricted or treated with SGLT2 inhibitor from 5 to 14 weeks of age</th>
<th>Ad-libitum fed PEPCK transgenic</th>
<th>Calorie-restricted PEPCK transgenic</th>
<th>Dapagliflozin-treated PEPCK transgenic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>6 hour fast</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood glucose (mmol/L)</td>
<td>6.6 ± 0.1 (6)</td>
<td>7.7 ± 0.2### (6)</td>
<td>4.9 ± 0.1### ††† (6)</td>
</tr>
<tr>
<td>Plasma insulin (ng/mL)</td>
<td>2.0 ± 0.1 (6)</td>
<td>2.2 ± 0.3 (6)</td>
<td>1.2 ± 0.3† (6)</td>
</tr>
<tr>
<td><strong>Overnight fast</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma glucose (mmol/L)</td>
<td>7.2 ± 0.2 (4)</td>
<td>7.9 ± 0.2 (5)</td>
<td>4.5 ± 0.2### ††† (6)</td>
</tr>
<tr>
<td>Plasma insulin (ng/mL)</td>
<td>2.6 ± 0.3 (4)</td>
<td>2.2 ± 0.2 (5)</td>
<td>0.5 ± 0.1### ††† (6)</td>
</tr>
<tr>
<td>Blood ketones (mmol/L)</td>
<td>1.13 ± 0.05 (7)</td>
<td>0.27 ± 0.03## (6)</td>
<td>3.03 ± 0.27### ††† (6)</td>
</tr>
<tr>
<td>Plasma triglycerides (mmol/L)</td>
<td>1.067 ± 0.004 (6)</td>
<td>0.622 ± 0.078### (4)</td>
<td>0.954 ± 0.063†† (6)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. The number of animals used for each strain is shown in parenthesis. ## *P* < 0.01 and ### *P* < 0.001 vs. *ad-libitum* fed PEPCK transgenic; †*P* < 0.05, ††*P* < 0.01 and †††*P* < 0.001 vs. calorie-restricted PEPCK transgenic.
Early intervention with calorie-restriction or dapagliflozin improves glucose tolerance in PEPCK transgenic rats.

In order to determine the contribution of obesity and glucose per se on glucose intolerance, an IPGTT in overnight fasted 14 week-old PEPCK transgenic rats that were either ad-libitum fed, calorie-restricted or treated with dapagliflozin was performed. The glucose tolerance test revealed that early intervention with either calorie-restriction or dapagliflozin significantly improved the blood glucose excursion over the 120 minute experiment (Figure 5.1A). These significant improvements are clearly observed when the data is represented by the incremental area under the glucose curve (Figure 5.1B).
Figure 5.1: Intraperitoneal glucose tolerance test in PEPCK transgenic rats fed *ad-libitum*, calorie-restricted or treated with dapagliflozin for 9 weeks starting from 5 weeks of age. (A) Blood glucose and (B) incremental area under the glucose curve for glucose in 14 week-old *ad-libitum* fed, calorie-restricted and dapagliflozin-treated PEPCK transgenic rats. Data are presented as mean ± SEM. Number of animals included: *n* = 6 for each group. *P* < 0.05, **P** < 0.01 and ***P*** < 0.001 vs. *ad-libitum* fed PEPCK transgenic; ††*P* < 0.01 vs. calorie-restricted PEPCK transgenic.
Early intervention with calorie-restriction or dapagliflozin does not restore glucose-stimulated insulin secretion in PEPCK transgenic rats.

Previous results from our laboratory and those outlined in Chapter 3 have shown that the PEPCK transgenic rat is characterised by mild obesity (Table 3.1) and hyperglycaemia (Table 3.2), and develops β-cell dysfunction around 14 weeks of age (Figure 3.2C and 3.3A) (Mangiafico et al., 2011). Since obesity has been shown to expedite hyperglycaemia, the relative contribution of obesity and a primary defect in glucose overproduction in the progression from normal to impaired GSIS in the PEPCK transgenic is currently not known. Therefore, we attempted to address the importance of the two in β-cell function by initiating calorie-restriction and dapagliflozin at 5 weeks of age before any metabolic defects were present. A one-step hyperglycaemic clamp was performed at 14 weeks of age in PEPCK transgenic rats fed ad-libitum, calorie-restricted or treated with dapagliflozin for 9 weeks duration. The GIR required to maintain glycaemia between 15-17 mmol/L throughout the clamp experiment were comparable between the ad-libitum fed and calorie-restricted PEPCK transgenic groups (Figure 5.2A and C). Interestingly, careful examination of the GIR in the calorie-restricted PEPCK transgenic group clearly shows a small, but not significant increase in the GIR during the first 45 minutes of the hyperglycaemic clamp which was not due to increased insulin secretion as plasma insulin levels were not raised at these time-points (Figure 5.2B). This is most likely due to a slight improvement in insulin sensitivity which required a small increase in the GIR to balance glycaemia between 15-17 mmol/L. The subsequent decline in GIR after 45 minutes back to a rate similar to the ad-libitum fed PEPCK group suggests that this fine balance was achieved. Dapagliflozin treatment on the other hand resulted in significantly raised GIR which were required to maintain hyperglycaemia, indicative of enhanced insulin sensitivity. Surprisingly, neither intervention improved the insulin secretory response in the PEPCK transgenic rat (Figure 5.2B and panel insert).

To assess whether preventing obesity and hyperglycaemia could improve the β-cell’s overall capacity to secrete insulin, glucose infusion was stopped and a bolus of arginine injected at 121 minutes. Arginine stimulated a robust insulin secretory response above that of glucose alone in all three groups; however, ad-libitum fed PEPCK transgenic rats trended to hypersecrete insulin compared with the calorie-
restricted and dapagliflozin-treated groups, but this did not reach statistical significance (Figure 5.2B dotted areas and D). Of note, the blood glucose excursion after an arginine bolus followed a similar pattern to that of the ad-libitum fed group irrespective of the intervention given (Figure 5.2A dotted areas).
Chapter 5

Relative contribution of obesity and glucose in β-cell dysfunction

A) Blood glucose (mmol/L) over time with Arginine injection.

B) Plasma insulin (ng/mL) over time with different treatments.

C) Glucose infusion rate (μmol/min/kg) over time.

