Regulation of renal brush-border glucose transport in response to metabolic dysregulation

A thesis submitted by Havovi Chichger

For the degree of Doctor of Philosophy in the Division of Biosciences, Faculty of Life Sciences, University College London

Department of Neuroscience, Physiology & Pharmacology
University College London
Royal Free Campus
Rowland Hill Street
London
NW3 2PF

2011
1 Abstract

Diabetic nephropathy is a consequence of hyperglycaemia-induced renal cell damage and a major contributor to end-stage renal disease in later stages of diabetes. Previous studies, in type I diabetes, observed PKC-βI-dependent GLUT2 recruitment to the proximal tubule brush-border membrane (BBM) as a direct result of elevated plasma glucose. The up-regulation of glucose transporters at the proximal tubule BBM causes a rise in intracellular glucose concentration; tubular injury in diabetic nephropathy is postulated to be a result of increased GLUT-mediated glucose entry into the proximal tubule cell. Augmented glucose transport across the proximal tubule BBM is likely to elevate glucose reabsorption and exacerbate hyperglycaemia during diabetes. Thus identification of regulators of this process may offer novel therapeutic targets to reduce renal glucose handling during hyperglycaemia. Studies described in this thesis were designed to elucidate the modulation of glucose transporters in models of metabolic syndrome associated with diabetes.

Type II diabetes, the prevalent form of the disease, was found to elevate both SGLT- and GLUT-mediated glucose transport across the proximal tubule BBM, with an accompanying increase in glucose transporter expression. In rodent models of metabolic syndrome, induced by feeding studies, an elevation in PKC-βI-dependent GLUT2 recruitment to the proximal tubule BBM was also observed. However SGLT1 expression at the proximal tubule BBM did not consistently mirror that of GLUT2. In studies presented in this thesis, the sweet taste receptor heterodimer (T1R2/3) was identified at the proximal tubule BBM and this sweet taste sensor exhibited a tight correlation with SGLT1 in all models of metabolic dyregulation studied. Furthermore, stimulation of T1R2/3, by the artificial sweetener saccharin, resulted in an increase in SGLT-mediated glucose transport across the proximal tubule BBM.

The data reported in this thesis provide evidence that GLUT2 expression at the proximal tubule BBM is not solely a response to hyperglycaemia but also a renal response to whole-body metabolic dysregulation. From these studies it is hypothesised that PKC-βI-mediated GLUT2 recruitment to the proximal tubule BBM occurs via a SGLT-T1R2/3-dependent and –independent pathway.
2 Acknowledgements

The work reported in this thesis was carried out in the Department of Neuroscience, Physiology & Pharmacology, UCL at the Royal Free campus, London, UK. I am grateful to Diabetes UK for funding the PhD project, without which these studies could not have been performed.

I would like to show my gratitude for my supervisors; Dr. Ted Debnam for his excellent supervision and encouragement, Professor Robert Unwin for his invaluable guidance and support and Professor Kaila Srai for all his advice.

In addition, I would like to acknowledge Dr. Mark Cleasby, Dr. Scott Wildman and Dr. Marco Bueter for their assistance and advice in setting up the high-fat, junk-food and RYGB rodent models described in this thesis. I would also like to show my gratitude to the members of the London Epithelial Group for their support, in particular, Dr. Joanne Marks for her never-ending patience and for teaching me everything I know, and Sobiya Nadaraja for her encouragement and support both in and out of the lab.

Last but not least, I would like to thank everyone who made me laugh when none of my experiments worked and specifically, my parents, my sister and Pete, for their endless encouragement, patience and support.
3 Contents

3.1 Table of contents

1 ABSTRACT 2

2 ACKNOWLEDGEMENTS 3

3 CONTENTS 4

3.1 Table of contents 4

3.2 Table of figures 12

3.3 Table of tables 15

4 ABBREVIATIONS 16

5 INTRODUCTION 21

5.1 Glucose homeostasis 22
  5.1.1 Utilisation of glucose 24
  5.1.2 Hepatic glucose production and storage 24
  5.1.3 Renal glucose production and release 25
  5.1.4 Adipocyte and skeletal muscle contribution to glucose homeostasis 25

5.2 Diabetes 26
  5.2.1 Type I diabetes 26
    5.2.1.1 Genetic and environmental factors in the onset of type I diabetes 26
  5.2.2 Type II diabetes 27
    5.2.2.1 Genetic and environmental factors in the onset of type II diabetes 28
    5.2.2.2 Incretins in type II diabetes 29
  5.2.3 Potential Type III diabetes 29
  5.2.4 Diabetic nephropathy 30
    5.2.4.1 Classification of diabetic nephropathy 30
    5.2.4.2 Glomerular signalling pathway in the onset of diabetic nephropathy 32
    5.2.4.3 Tubular injury in diabetic nephropathy 33
  5.2.5 Current treatment for diabetes 34
    5.2.5.1 Treatment for type I diabetes 34
5.2.5.2  Treatment for type II diabetes
5.2.5.2.1  Pharmaceutical treatments of type II diabetes
5.2.5.2.2  Newer treatments for type II diabetes
5.2.5.2.3  Drug-combination treatment in type II diabetes