D) iAUC insulin (ng/mL x min) with different treatments.
Figure 5.2: One-step hyperglycaemic clamp in PEPCK transgenic rats fed *ad-libitum*, calorie-restricted or treated with dapagliflozin for 9 weeks starting from 5 weeks of age. (A) Blood glucose, (B) plasma insulin levels and incremental area under the insulin curve (C) glucose infusion rate during the course of the one-step hyperglycaemic clamp, and (D) incremental area under the insulin curve following a bolus of arginine at the conclusion of the one-step hyperglycaemic clamp in 14 week-old *ad-libitum* fed, calorie-restricted and dapagliflozin-treated PEPCK transgenic rats. Incremental area under the insulin curve for GSIS during the one-step hyperglycaemic clamp is illustrated as corresponding panel insert. An arginine bolus (1 g/kg) was administered one minute after last sampling (120 minute time-point) and is represented by dotted areas. Data are presented as mean ± SEM. Number of animals included: *n* = 6 for each group. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. *ad-libitum* fed PEPCK transgenic; ††*P < 0.01 and †††*P < 0.001 vs. calorie-restricted PEPCK transgenic.
To confirm the results of the *in vivo* GSIS, pancreatic islets were isolated from 14 week-old PEPCK transgenic rats fed *ad-libitum*, calorie-restricted or treated with dapagliflozin for 9 weeks duration and *in vitro* GSIS in the presence of 20 mmol/L glucose was performed. When compared to the *ad-libitum* fed PEPCK transgenic rat islets, both early intervention with calorie-restriction and dapagliflozin showed similar insulin secretion in response to 20 mmol/L glucose (Figure 5.3A). Furthermore, there were no differences in the insulin content of *ad-libitum* fed, calorie-restricted or dapagliflozin-treated PEPCK transgenic rat islets (Figure 5.3B). These data are consistent with the one-step hyperglycaemic clamp where calorie-restriction or dapagliflozin treatment does not improve GSIS (Figure 5.2A and B).
Figure 5.3: Glucose-stimulated insulin secretion in isolated pancreatic islets from PEPCK transgenic rats fed ad-libitum, calorie-restricted or treated with dapagliflozin for 9 weeks starting from 5 weeks of age. (A) Insulin release from pancreatic islets stimulated with 20 mmol/L glucose, and (B) insulin content in islets isolated from 14 week-old ad-libitum fed, calorie-restricted or dapagliflozin-treated PEPCK transgenic rats. Data are presented as mean ± SEM. Number of animals included: n = 3-16 for each group.
Early intervention with calorie-restriction or dapagliflozin reduces islet size and preserves islet number in PEPCK transgenic rats.

As shown in Chapter 3, 14 week-old PEPCK transgenic rats possess significantly larger but fewer pancreatic islets than their control counterparts (Table 3.3). To determine how much adiposity and mild hyperglycaemia in the PEPCK transgenic rat contributes to the size and number of pancreatic islets, we compared insulin stained sections taken from PEPCK transgenic rats calorie-restricted or treated with dapagliflozin from 5 weeks of age for 9 weeks duration with those from 14 week-old ad-libitum fed PEPCK transgenic rats (Table 5.3). Representative images of insulin immunohistochemical staining of the pancreas from PEPCK transgenic rats fed ad-libitum, calorie-restricted or treated with dapagliflozin at 8x magnification (scale bar, 300 µm) are shown in Figure 5.4. Pancreas was weighed prior to formalin fixation as an approximate measure of pancreatic growth. Both calorie-restriction and dapagliflozin administration did not affect pancreatic growth during the intervention period. Results from image analysis show that early intervention with calorie-restriction and dapagliflozin treatment increases islet number by 38 % and 18 % compared to ad-libitum fed PEPCK transgenic rats, respectively. Calorie-restriction significantly reduced the average islet size with fewer islets being larger than 20,001 µm² and a trend for a larger proportion of islets less than 1,000 µm² compared to PEPCK transgenic rats fed ad-libitum. Initiating dapagliflozin at weaning did not significantly alter islet size although there was a trend for a decrease in size of islets being distributed over 20,001 µm² when compared to ad-libitum fed PEPCK transgenic rats. Neither intervention altered insulin positive staining nor β-cell mass (or β-cell area) even though resolving both obesity and hyperglycaemia have been shown to promote insulin sensitivity and thus, alleviate the demand for the β-cell to compensate by hypersecreting insulin and β-cell hyperplasia. These data indicate that significantly reducing the amount of weight gained through calorie-restriction restores both islet size and islet number in the PEPCK transgenic rat, but does not alter β-cell mass or area. A similar conclusion can be made with early intervention of dapagliflozin, but not to the level of calorie-restriction and excessive reduced weight gain.
Figure 5.4: Insulin immunohistochemical staining of the pancreas of PEPCK transgenic rats fed *ad-libitum*, calorie-restricted or treated with dapagliflozin for 9 weeks starting from 5 weeks of age. Representative images of insulin immunohistochemical staining of pancreatic sections from PEPCK transgenic rats fed either (A) *ad-libitum*, (B) calorie-restricted or (C) treated with dapagliflozin for 9 weeks starting from 5 weeks of age. The insulin-containing β-cells stain brown and the vascular endothelial cells and exocrine portion of the pancreas appear blue. 8x magnification; scale bar, 300 μM.
Table 5.3: Quantitative insulin immunohistochemical staining of pancreatic sections from PEPCK transgenic rats fed *ad-libitum*, calorie-restricted or treated with dapagliflozin for 9 weeks starting from 5 weeks of age.

<table>
<thead>
<tr>
<th></th>
<th>Ad-libitum fed PEPCK transgenic</th>
<th>Calorie-restricted PEPCK transgenic</th>
<th>Dapagliflozin-treated PEPCK transgenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreas weight (g)</td>
<td>1.47 ± 0.08 (4)</td>
<td>1.52 ± 0.04 (6)</td>
<td>1.40 ± 0.16 (5)</td>
</tr>
<tr>
<td>Average islet size (µm²)</td>
<td>9743 ± 838 (6)</td>
<td>6150 ± 1193# (4)</td>
<td>6697 ± 522 (3)</td>
</tr>
<tr>
<td>Islet number (per mm² pancreas area)</td>
<td>0.84 ± 0.05 (6)</td>
<td>1.35 ± 0.19# (4)</td>
<td>1.02 ± 0.14 (3)</td>
</tr>
<tr>
<td>Islet size distribution (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1,000 µm²</td>
<td>34.5 ± 2.9</td>
<td>41.6 ± 3.8#</td>
<td>34.0 ± 2.3</td>
</tr>
<tr>
<td>1,001 – 3,000 µm²</td>
<td>24.2 ± 1.6</td>
<td>21.6 ± 1.7</td>
<td>25.8 ± 2.7</td>
</tr>
<tr>
<td>3,001 – 5,000 µm²</td>
<td>9.1 ± 1.4</td>
<td>9.4 ± 3.0</td>
<td>9.0 ± 1.0</td>
</tr>
<tr>
<td>5,001 – 10,000 µm²</td>
<td>10.2 ± 1.5</td>
<td>10.2 ± 0.6</td>
<td>11.2 ± 0.3</td>
</tr>
<tr>
<td>10,001 – 20,000 µm²</td>
<td>7.3 ± 0.8</td>
<td>9.7 ± 3.5</td>
<td>10.1 ± 1.2</td>
</tr>
<tr>
<td>&gt;20,001 µm²</td>
<td>14.9 ± 1.0</td>
<td>7.6 ± 2.4#</td>
<td>9.9 ± 2.1</td>
</tr>
<tr>
<td>Insulin⁺ area (% islet area)</td>
<td>68.2 ± 1.9 (6)</td>
<td>68.9 ± 1.8</td>
<td>61.3 ± 2.5</td>
</tr>
<tr>
<td>β-cell area (% pancreas area)</td>
<td>0.5 ± 0.1 (6)</td>
<td>0.5 ± 0.1 (4)</td>
<td>0.4 ± 0.1 (3)</td>
</tr>
<tr>
<td>β-cell mass (mg)</td>
<td>6.7 ± 0.2 (4)</td>
<td>8.3 ± 1.9 (4)</td>
<td>5.4 ± 1.2 (3)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. The number of animals used for each strain is shown in parenthesis. #P < 0.05 vs. *ad-libitum* fed PEPCK transgenic. ImageScope software was used for quantification of pancreatic images.
**Discussion**

Due to the interdependent nature of obesity and hyperglycaemia in T2D, it is difficult to accurately determine the influence of obesity and glucose *per se* in the pathogenesis of β-cell dysfunction. The PEPCK transgenic rat is characterised by mild obesity and primary chronic hyperglycaemia which in turn is associated with impaired insulin secretion. The major advantage of this model is that the appearance of hyperglycaemia is not dependent on obesity, thereby allowing us to investigate the effects of obesity and glucose *per se* in the progression of β-cell dysfunction without any of the complexities that would be encountered if we were to use currently available pre-clinical models of diabetes. Therefore, the primary aim of this study was to determine the relative contribution of obesity and glucose *per se* in the development of β-cell dysfunction in the PEPCK transgenic rat. In order to look at both of these parameters in isolation, we devised an intervention protocol using calorie-restriction and dapagliflozin treatment at a time where β-cell function in the PEPCK transgenic rat was normal, i.e., at 5 weeks of age. In addition, since initiating dapagliflozin treatment in glucose intolerant PEPCK transgenic rats with established β-cell dysfunction did not improve insulin secretory function when assessed by a one-step hyperglycaemic clamp (Chapter 4, Figure 4.2), this study also sought to determine whether early intervention with dapagliflozin is necessary to improve or preserve the insulin secretory capacity of the β-cell.

The loss of fat mass is considered to be one of the most striking phenotypic changes with calorie-restricting animals (Barzilai & Gabriely, 2001; Bertrand, Lynd, Masoro, & Yu, 1980). Indeed, restricting food intake in a subset of PEPCK transgenic rats to a level below that which would be consumed voluntarily resulted in significant reductions in total WAT (the total of subcutaneous, infra-renal and epididymal fat) and body weight when compared to *ad-libitum* fed PEPCK transgenic rats. The reduction in fat mass due to calorie-restriction has also been shown to sufficiently lower plasma glucose levels in rodent models of T2D, regardless of when intervention is commenced. For example, starting calorie-restriction in 12 week-old C57BL/Ks-db/db mice, an age when T2D is firmly established (Aasum, Hafstad, Severson, & Larsen, 2003), completely normalises glucose levels back to the control db/m mice (Sheng et al., 2016). Similarly, calorie-restricting 10 week-old ZDF rats, prevents the development of hyperglycaemia (Lezcano et al., 2014). The effect of calorie-restriction on glucose
homeostasis is even more remarkable given that introducing calorie-restricted lean ZDF rats to unrestricted food access for only 2 weeks results in significant weight gain that culminates to a final body weight that exceeds obese rats on an ad-libitum diet, while increasing glucose levels by approximately 4.2 mmol/L (Ohneda et al., 1995). Based on these findings, one would predict that implementing a similar intervention, especially before the onset of hyperglycaemia, would result in reduced glucose levels. It is important to reiterate that our transgenic rat is unique to other rodent models of glucotoxicity primarily because the PEPCK transgene is driven by a promoter that does not respond to the suppressive effects of insulin, so glucose overproduction is unable to be “switched off”. Thus, we were not entirely expecting that resolving adiposity through calorie-restriction would have any beneficial effect on glucose metabolism. In fact, our results are similar to those in Chapter 4 (Table 4.2) whereby calorie-restriction caused significantly elevated six hour FPG concentrations that were accompanied with a trend for increased overnight FPG levels. As it is widely accepted that fasting glucose is a reflection of hepatic glucose production, and to a smaller extent, renal glucose production (Ekberg et al., 1999), it would follow that the PEPCK transgene is driving glucose production during fasting conditions despite the prevention of significant weight gain that was afforded with calorie-restriction. It is common knowledge that the energy status of an animal, i.e., whether they are fed or fasted, and the length of fasting, can influence the degree of gluconeogenesis (Rui, 2014). As calorie-restricted PEPCK transgenic rats ate their allocated food within a couple of hours, we attempted to control for this confounding factor by introducing a re-feed on the day prior to experimentation to ensure accurate fasting times with other experimental groups. However, because these animals spent a large proportion of their life span in a fasted state, it might be predicted that hepatic gluconeogenic flux is increased. Studies investigating the major metabolic pathways involved in fuel usage of calorie-restricted rodents have reported increased gene expression of all three of the gluconeogenic enzymes; PEPCK, G6Pase and FBPase, in addition to increased enzymatic activity of PEPCK in the liver, while genes and enzymes pertinent to glycolysis, such as liver pyruvate kinase and pyruvate dehydrogenase, were reduced by approximately 50-60 % (Dhahbi et al., 2001; Dhahbi et al., 1999; Hagopian et al., 2003). Therefore, these studies in combination with ours,
suggest that the enzymatic capacity for gluconeogenesis is even higher with calorie-restriction than with *ad-libitum* feeding in the PEPCK transgenic rat.