5.3  Metabolic models related to diabetes
5.3.1  Obesity
5.3.2  The treatment of obesity with gastric bypass surgery
5.3.2.1  Gastric bypass surgery in type II diabetics
5.3.2.1.1  Foregut hypothesis
5.3.2.1.2  Hindgut hypothesis
5.3.3  Metabolic syndrome
5.3.4  Pre-diabetes

5.4  Sweeteners in the Western diet
5.4.1  Fructose
5.4.2  Artificial sweeteners

5.5  Animal models of metabolic dysregulation
5.5.1  Models of type I diabetes
5.5.2  Models of metabolic syndrome and type II diabetes
5.5.2.1  Rat models of metabolic syndrome
5.5.2.2  Mouse models of metabolic syndrome
5.5.2.3  Rodent models of type II diabetes
5.5.2.3.1  Zucker rat model of obesity and type II diabetes
5.5.2.3.2  Goto-Kakizaki rat model of type II diabetes

5.6  Glucose transporters
5.6.1  Facilitative glucose transporters
5.6.1.1  Class I GLUT transporters
5.6.1.1.1  GLUT1
5.6.1.1.2  GLUT2
5.6.1.1.2.1  GLUT2 as a glucose sensor
5.6.1.1.2.2  Genetic regulation of GLUT2
5.6.1.1.3  GLUT3
5.6.1.1.4  GLUT4
5.6.1.2  Class II and III GLUT transporters
5.6.1.2.1  GLUT5
5.6.1.3  Inhibitors of GLUT-mediated transport
5.6.2 Active glucose transporters
  5.6.2.1 SGLT family
    5.6.2.1.1 SGLT1
    5.6.2.1.2 SGLT2
    5.6.2.1.3 SGLT3
  5.6.2.2 SGLT inhibitors
    5.6.2.2.1 Phlorizin
    5.6.2.2.2 SGLT2 inhibitors in type II diabetes treatment
      5.6.2.2.2.1 Potential treatment of diabetes by SGLT2 inhibitors

5.7 Glucose handling in the kidney
  5.7.1 Renal glucose filtration and reabsorption
    5.7.1.1 Glucose filtration
    5.7.1.2 Glucose reabsorption
    5.7.1.3 Renal glucose production and storage
  5.7.2 Regulation of renal glucose handling
    5.7.2.1 Renal glucose filtration, production and storage in diabetes
      5.7.2.1.1 Filtration and tubuloglomerular feedback
      5.7.2.1.2 Renal glucose production and release
      5.7.2.1.3 Renal glucose storage
      5.7.2.1.4 Renal glucose reabsorption
        5.7.2.1.4.1 Renal glucose transporters involved in glucose reabsorption
        5.7.2.1.4.2 Involvement of PKC in glucose transporter expression
    5.7.2.2 Other factors affecting renal glucose transport

5.8 Glucose absorption across the small intestine
  5.8.1 Before a meal
  5.8.2 Following a meal
    5.8.2.1 Breakdown of carbohydrates at the enterocyte BBM
    5.8.2.2 Sodium-dependent glucose absorption into the enterocyte
    5.8.2.3 GLUT2-mediated glucose absorption and its regulation
    5.8.2.4 PKC-βII and calcium signalling
  5.8.3 Sweet taste receptors in the enterocyte
  5.8.4 Revised model of glucose absorption across the enterocyte
  5.8.5 Glucose transport at the small intestine in diabetes
    5.8.5.1 Other regulators of intestinal glucose absorption
      5.8.5.1.1 Flavonoids
      5.8.5.1.2 Stages of development
      5.8.5.1.3 Stress
5.8.5.1.4 AMPK 95
5.8.5.1.5 RELMβ 96
5.8.5.1.6 Hormones 97
5.8.5.1.6.1 Incretin hormones 97
5.8.5.1.6.2 Leptin 98

5.9 Aims of thesis 99

6 MATERIALS AND METHODS 100

6.1 Short-term intravenous (IV) infusions 101

6.2 Animal models 101
6.2.1 Insulin-dependent diabetes mellitus (IDDM) 101
6.2.2 Non insulin-dependent diabetes mellitus (NIDDM) 102
6.2.3 High-fat diet 102
6.2.4 Junk-food diet 103
6.2.5 Roux-en-Y Gastric Bypass (RYGB) surgery 104

6.3 LLC-PK₁ cell culture 104

6.4 Membrane preparation 105
6.4.1 Whole membrane preparation 105
6.4.2 Renal BBM vesicle preparation 105
6.4.3 Small intestinal BBM vesicle preparation 106