One of the classic characteristics of obesity in humans is dyslipidaemia characterised by high levels of triglycerides and low HDL-C (Klop, Elte, & Cabezas, 2013). Reducing the amount of weight gained by calorie-restriction significantly mitigated fasting triglycerides, thus confirming a link between hypertriglyceridaemia in the PEPCK transgenic rat with mild obesity and excessive food consumption. We also found that calorie-restriction did not shift metabolism from glucose to the utilisation of ketone bodies, an otherwise consistent phenomenon observed in rodents maintained on a dietary-restricted regimen (Anson et al., 2003; Mahoney, Denny, & Seyfried, 2006; Seyfried, Sanderson, El-Abbadi, McGowan, & Mukherjee, 2003). This is most likely due to the increased gluconeogenic capacity provided by the PEPCK transgene and the readily available substrate for gluconeogenesis from the breakdown of non-glucose precursors such as amino-acids and pyruvate from skeletal muscle and glycerol from adipose tissue in order to maintain blood glucose at an optimal level for normal brain function when food is scarce. In contrast, a modest reduction in weight gain and the prevention of hyperglycaemia from early intervention with dapagliflozin did not alter fasting plasma triglycerides. Previous studies in pre-clinical models of diabetes that were fed a standard laboratory chow diet have shown that the effect of SGLT2 inhibitors on triglyceride levels to be inconsistent. Specifically, treatment of C57BL/Ks-db/db mice with remogliflozin etabonate results in improved fasting triglycerides (Fujimori et al., 2008), whilst treatment in the same strain of mice, but with a different SGLT2 inhibitor, BI-38335, had no effect (L. Chen et al., 2012). From the current literature, it appears that the effect of SGLT2 inhibitors in lowering triglyceride levels is enhanced when a high-fat diet is used (Fujimori et al., 2008; F. R. Macdonald et al., 2010; Suzuki et al., 2014). This may be due to the fact that a conventional high-fat diet artificially raises plasma lipids and so any change in whole-body metabolism from SGLT2 inhibition is more than likely to elicit a beneficial effect.

In the context of reduced glucose and insulin, we speculate that the rate of lipolysis would be increased and thus provide a high rate of free fatty-acid flux to the liver. This would promote the β-oxidation of fatty-acids and the use of ketone bodies as a fuel source to the heart and brain, whilst potentially sparing triglycerides for other tissues.
This could explain the unaltered levels in the plasma triglyceride and the significant increase in blood beta-hydroxybutyrate during fasting conditions. Due to the safety concerns made by both the FDA and the EMA with respect to the increased incidence of euglycaemic diabetic ketoacidosis in diabetic patients who use SGLT2 inhibitors as adjunct therapy (Rosenstock & Ferrannini, 2015), it is imperative that the mechanism/s underlying these increases in ketone levels are investigated further. The increased incidence of diabetic ketoacidosis in type 1 diabetics prescribed SGLT2 inhibitors off-label by experienced practitioners in diabetes care is also a concern. However, considering that the T1D population are in desperate need of therapies other than exogenous insulin, the benefit of using SGLT2 inhibitors far outweigh the risk of euglycaemic diabetic ketoacidosis which can be greatly reduced by doctor-initiated drug titration and education.

As previously mentioned in this thesis, the relative contribution of obesity and glucose per se in T2D has been difficult to ascertain since the majority of diabetic rodent models rely on the presence of obesity to induce insulin resistance and subsequently, hyperglycaemia. The PEPCK transgenic rat is unlike these rodent models in that the presence of chronic mild hyperglycaemia is a reflection of a primary defect in glucose overproduction that arises from the over-expression of PEPCK in the kidney and liver (Rosella et al., 1995). This unique transgenic rat therefore serves as a useful tool to dissect the influence that obesity and glucose per se may have in the progression from NGT to IGT amongst other defects pertinent to T2D. Our results clearly show that despite possessing the required machinery for increased gluconeogenesis which would be expected to lead to IGT, calorie-restriction significantly enhanced glucose tolerance. Similarly, the early use of dapagliflozin also improved glucose tolerance. These improvements in glucose tolerance are in agreement with other obese rodent models of pre-hyperglycaemia that were either calorie-restricted (Kanda et al., 2015; Ohneda et al., 1995) or treated with a SGLT2 inhibitor (L. Chen et al., 2012) prior to hyperglycaemia being established. Interestingly, this current study did not recapitulate our previous findings in which calorie-restriction failed to improve glucose tolerance in the PEPCK transgenic rat (Mangiafico et al., 2011). Reviewing the final body weights of the calorie-restricted PEPCK transgenic rats used in both studies clearly show that our previously published study achieved slightly more weight gain than what was
observed in the current study. Although the exact relationship between the percentage of weight gain and the extent to which it influences glucose tolerance in age-matched rodents is not well defined, our results suggest that limiting weight gain further translates into significantly improved glucose tolerance. This is supported by studies in humans which show that skeletal muscle insulin sensitivity does not change after moderate weight loss (6-8%) (Petersen et al., 2005; F. Sato et al., 2007), yet increases markedly after greater weight loss (16%) is achieved (Niskanen et al., 1996).

Obesity in concert with chronically elevated glucose levels has been shown to contribute to the progressive loss of β-cell function. Due to the complex nature in dissociating the relationship between obesity and excess glucose in currently available pre-clinical models of diabetes, the primary aim of this chapter was to determine the relative contribution that obesity and glucose per se have in the development of β-cell dysfunction in the PEPCK transgenic rat. In this regard, we measured the insulin secretory response to a glucose stimulus by using two methods; the one-step hyperglycaemic clamp and static incubations with isolated pancreatic islets. Additionally, since initiating dapagliflozin treatment in PEPCK transgenic rats with established impaired insulin secretion did not improve insulin secretion (Chapter 4, Figure 4.2B), we also sought to determine whether early intervention, i.e., at 5 weeks of age when normal insulin secretion is present, is necessary to improve or preserve β-cell function. The utilisation of calorie-restriction in type 2 diabetic rodent models has been shown to reduce hyperglycaemia and be beneficial to β-cell function and morphology. For example, reducing food intake in C57BL/Ks-db/db mice by 50% for 6 weeks improves glucose-mediated insulin secretion and insulin content in isolated islets and increases β-cell mass, which is in contrast to the decline that is seen in ad-libitum fed C57BL/Ks-db/db mice (Kanda et al., 2015). In another experiment using the same strain, limiting the daily food intake to 0.1 g/kg body weight/day for 3 months significantly ameliorated β-cell function and reduced β-cell dedifferentiation (Sheng et al., 2016). Calorie-restriction in obese and pre-diabetic male ZDF rats also prevents the loss of GSIS in isolated pancreatic islets and improved β-cell volume fraction (Ohneda et al., 1995). These findings are in contrast to our study in calorie-restricted PEPCK transgenic rats which clearly showed no significant improvements in insulin secretion in vivo or in vitro using isolated islets in static experiments, or in β-cell mass. Our
laboratory has previously shown that chronic high glucose treatment of islets can impair insulin secretory function via the generation of advanced glycation end products and oxidative stress (Kooptiwut et al., 2005; Zraika et al., 2006). It therefore makes sense that treatment with a SGLT2 inhibitor should be beneficial in improving β-cell function in the glucose intolerant PEPCK transgenic rat, especially when given at an age when no impairment in β-cell function is present. Such preventative experiments have already been performed in type 2 diabetic rodent models with varying results. For example, preventing hyperglycaemia from occurring in C57BL/Ks-db/db mice with early treatment of the SGLT2 inhibitor, BI-38335, significantly enhances glucose-mediated insulin secretion in isolated islets in static experiments (L. Chen et al., 2012). This is in part due to islet morphology being maintained through preserved β-cell/α-cell ratio and decreased islet cell apoptosis (L. Chen et al., 2012). This observation does not corroborate with the results in our study showing no changes in glucose-mediated insulin secretion in pancreatic islets. In spite of this difference, a study using C57BL/Ks-db/db mice that were crossed to SGLT2 whole-body knockout mice showed that insulin secretion was not improved in vitro using isolated islets in static or perifusion experiments (Jurczak et al., 2011). Similarly, a study using the hyperglycaemic clamp in ZDF rats treated with dapagliflozin prior to the onset of hyperglycaemia showed reduced plasma insulin and C-peptide levels and no effect on β-cell mass compared with the vehicle treated group (F. R. Macdonald et al., 2010), which support the findings of our study. This study also showed that the disposition index, which takes into account the relationship between prevailing insulin resistance and insulin secretion, was significantly improved with early treatment of dapagliflozin (F. R. Macdonald et al., 2010). Unfortunately, we did not measure the C-peptide concentration during the hyperglycaemic clamp and so were unable to calculate or compare the disposition index to other studies. Instead, we attempted to use the GIR as a marker of insulin sensitivity; however, doing so proved technically difficult as we did not eliminate the potential for SGLT2 inhibitor-induced glycosuria by performing a wash-out period prior to experimentation. A number of methods used by other groups to combat this problem have been discussed at length in Chapter 4. Considering that our pre-defined steady-state of 15-17 mmol/L glucose for the hyperglycaemic clamp is considerably higher than what would otherwise be seen in the fed-state of PEPCK
transgenic rats, this most likely resulted in the gross inflation of the GIR and hence, increased the magnitude of insulin sensitivity that we saw with dapagliflozin treatment. Despite our difficulty in accurately determining the level of insulin sensitivity in the dapagliflozin group, it is important to note that we did perform a hyperinsulinaemic-euglycaemic clamp in PEPCK transgenic rats that were treated with calorie-restriction or dapagliflozin after metabolic defects such as insulin resistance and impaired insulin secretion were present (Chapter 4, Figure 4.4). Based on our findings of resolved insulin resistance in these older PEPCK transgenic rats due to improved muscle and fat insulin resistance, we would hypothesise that early intervention with either calorie-restriction or dapagliflozin in PEPCK transgenic rats would provide similar or even better insulin sensitivity compared to late treatment. Therefore, while there may not have been a direct increase in the insulin secretory capacity in the one-step hyperglycaemic clamp or the isolated pancreatic islet static incubation experiments with either intervention, the current findings in conjunction with our results from Chapter 4 suggest that preventing excessive weight gain and hyperglycaemia could potentially increase insulin sensitivity and provide an islet β-cell sparing effect. A caveat worth mentioning is that PEPCK transgenic rats begin with significantly fewer islets (Chapter 3, Table 3.3) compared to control and this continues throughout life. This suggests that altered β-cell development and function is occurring and could potentially offset any dapagliflozin-mediated improvements in β-cell function. Further experiments looking at the expression of genes implicated in β-cell development such as Pdx-1 and MafA would be worth performing. Of note, one of the major limitations of the current study is that we did not consider the effect of lipotoxicity on β-cell function in the setting of chronic mild hyperglycaemia (calorie-restriction) or resolved mild hyperglycaemia (dapagliflozin treatment) in the PEPCK transgenic rat. Indeed, the effect of lipotoxicity in the absence of chronic high glucose and its contribution to the demise of the β-cell in vivo are quite controversial. For example, mimicking lipotoxic conditions by infusing male Wistar rats for 72 hours with 20 % Intralipid showed no differences in insulin secretion when a hyperglycaemic clamp was performed (Hagman et al., 2008). Other experiments, by contrast, have shown that Intralipid infusion for just 48 hours actually leads to increased β-cell responsiveness in vivo, but not in vitro, when the stimulatory effect of 5.5, 11 and 16.6 mmol/L glucose was investigated (Magnan et al., 1999).
Moreover, a 48 hour infusion of oleate decreased insulin and C-peptide concentrations to a hyperglycaemic clamp and induced oxidative stress in isolated islets (Oprescu et al., 2007). Both of these effects were restored to normal when the antioxidants N-acetylcysteine and taurine were co-infused (Oprescu et al., 2007). Of particular interest to this thesis, a study investigating the detrimental effects of lipotoxicity in obese pre-diabetic and diabetic ZDF rats found that in the scenario of mild hyperglycaemia, the β-cell is able to partially compensate for the lipotoxic-induced insulin resistance whereas with more marked hyperglycaemia, this compensation is lost (Goh et al., 2007). Based on the mixed findings above, an experiment using a lipid-lowering agent such as one of the fibrates should be performed in the future with and without dapagliflozin in order to address whether or not lipotoxicity may play an important role in the progression from normal to impaired β-cell function in the PEPCK transgenic rat.