6.5 Renal glucose uptake studies 107
6.5.1 Validation of BBM vesicles for glucose uptake studies 107
6.5.1.1 Intravesicular volume 107
6.5.1.2 Time-dependent overshoot 107
6.5.2 SGLT- and GLUT-mediated glucose uptake 108
6.5.3 Glucose uptake calculations 108

6.6 Determination of protein concentration 109

6.7 Determination of alkaline phosphatase 110
6.7.1 BBM vesicle enrichment of alkaline phosphatase 111

6.8 Determination of plasma and urine glucose concentration 111
6.8.1 Plasma 111
6.8.2 Urine 111
6.8.3 Glucose oxidase assay

6.9 Insulin assay

6.10 Osmolality measurements

6.11 Protein analysis
  6.11.1 Dot blots
  6.11.2 Western blotting

6.12 Real time PCR
  6.12.1 cDNA preparation
  6.12.2 Primers

6.13 Statistics

7 ACUTE REGULATION OF GLUT2 AT THE PROXIMAL TUBULE BRUSH-BORDER

7.1 Introduction

7.2 Aims of chapter

7.3 Results
  7.3.1 Acute hyperglycaemia: time course in vivo infusion studies
  7.3.1.1 Effect of infusion studies on plasma glucose
  7.3.1.2 Glycosuria in acute hyperglycaemia
  7.3.1.3 Effect of acute hyperglycaemia on GLUT2 expression at the proximal tubule BBM
  7.3.1.4 Effect of plasma osmolality on renal GLUT2 expression
  7.3.2 Chronic hyperglycaemia: type I diabetes induced with streptozotocin
  7.3.2.1 Effects of short-term STZ-induced diabetes
  7.3.2.2 Effect of 7-day STZ-induced diabetes on GLUT2 expression at the proximal tubule BBM
  7.3.3 Effect of STZ-induced diabetes on GLUT2 intracellular pool in proximal tubule cells

7.4 Discussion

7.5 Conclusions
8 RENAL GLUCOSE TRANSPORT ADAPTATIONS IN RODENT MODELS ASSOCIATED WITH METABOLIC SYNDROME

8.1 Introduction
  8.1.1 Type II diabetes – the Goto-Kakizaki model
  8.1.2 Obesity and metabolic syndrome
    8.1.2.1 Junk-food diet
    8.1.2.2 High-fat diet
    8.1.2.3 RYGB surgery

8.2 Aims of chapter

8.3 Results
  8.3.1 Type II diabetes
    8.3.1.1 Animal parameters of the Goto-Kakizaki (GK) rat
    8.3.1.2 Renal BBM vesicle glucose uptakes in GK type II diabetic rat
      8.3.1.2.1 Validation of BBM vesicles
      8.3.1.2.2 Renal BBM glucose transport in GK type II diabetic rats
    8.3.1.3 Effect of type II diabetes on BBM expression of renal glucose transporters
  8.3.2 Junk-food model
    8.3.2.1 Animal parameters following a junk-food diet
    8.3.2.2 Effect of junk-food diet on glucose transporter expression at the proximal tubule BBM
  8.3.3 High-fat diet
    8.3.3.1 Animal parameters following a high-fat diet
    8.3.3.2 Effect of high-fat diet on glucose transporter expression at the proximal tubule BBM
  8.3.4 Roux-en-Y Gastric Bypass (RYGB) surgery
    8.3.4.1 Animal parameters following RYGB surgery
    8.3.4.2 Effect of RYGB surgery on glucose transporter expression at the proximal tubule BBM
    8.3.4.3 Effect of RYGB surgery on gene expression of glucose transporters in the renal cortex
    8.3.4.4 Effect of RYGB surgery on glucose transporter expression at the enterocyte BBM
    8.3.4.5 Effect of RYGB surgery on gene expression of glucose transporters in the small intestine

8.4 Discussion
8.5 Conclusions

9 MODULATION OF RENAL GLUCOSE TRANSPORT BY SWEET TASTE SENSING AT THE PROXIMAL TUBULE BRUSH-BORDER

9.1 Introduction

9.2 Aims of chapter

9.3 Results

9.3.1 Taste receptor mRNA expression in the kidney

9.3.2 Detection of sweet taste dimer component proteins in the kidney

9.3.2.1 Detection of T1R2 and T1R3 protein in whole membrane from kidney

9.3.2.2 Detection of renal sweet taste receptors in other renal cell types

9.3.2.3 Detection of renal sweet taste receptors at the proximal tubule BBM

9.3.3 Effects of acute high plasma glucose and osmolality on expression of sweet taste receptors at the proximal tubule BBM

9.3.4 Effect of chronic hyperglycaemia on sweet taste receptors at the proximal tubule BBM

9.3.5 Effects of different metabolic models on the expression of sweet taste receptors at the proximal tubule BBM

9.3.6 Effect of artificial sweetener infusion on glucose transport and sweet taste receptor expression in the proximal tubule