**Summary**

In conclusion, since both obesity and glucose *per se* are considered to be key contributors in the progression of normal to impaired β-cell dysfunction, the primary aim of this study was to investigate the relative contribution of obesity and excess glucose in the progression of β-cell dysfunction in the PEPCK transgenic rat. In keeping with our findings from Chapter 4 where we showed that late treatment of dapagliflozin does not improve β-cell function, we also wanted to determine whether implementing an intervention protocol prior to any apparent defects would be beneficial to β-cell function. The findings in this study clearly show that preventing obesity or hyperglycaemia in the PEPCK transgenic rat by calorie-restriction or dapagliflozin treatment did not directly increase the insulin secretory capacity *in vivo* or *in vitro*, nor did it increase β-cell mass. These data therefore suggest that both obesity and glucose *per se* play a contributing role in the development of glucose intolerance, insulin resistance and β-cell dysfunction in the PEPCK transgenic rat, and that improving insulin sensitivity with either intervention can effectively reduce the burden on the β-cell.
Chapter 6 Summary, Conclusions and Future Directions

Introduction
Insulin resistance and impaired insulin secretion are cardinal defects that contribute to the development of T2D (S. E. Kahn, 2003). Obesity is considered to be one of the major drivers in exacerbating insulin resistance, which in turn places unnecessary stress on the β-cell. Due to the strong association between obesity and T2D in humans (Sims et al., 1973), it is necessary for pre-clinical models to incorporate both of these disease states so that potential drug therapies can be evaluated and translated to humans. However, one of the biggest drawbacks with most of the commonly used diabetic rodent models of T2D such as the ZDF rat, the C57BL/Ks-db/db mouse and the NZO mouse, is that they possess several limitations such as severe obesity due to leptin mutations and the early-onset of β-cell dysfunction which are in contrast to the insidious nature of obesity and T2D in humans (H. Chen et al., 1996; Phillips et al., 1996; Veroni et al., 1991). Moreover, their dependence on obesity to drive hyperglycaemia and β-cell dysfunction makes it difficult to delineate the contribution that obesity and glucose per se have in the development of IGT and T2D. The PEPCK transgenic rat is unique to the rodent models of T2D described herein as it does not rely on obesity-induced insulin resistance to cause glucotoxicity. Instead, mild chronic excess glucose occurs as a consequence from a 2-3 fold induction of the gluconeogenic enzyme, PEPCK, in both the kidney and liver (Mangiafico et al., 2011). The main objective of this thesis was to characterise the PEPCK transgenic rat as a suitable model of β-cell dysfunction and then to use this model to dissociate the relative contribution of obesity and chronic excess glucose in the pathogenesis of T2D using dietary and pharmacological interventions.

Overall Aim
The overall aim of this thesis was to investigate the relative contribution of obesity and glucose per se in the development of NGT to IGT, insulin resistance and β-cell dysfunction in the PEPCK transgenic rat.
Overall Hypothesis
The overall hypothesis of this thesis was that both obesity and chronic glucose excess due to the PEPCK transgene contribute to the development of glucose intolerance, hyperglycaemia, insulin resistance and β-cell dysfunction in the PEPCK transgenic rat.

Experimental Approach
To explore the hypothesis in this thesis, it was necessary to do the following:

1. Characterise the PEPCK transgenic rat as a suitable model of mild obesity, glucose intolerance, hyperglycaemia and β-cell dysfunction (Chapter 3).
2. Assess whether initiating the SGLT2 inhibitor, dapagliflozin, in PEPCK transgenic rats with established glucose intolerance, hyperglycaemia, insulin resistance and β-cell dysfunction can resolve these defects (Chapter 4).
3. Determine the relative contribution of obesity and glucose per se by using calorie-restriction and dapagliflozin treatment in the development of β-cell dysfunction in the PEPCK transgenic rat (Chapter 5).

Summary of Results
The PEPCK transgenic rat was used to test our hypothesis as it is a robust model of chronic mild glucose excess that acquires several metabolic defects commonly found in pre-diabetes, such as mild obesity, glucose intolerance, peripheral insulin resistance and early signs of β-cell dysfunction, which gradually worsen with age (Lamont et al., 2003; Mangiafico et al., 2011; Rosella et al., 1995; Thorburn et al., 1999). Prior to characterising the PEPCK transgenic rat as an appropriate model of glucose intolerance, hyperglycaemia and β-cell dysfunction, we sought to firstly confirm its mild obese phenotype. This was particularly important as we wanted to ensure that the obesity element in our transgenic model represented the majority of the current human population with T2D. We therefore measured both body weight and food intake on a weekly basis from 5 to 20 weeks of age. We found that PEPCK transgenic rats became progressively heavier compared to their control counterparts with age and this was largely due to an increase in food intake. Of particular importance to the translational aspect of our rodent model to humans, the differences between the PEPCK transgenic and control rat strains at 14 and 20 weeks of age were only a modest 28 % and 33 % respectively, which is in stark contrast to the 78 % difference observed in the
C57BL/Ks-db/db mouse when compared to its C57BL/6J control. Our previous findings using the IVGTT showed that the PEPCK transgenic rat develops an acquired defect in second-phase GSIS at 12-14 weeks-old (Mangiafico et al., 2011). However, as circulating glucose levels in this experimental test often reach non-physiological levels in excess of 30 mmol/L following glucose injection, and the relationship between insulin secretion and sensitivity is not taken into account, in this thesis we decided to employ the more informative hyperglycaemic clamp. The GIR required to maintain glycaemia between 15-17 mmol/L during the experiment was significantly decreased by 40-47 % in PEPCK transgenic rats at both ages, indicative of profound insulin resistance. Moreover, hyperglycaemia did not result in increased insulin secretory capacity and was in fact significantly less than age-matched controls in both first- and second-phases of GSIS. We next looked at the expression profile of genes of significant importance to β-cell function, namely Glut2, Gck, Sur1 and Kir6.2. Our results show that islet gene expression is normal in normoglycaemic 5 week-old PEPCK transgenic rats, but that the expression of these genes begin to decline as the PEPCK transgenic rat develops mild obesity, glucose intolerance and hyperglycaemia with age. Our in vitro experiments clearly show that in the first instance, the defect in GSIS arises from a combination of reduced Glut2 expression and the inability of the K⁺ATP channel to function properly. With age, progressive reductions in Glut2, Sur1 and Kir6.2 as well as β-cell mass occur, further exacerbating the decline in β-cell function. Of particular interest was the finding that gene expression levels of the subunits that make up the K⁺ATP channel, Sur1 and Kir6.2, were normal in 14 week-old PEPCK transgenic rats even though tolbutamide-induced secretion in isolated islets was significantly blunted. This indicates that there are other mechanisms besides gene expression which may be affected, such as the channel assembly or the translocation to the plasma membrane. The above findings clearly show that the PEPCK transgenic rat is an excellent model to investigate the link between mild obesity and glucose excess in the progression from normal to impaired β-cell function.