9.3.6.1 Animal parameters following saccharin infusion
9.3.6.2 Effect of saccharin infusion on the expression of renal sweet taste dimer components 222
9.3.6.3 Renal BBM glucose transport in response to saccharin infusion 223
9.3.6.4 Effect of saccharin infusion on the expression of renal glucose transporters 224
9.3.7 Relationship between T1R2 and T1R3, and SGLT1 expression at the proximal tubule BBM 226
9.3.8 Relationship between expression of T1R2 and T1R3 at the proximal tubule BBM 227

9.4 Discussion

9.4.1 The umami taste sensor 229
9.4.2 The sweet taste sensor 231
9.4.3 Sweet taste sensing and hormonal regulation 233
9.4.4 Sweet taste sensing and renal glucose transport 239
9.4.5 Artificial sweeteners 240
9.4.6 Sweet taste sensing and glucose absorption in response to gastric bypass surgery 242
9.4.7 Characteristics of renal sweet taste sensing 244
9.4.8 Novel locations and roles for taste sensors 245

9.5 Conclusion 246

10 DISCUSSION

10.1 Aims of thesis 248
10.2 Discussion 249

11 CONCLUSIONS 255

12 REFERENCES 256
3.2 Table of figures

**Figure 5.1** – The regulation of glucose homeostasis including the main contributors to the control of the whole-body glucose pool 23
**Figure 5.2** – The development of diabetic nephropathy in glomerular cells following high extracellular glucose stimulation 31
**Figure 5.3** – The complex interplay between the onset of obesity, metabolic syndrome and type II diabetes 38
**Figure 5.4** – Schematic diagram of the specific regions of the proximal tubule associated with glucose reabsorption from tubular fluid 71
**Figure 5.5** – Schematic diagram of the sugar transporters involved in glucose reabsorption across the proximal tubule cell during diabetes 79
**Figure 5.6** – Schematic diagram of the sugar transporters involved in glucose absorption across the enterocyte before and after a meal 83
**Figure 5.7** – Original model of the regulation of GLUT2 recruitment to the enterocyte BBM mediated by the calcium-induced terminal web 86
**Figure 5.8** – The signalling cascade activated by the stimulation of the sweet taste heterodimer 89
**Figure 5.9** – Revised model of the regulation of GLUT2 recruitment to the enterocyte BBM mediated by sweet taste receptors and intracellular calcium 91
**Figure 7.1**– Effect of 1 hour 1.1 M glucose infusion on plasma glucose concentrations 124
**Figure 7.2** – Effect of 2 hour infusion of 1.1 M glucose with initial bolus, on plasma glucose concentrations 124
**Figure 7.3** – Effect of acute hyperglycaemia on GLUT2, PKC-βI and SGLT1 expression at the proximal tubule BBM 127
**Figure 7.4** – Effect of 1.09 M mannitol infusion on the expression of GLUT2, PKC-βI and SGLT1 at the proximal tubule BBM 129
**Figure 7.5** – Effects of 2 – 7 days of STZ-induced diabetes on GLUT2, PKC-βI and SGLT1 expression at the proximal tubule BBM 132
**Figure 7.6** – Effects of 10- and 30-day STZ-induced type I diabetes on GLUT2 mRNA expression in renal cortex 133
**Figure 8.1** – Schematic diagram of the physiological intestinal alterations in RYGB surgery 148
**Figure 8.2** – Scattergraph of the relationship between glycosuria and hyperglycaemia in 8-9 week lean and type II diabetic GK rats 150
**Figure 8.3** – Representative graph of the time-dependent overshoot seen for renal BBM vesicle glucose uptake at 100μM glucose concentration 152
Figure 8.4 – Effect of type II diabetes in GK rats on SGLT-mediated glucose uptake

Figure 8.5 – Effect of type II diabetes on GLUT-mediated glucose uptake

Figure 8.6 – Effects of type II diabetes in GK animals on expression of GLUT2 and GLUT5, PKC-βI and SGLT1 at the proximal tubule BBM

Figure 8.7 – Daily weight of chow consumed by control and experimental animals and components of junk-food diet consumed by experimental rats

Figure 8.8 – Body weight increase in response to 8 weeks junk-food diet

Figure 8.9 – Effect of chow and junk-food diet on plasma and urine glucose concentration and plasma insulin levels

Figure 8.10 – Effects of 8-week junk-food diet on expression of GLUT2 and GLUT5, PKC-βI and SGLT1 expression at the proximal tubule BBM

Figure 8.11 – Final body and kidney weights in rats fed chow or high-fat diet

Figure 8.12 – Effects of 5-week high-fat diet on GLUT2 and GLUT5, PKC-βI and SGLT1 expression at the proximal tubule BBM

Figure 8.13 – Body weight and fasted basal plasma insulin levels in animals 60 days after sham or RYGB surgery