Previous findings from our laboratory and in this thesis have shown that insulin resistance and β-cell dysfunction are firmly established in the glucose intolerant PEPCK transgenic rat by 14 weeks of age. In the current literature, it is not entirely clear whether treatment with a SGLT2 inhibitor can directly or indirectly improve β-cell function.
function as studies have chosen to only use techniques that look at one or the other, and not both. Therefore, we investigated whether or not treatment for 6 weeks with the SGLT2 inhibitor, dapagliflozin, could resolve insulin resistance or β-cell dysfunction (or both) in the PEPCK transgenic rat by using the hyperinsulinaemic-euglycaemic and hyperglycaemic clamp, respectively. Furthermore, since SGLT2 inhibitors have been shown to cause substantially less weight loss than what was initially expected from the energy excreted via glycosuria, we addressed this phenomenon by monitoring body weight and food intake during dapagliflozin treatment. In line with other human (Bailey et al., 2010; Millar et al., 2016; Nauck, 2014) and pre-clinical studies (Devenny et al., 2012; Fujimori et al., 2008; Katsuno et al., 2009), we found that dapagliflozin resulted in a modest, but not significant, reduction in body weight gain that was offset by the significant increase in average food intake over the treatment period. The ketone body, β-hydroxybutyrate, was also found to be significantly elevated in PEPCK transgenic rats treated with dapagliflozin which was counter-intuitive to the hyperphagia that was observed. Our data shows that the excess loss of glucose from the urine with SGLT2 inhibitors shift energy metabolism from glucose to fatty-acid oxidation in order to maintain energy balance. In order to determine whether established defects in insulin sensitivity and secretion could be corrected with dapagliflozin, we implemented the “gold-standard” techniques, the hyperinsulinaemic-euglycaemic clamp and hyperglycaemic clamp, respectively. We show that dapagliflozin treatment does not increase the insulin secretory capacity. In addition, treatment with dapagliflozin did not alter islet morphology or preserve β-cell mass. However, dapagliflozin did improve glucose tolerance and this was associated with enhanced insulin sensitivity due to improved glucose uptake in muscle and fat. A subset of PEPCK transgenic rats were also calorie-restricted in order to prevent further weight gain so that they could be used to account for any potential weight-induced insulin-sensitising benefits seen in the dapagliflozin-treated PEPCK transgenic group. Preventing weight gain in these PEPCK transgenic rats greatly enhanced peripheral insulin sensitivity to levels comparable with dapagliflozin treatment. Our results indicate that although dapagliflozin treatment may not have had a direct effect on the β-cell, improvements in glycaemia and insulin sensitivity would lessen the burden and provide an islet β-cell sparing effect.
To establish the relative contribution of obesity and glucose in the progression from NGT to IGT and its effect on β-cell function, we prevented obesity and hyperglycaemia from occurring in the PEPCK transgenic rat by starting calorie-restriction and dapagliflozin treatment at 5 weeks of age before any metabolic defects were present. In addition, since late treatment with dapagliflozin did not improve insulin secretion, we also sought to determine whether earlier intervention was necessary to preserve or improve the insulin secretory capacity of the β-cell. We show that early intervention with either treatment adequately improves glucose tolerance but does not directly increase the insulin secretory capacity, as assessed by the one-step hyperglycaemic clamp and static incubation of isolated islets. This was surprising, particularly because treatments that improve hyperglycaemia have been suggested to be beneficial to impairments in insulin secretion in T2D. Although we did not perform the hyperinsulinaemic-euglycaemic clamp in PEPCK transgenic rats given early treatment, our previous results from late treatment would suggest initiating either calorie-restriction or dapagliflozin before any defects in insulin action or secretion are present may result in similar or even better insulin sensitivity than late treatment. These results suggest that both obesity and glucose *per se* may play a role in the insulin resistant phenotype of the PEPCK transgenic rat and that enhancing insulin sensitivity with either intervention could effectively reduce the secretion pressure that is placed on the β-cell.

**Overall Conclusion**

This thesis builds upon the current literature of SGLT2 inhibitors and investigates whether they directly or indirectly exert their effects on the β-cell by being the first to measure both insulin sensitivity and insulin secretory function using the “gold-standard” techniques, the hyperinsulinaemic-euglycaemic and hyperglycaemic clamp. We clearly show that dapagliflozin treatment reduces blood glucose and insulin levels, enhances glucose tolerance due to improved muscle and fat insulin resistance but does not improve insulin secretory function. These findings suggest that dapagliflozin has an indirect effect on the β-cell by enhancing insulin sensitivity and alleviating the pressure on the β-cell to secrete insulin. Overall, our results support the hypothesis that both obesity and glucose *per se* play a contributing role in the progression of glucose intolerance, insulin resistance and β-cell dysfunction in the PEPCK transgenic rat.
Future Directions

Does dapagliflozin treatment actually preserve or improve β-cell function in the PEPCK transgenic rat?