Figure 8.14 – Effects of RYGB surgery on GLUT2 and GLUT5, PKC-βI and SGLT1 expression at the proximal tubule BBM

Figure 8.15 – Effects of RYGB surgery on SGLT1, SGLT2, GLUT2 and GLUT5 mRNA expression in renal cortex

Figure 8.16 – Effects of RYGB surgery on GLUT2 and GLUT5, PKC-βII and SGLT1 BBM protein expression in the small intestine

Figure 8.17 – Regional mRNA expression profile of GLUT2 and GLUT5 in the small intestine following RYGB surgery

Figure 9.1 – Detection of taste receptor mRNA in the jejunum and kidney cortex

Figure 9.2 – Dot blot detection of sweet taste heterodimer components T1R2 and T1R3 in whole membrane prepared from control rat kidney cortex and jejunal mucosa

Figure 9.3 – Detection of sweet taste receptor heterodimer components T1R2 and T1R3 by western blotting of control mouse kidney cortex and porcine LLC-PK1

Figure 9.4 – Detection of sweet taste heterodimer components, T1R2 and T1R3 in BBM prepared from the kidney and jejunum from control rats

Figure 9.5 – Effect of acute hyperglycaemia or high osmolality on sweet taste heterodimer components, T1R2 and T1R3 at the proximal tubule BBM

Figure 9.6 – Effect of chronic hyperglycaemia studied in 10- and 30-day STZ-induced type I diabetic rats on mRNA expression of sweet taste receptors T1R2 and T1R3 in the kidney cortex and jejunum

Figure 9.7 – Effect of chronic hyperglycaemia studied with 7-day STZ-induced diabetes on sweet taste receptors T1R2 and T1R3 at the proximal tubule BBM
Figure 9.8 – Effect of type II diabetes on sweet taste receptors T1R2 and T1R3 at the proximal tubule BBM in lean control rats and GK type II diabetic rats 211

Figure 9.9 – Effect of high-fat diet on sweet taste receptor expression at the proximal tubule BBM 212

Figure 9.10 – Effects of junk-food diet on sweet taste receptor expression at the proximal tubule BBM 213

Figure 9.11 – Effects of RYGB surgery on sweet taste receptor mRNA components T1R2 and T1R3 in the renal cortex 214

Figure 9.12 – Effects of RYGB surgery on sweet taste receptor components T1R2 and T1R3 at the proximal tubule BBM 215

Figure 9.13 – Effects of RYGB surgery on sweet taste receptor mRNA components T1R2 and T1R3 in specific regions of the small intestine 217

Figure 9.14 – Effects of RYGB surgery on expression of sweet taste receptor T1R2 protein in the specific regions of the small intestine 219

Figure 9.15 – Effects of RYGB surgery on expression of sweet taste receptor T1R3 protein in specific regions of the small intestine 220

Figure 9.16 – Effect of saccharin on expression of sweet taste receptors, T1R2 and T1R3, at the proximal tubule BBM 222

Figure 9.17 – Effect of saccharin on SGLT-mediated glucose uptake 223

Figure 9.18 – Effect of saccharin on GLUT-mediated glucose uptake 224

Figure 9.19 – Effect of saccharin on expression of GLUT2 and GLUT5, PKC-βI and SGLT1 at the proximal tubule BBM 225

Figure 9.20 – Effect of different metabolic models on the expression of T1R2, T1R3 and SGLT1 at the proximal tubule BBM 226

Figure 10.1 – Proposed signalling pathways responsible for the regulation of glucose transport across the proximal tubule BBM in response to metabolic dysregulation and stress models 254
### 3.3 Table of tables

Table 5.1 - Table of rodent models of type I diabetes induced by chemicals, surgical treatment or spontaneous genetic models 50

Table 5.2 - Table of obese and non-obese rodent models for spontaneous or genetically-derived type II diabetes 53

Table 6.1 - Nutritional Information for chow and junk-food diet given to animals over an 8 week period 103

Table 6.2 - Table of the primary antibodies used to detect proteins analysed in western blotting 115

Table 6.3 - Table of the secondary antibodies used to bind to and detect primary antibodies analysed in western blotting 115

Table 6.4 - Table of primers designed for use in mRNA expression analysis 117

Table 7.1 – Body and kidney weights and renal BBM vesicle enrichment of alkaline phosphatase marker enzyme, in rats infused with 154 mM saline, 1.1 M glucose, 0.08 M and 1.09 M mannitol 125

Table 7.2 – Infusate osmolality and its effect on plasma osmolality for 154 mM saline, 0.08 M mannitol, 1.1 M glucose and 1.09 M mannitol 125

Table 7.3 – Body and kidney weights and renal BBM vesicle enrichment of alkaline phosphatase marker enzyme in rats with 7-day STZ-induced type I diabetes 131

Table 8.1 – Body and kidney weight and plasma glucose, osmolality and basal circulating insulin measurements in 8-9 week lean and type II diabetic GK rats 151

Table 8.2 – Fasted plasma concentrations of glucose, basal insulin and insulin concentration following the insulin tolerance test 164

Table 8.3 - Summary table of effects of type II diabetes, and metabolic models associated with the disease, on animal parameters and expression of glucose transporters at the proximal tubule BBM 176

Table 9.1 – Infusate osmolality and its effect on plasma osmolality, and body and kidney weights and renal BBM vesicle enrichment of alkaline phosphatase marker enzyme in rats following IV infusion of 1.37 mM saccharin 221

Table 9.2 – Relationship between the expression of renal sweet taste receptors T1R2 and T1R3 in response to various metabolic models quantified as percentage expression of T1R2 to T1R3 227

Table 9.3 - Summary table of effects of type II diabetes, and metabolic models associated with the disease, on expression of sweet taste receptors and SGLT1 at the proximal tubule BBM 228
4 Abbreviations

3T3-L1 cells – fibroblast-like adipocyte cell line
ACCORD – action to control cardiovascular risk in diabetes
ADP – adenosine diphosphate
AGE – advanced glycation end-products
AICAR - aminoimidazole carboxamide ribonucleotide
ALS – alloxan susceptible mice
AMP – adenosine monophosphate
AMPK – adenosine monophosphate protein kinase
AP1 – activator protein 1 transcription factor
ATP – adenosine triphosphate
BB – biobreeding rat model of type I diabetes
BBM – brush-border membrane
BLM – basolateral membrane
BMI – body mass index
BP – blood pressure
BPD – biliopancreatic diversion
BSA - bovine serum albumin
BTBR ob/ob – leptin-deficient, obese model of type II diabetes
C57BL/6J - C57 black 6 mouse strain (common inbred mouse strain)
C57BL/KsJ - genetically obese mice carrying db/db mutation
Caco-2 cells - human epithelial colorectal adenocarcinoma cells
cAMP – cyclic adenosine monophosphate
CaSR – calcium-sensing receptor
Ca,1.3 – L-type calcium channel
cDNA - complementary DNA
CJC-1131 – GLP-1 analog
CNS – central nervous system
cPLA2 - cytosolic phospholipase A2
CREBH – cAMP-responsive element binding protein
DAG - diacylglycerol
$db/db$ – mouse model of type II diabetes
d$H_2$O – distilled water
DNA – deoxyribonucleic acid
DOCA - deoxycorticosterone salt
DOG - deoxyglucose
DPM – disintegrations per minute
DPP-IV – dipeptidyl peptidase IV
DTT - dithiothreitol
ECL - enhanced chemiluminescence
ECM – extracellular matrix
EDTA – ethylene diaminetetraacetic acid
EGTA - ethylene glycoltetraacetic acid
ELISA – enzyme-linked immunosorbent assay
EPI – epididymal
ERK – extracellular signal-regulated kinase
ESRD – end-stage renal disease
$fa/fa$ – recessive trait of leptin receptor mutation in obese Zucker rats
FBS – fetal bovine serum
FFA – free fatty acids
FGF – fibroblast growth factor
FRG – familial renal glycosuria
G-6-P – glucose-6-phosphate
$G\alpha_{gustducin}$ – $\alpha$-gustducin G protein
GDP – guanosine diphosphate
GFR – glomerular filtration rate
GIP – gastric inhibitory polypeptide
GK – Goto-Kakizaki rat
GLP-1 – glucagon-like peptide-1
GLP-2 - glucagon-like peptide-2
GLUT – facilitative glucose transporter
GOR - glucose oxidase reagent
GTP – guanosine triphosphate
HDL – high-density lipoprotein
HEPES – hydroxyethyl piperazine ethanosulfonic acid
HFCS – high-fructose corn syrup
HMIT - H⁺-myoinositol transporter
HNF – hepatic nuclear factor
HRP - horseradish peroxidase
IDDM – insulin-dependent diabetes mellitus
IDF – International Diabetes Foundation
IKKβ - IκB kinase β
IL-6 – interleukin-6
IP₃ – inositol phosphate
IP – intraperitoneal
IPIST - intra-peritoneal insulin sensitivity test
IRS-1 - insulin receptor substrate-1
IV – intravenous
JAK/STAT - janus kinase/signal transducers and activators of transcription
JNK - c-Jun N-terminal kinase
kDa - kilodaltons
KIU - kallikrain inactivator units
KO – knock out rodent model
K_T – transport constant, glucose concentration at half V_max
Lep^ob/ob (or ob/ob) - leptin-deficient mouse model of obesity
LepR^db/db (or db/db) - leptin receptor-deficient mouse model of type II diabetes
LETL rat - Long Evans Tokushima Lean rat
LLC-PK₁ cells - porcine kidney proximal tubule cell line
LPS - lipopolysaccharides
M16 – obese mouse model of type II diabetes
MAPK- mitogen-activated protein kinase
MCP1 - monocyte chemotactic protein-1
MIP3α – macrophage inflammatory protein-3α
MLCK – myosin light chain kinase
mOsm - milliosmole
mRNA – messenger ribonucleic acid
Na+/K+- ATPase – sodium-potassium adenosine triphosphatase
NADH – reduced nicotinamide adenine dinucleotide
NaPiIIa - renal Na+/phosphate cotransporter
NaSCN – sodium thiocyanate
NF-κB - nuclear factor-kappa B
NHE3 - sodium-hydrogen exchanger-3
NIDDM – non insulin-dependent diabetes mellitus
NOD – non-obese diabetic mouse model
NP-40 – nonyl phenoxylpolyethoxylethanol
Ob-R – leptin receptor
ODB-1 – diabetogenic gene
OK cells – opossum kidney cells
OLETF – Otsuka Long Evans Tokushima Fatty rat
PBS – phosphate-buffered saline
PBS-T – phosphate-buffered saline with 0.1% Tween 20
PCR – polymerase chain reaction
PDGF – platelet-derived growth factor
PDX-1 – pancreatic and duodenal homeobox-1 (insulin promoter factor-1)
PKA – protein kinase A
PKC – protein kinase C
PLCβ2 – phospholipase C β2
PMA – phorbol 12-myristate 13-acetate
PPARγ - peroxisome proliferator-activated receptor-γ
PVDF - polyvinylidene fluoride membrane
PYY – peptide YY
RAS – renin-angiotensin system
RELMβ – resistin-like molecule-β
RIPA – radioimmunoprecipitation assay buffer
RLC20 – regulatory light chain-20
RNA – ribonucleic acid
**ROS** – reactive oxygen species