Given that glucotoxicity has been shown to elicit deleterious effects on the β-cell through various mechanisms such as oxidative and ER stress, we hypothesised that treatment with the SGLT2 inhibitor, dapagliflozin, would provide beneficial effects on impaired insulin secretion in the PEPCK transgenic rat. It was therefore surprising that the insulin secretory capacity as assessed by the hyperglycaemic clamp was not improved when dapagliflozin was given in the treatment group (Chapter 4, Figure 4.2) and was not protected in the intervention group (Chapter 5, Figure 5.2) in our study. The lack of increased insulin secretory capacity and β-cell mass in both of these groups does not necessarily mean that β-cell function was not improved. The relationship between insulin sensitivity and secretion is hyperbolic in nature which means that as insulin sensitivity falls, insulin secretion must increase in order to maintain normoglycaemia. Our findings clearly show that initiating dapagliflozin significantly enhances insulin sensitivity through increased glucose uptake in muscle and fat, which suggests that the β-cell is not under any pressure to increase insulin output. We did attempt to isolate pancreatic islets from PEPCK transgenic rats that were ad-libitum fed, calorie-restricted and treated with dapagliflozin from both treatment and intervention groups on several occasions. However, we found great difficulty in isolating a suitable number of islets to adequately perform experiments as there were significant amounts of adipose tissue surrounding and throughout the pancreas making the isolation process tedious and the survival of islets very low. Our failure to measure the expression profile of genes pertinent to β-cell function such as Glut2, Gck, Sur1 and Kir6.2 after calorie-restriction and dapagliflozin treatment is one of the biggest limiting factors of this thesis. Therefore, repeating the same treatment and intervention protocols, isolating pancreatic islets and performing gene expression analyses would be the most appropriate future experiment. The results from this experiment will definitively show whether or not calorie-restriction and dapagliflozin treatment can actually improve β-cell function, and more importantly, provide insight in our understanding of the direct effect that obesity and excess glucose have on the β-cell in vivo.
What is the mechanism that drives compensatory hyperphagia with chronic dapagliflozin treatment?

The precise mechanism that drives compensatory hyperphagia during chronic SGLT2 inhibitor treatment is currently unknown. Our study showed that PEPCK transgenic rats treated with dapagliflozin had a modest decrease in weight loss that was associated with significant reductions in adiposity despite higher food intake and blood $\beta$-hydroxybutyrate levels when compared to ad-libitum fed PEPCK transgenic rats. The increase in ketone levels is associated with reduced appetite in obese individuals (Chearskul et al., 2008; Sumithran et al., 2013) and in transgenic mice with a lean phenotype (Visinoni et al., 2012), which makes our finding counter-intuitive. There is obviously a sensing mechanism that is activated with glycosuria that causes an increase in energy intake and is worth investigating further as greater weight loss would lead to even more marked improvements in blood glucose levels. In order to investigate this experimental question, a good starting point would be to analyse the hormonal and neuronal regulators involved in hunger and satiety in PEPCK transgenic rats chronically treated with dapagliflozin. The measurement of gut hormones such as ghrelin and cholecystokinin, and neuropeptides such as neuropeptide Y and agouti-related peptide, may provide a better understanding of the mechanism that drives compensatory hyperphagia with chronic SGLT2 inhibitor treatment.

Dapagliflozin and kidney hypertrophy

One of the most striking findings in this thesis was that chronic administration of dapagliflozin significantly increased kidney wet weight when compared to ad-libitum fed PEPCK transgenic rats. As the main focus of this thesis was to dissociate the relative contribution of obesity and glucose in insulin action and secretion in the PEPCK transgenic rat, we did not pursue any additional experiments as to how and why hypertrophy of the kidney occurred with dapagliflozin treatment. We hypothesise that SGLT2 inhibitor-induced glycosuria promotes increased water retention in the tubular lumen due to osmotic effects of unabsorbed glucose. The first experiment should therefore address this hypothesis by determining the kidney dry weight which can be achieved by incubating the kidney for 24 hours at 80°C in the presence of a desiccant. If there is still a significant difference in kidney weight between ad-libitum fed and
dapagliflozin-treated PEPCK transgenic rats, then the next experiment should focus on histology of the kidney to show if any particular regions have undergone expansion.
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APPENDICES

Appendix I – Determining the Dosage of Dapagliflozin (Pilot Study)

Figure 6.1: Fluid intake in 10-12 week-old female PEPCK transgenic rats given either water or dapagliflozin over 20 days. Fluid intake was assessed by periodically weighing bottles and determining the average difference in weight over the duration of treatment. We assumed that 1 g was equivalent to 1 mL when making our calculations. Data are presented as mean ± SEM. Number of animals included: \(n = 3\) for each group. *\(P < 0.05\) and **\(P < 0.01\) vs. water PEPCK transgenic.

To determine the appropriate dosage of dapagliflozin for rodent studies, a pilot study was performed in a subset of 10-12 week-old female PEPCK transgenic rats given either water or dapagliflozin. We found that 60 mg/L of dapagliflozin resulted in a significant increase in fluid intake, whereas halving this dosage to 30 mg/L on day 13 returned fluid intake levels back to water-treated PEPCK transgenic rats. The subsequent increase of dapagliflozin back to 60 mg/L from day 13 onwards saw this significant increase in fluid intake restored. Since the SGLT2 inhibitor class of drugs have a mild diuretic effect, we chose 60 mg/L of dapagliflozin as the working concentration for all rodent experiments performed in this thesis.
Appendix II – Gene Expression Analysis of Pck1 in Other Tissues

Figure 6.2: Gene expression analysis of Pck1 in tissues from 4 week-old fed control and PEPCK transgenic rats. Samples were prepared for Real-Time PCR analysis as previously described in Chapter 2. Data are presented as mean ± SEM. Number of animals included: \( n = 5 \) for each group. *\( P < 0.05 \) vs. control (Mangiafico, S. P., unpublished data, 2011).
Appendix III – Intravascular Glucose Tolerance Test

Figure 6.3: Intravascular glucose tolerance tests in control and PEPCK transgenic rats fed ad-libitum, and PEPCK transgenic rats treated with dapagliflozin. (A) Plasma glucose and (B) plasma insulin levels during the course of the intravascular glucose tolerance test in intervention (for 9 weeks duration starting from 5 weeks of age) and treatment groups (for 6 weeks duration starting at 14 weeks of age). Following an overnight fast (approximately 16 hours), a 200 µL blood sample was collected to measure basal blood glucose and plasma insulin concentrations. A glucose bolus (1 g/kg body weight) was then injected into a jugular catheter and blood samples collected at 2, 5, 10, 15, 30 and 45 minute time-points. Data are presented as mean ± SEM. Number of animals included: Intervention, \( n = 4 \) for ad-libitum fed control, \( n = 5 \) ad-libitum fed PEPCK transgenic and \( n = 6 \) for dapagliflozin-treated PEPCK transgenic, and treatment, \( n = 7 \) for ad-libitum fed control, \( n = 4 \) ad-libitum fed PEPCK transgenic and \( n = 4 \) for dapagliflozin-treated PEPCK transgenic. \(*P < 0.05\), \(**P < 0.01\) and \(***P < 0.001\) vs. ad-libitum fed PEPCK transgenic.
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