**rpm** - revolutions per minute

**RT-PCR** – reverse transcription polymerase chain reaction

**RYGB** – roux-en-Y gastric bypass

**SDS** – sodium dodecyl sulfate

**SDS-PAGE** - sodium dodecyl sulfate polyacrylamide gel electrophoresis

**S.E.M.** - standard error of the mean

**SGK1** – serum and glucocorticoid-inducible kinase-1

**SGLT** – sodium-dependent glucose cotransporter

**SHROB** – spontaneously hypertensive, obese rat

**SP1** - specificity protein-1 transcription factor

**STZ** – streptozotocin

**T1R** - family of taste-specific class C G protein-coupled receptors

**TGF-β** – transforming growth factor-β

**T_m** - glucose concentration at half $V_{\text{max}}$

**TMB** - tetramethylbenzidine

**TNF-α** – tumour necrosis factor-α

**Tris HCl** – tris hydrochloride

**VEGF** - vascular endothelial growth factor

**VLDL** – very low density lipoproteins

**$V_{\text{max}}$** – maximum transport capacity

**UKPDS** – UK prospective diabetes study

**ZDF** – Zucker diabetic fatty rat

**Zn(O_2CCH_3)_2** – zinc acetate

**ZSF1** – Zucker fatty/spontaneously hypertensive heart failure rat
5 Introduction
5.1 Glucose homeostasis

Glucose is a vital fuel for all organisms, from bacteria to mammals, as glucose is a metabolic substrate in aerobic or anaerobic respiration, or fermentation. It is fundamental for mammals to maintain a constant blood glucose level for physiological processes such as cerebral metabolism (Figure 5.1). Glucose homeostasis is maintained in mammals by glucose absorption from dietary carbohydrates in the small intestine and glucose filtration and reabsorption by the kidney. Glucose is stored in the kidney, liver and adipocytes and regulated by pancreatic hormones, and utilised throughout the body, especially skeletal and cardiac muscle. Glucose sensing, which is an important survival mechanism, enables mammals to mobilise tissue energy stores in response to the metabolic needs of the body. Intracellular glucose is sensed and the resulting signal is relayed by metabolic messengers in tissues; for example, glucokinase and mitochondrial oxidative fluxes (ADP:ATP ratio) are involved in regulation of ATP-sensitive potassium channels controlling insulin secretion and thus are well-established glucose sensors in pancreatic β-cells (Matschinsky et al., 1998; Schuit et al., 2002). The inhibition of glucose sensing, by metabolic modulators, would likely be fatal.

Any given concentration of glucose in plasma is the result of simultaneous release of glucose into circulation and uptake of glucose from plasma by peripheral tissue. The liver is the primary source of endogenous glucose production, although the kidneys also contribute approximately 5% to 20% in the basal state. Following carbohydrate ingestion, a rise in plasma glucose leads to insulin release from β-cells and the resulting hyperinsulinemia stimulates glucose uptake by the liver, gut and peripheral tissue, primarily muscle. From a physiological perspective, it is clear that any defects in insulin secretion in response to a meal or defects in insulin action in peripheral tissue will lead to increased plasma glucose concentration.
Figure 5.1 – The regulation of glucose homeostasis including the main contributors to the control of the whole-body glucose pool. Glucose is absorbed from the diet by the small intestine, filtered and reabsorbed by the kidneys, utilised by the brain and skeletal muscle for ATP production and stored and generated by the liver, adipose tissue and kidney. In addition, the glucose pool is regulated by factors such as insulin released from pancreatic β-cells and leptin and tumour necrosis factor-α (TNF-α) secreted by adipocytes.
5.1.1 Utilisation of glucose

Glucose enters the glycolysis pathway, forming pyruvate to release NADH and ATP, and the citric acid cycle, oxidising into carbon dioxide and water and yielding energy, mostly ATP, as well as precursors for compounds including some amino acids. Glycogenolysis is the breakdown of glycogen to glucose, with adrenaline and glucagon both stimulators of the process. The glucose cycle maintains the dynamic equilibrium between glucose and glucose-6-phosphate to preserve constant blood glucose levels. Gluconeogenesis, the production of glucose from non-carbohydrate sources such as lactate, pyruvate, glycerol and amino acids, also primarily occurs in the liver. Fasting has been shown to promote the hepatic production of glucose via cAMP-dependent mechanisms whereas postprandial glucose production is down-regulated by insulin. Glucose production, traditionally viewed to occur solely in the liver has now also been shown to involve the kidney; however, under normal conditions, renal glucose production cannot be due to glycogenolysis since glycogen levels in the kidney are low (Biava et al., 1966; Greven et al., 1975). Therefore, glucose is produced in the kidney by gluconeogenesis and in vivo studies have shown that this process contributes to the maintenance of constant blood glucose levels (Kida et al., 1978).

5.1.2 Hepatic glucose production and storage

Hepatic glucose production is determined by the rate of net glycogenolysis, gluconeogenesis and glucose cycling between glucose and glucose-6-phosphate; all of which are altered postprandially, resulting in the 60% reduction in net hepatic glucose output seen following a meal (Shulman et al., 1978; Sacca et al., 1978; Rossetti et al., 1993; Clore et al., 1995; Hellerstein et al., 1997). The regulation of hepatic glucose production is hormonal, for example, insulin is important in controlling glucokinase and is involved in glucose cycling, glycogen synthase activity, and in the formation of glycogen (Davidson, 1981; Vaulont and Kahn, 1994).
5.1.3 Renal glucose production and release

In both rodent models and man, renal glucose release accounts for approximately 25% of all glucose released into the circulation (Kida et al., 1978; Stumvoll et al., 1995). Renal glucose handling will be discussed in more detail in section 5.7.1.3; however in brief, as one of the two only two organs in the body that expresses glucose-6-phosphatase, the other being the liver, renal glucose production is vital for glucose homeostasis (Mayer PA, 1993). Under normal conditions, the kidney stores glycogen at low levels, therefore most glucose production occurs via gluconeogenesis (Biava et al., 1966). Indeed, the renal uptake of gluconeogenic precursors, such as lactate and glutamine, was shown to be sufficient to account for glucose release by the kidney (Bjorkman et al., 1980; Bjorkman and Felig, 1982; Brundin and Wahren, 1994).

5.1.4 Adipocyte and skeletal muscle contribution to glucose homeostasis

Between 2 to 4% of whole body glucose uptake is performed by adipose tissue (Gerich, 1993). Despite this relatively low contribution, adipocytes play an important role in glucose homeostasis as seen with elevated fat levels in obesity or lowered fat in lipodystrophy, both of which are associated with insulin resistance and hyperglycaemia (Rosen and Spiegelman, 2006). Adipocytes secrete both pro- and anti-hyperglycaemic factors; examples of the former include resistin and TNF-α (Hotamisligil and Erbay, 2008; Banerjee et al., 2009) whilst the latter includes leptin and visfatin (Shimomura et al., 1999; Stephens and Vidal-Puig, 2006).

Skeletal muscle is responsible for 15 to 20% of glucose uptake in the body however this is elevated following consumption of a meal (Gerich, 1993; Taylor et al., 1993). Glucose is utilised by skeletal muscle for energy during exercise and muscle contraction, indeed glucose uptake increases as much as 50-fold during dynamic exercise (Katz et al., 1986).
5.2 Diabetes

In healthy individuals, plasma glucose levels are maintained within a very narrow range between 4 to 9 mM, by mechanisms described in section 5.1, despite the fluctuations which occur postprandially and following exercise. A reduction of glucose concentration in the plasma results in hypoglycaemic consequences such as limited fuel supply to the brain which, as an organ which cannot store glucose or uptake nutrients, is dependent on circulating glucose for its metabolic needs (Owen et al., 1967). An elevation in plasma glucose levels results in hyperglycaemic consequences often seen in patients suffering diabetes, such as retinopathy, neuropathy and nephropathy (UK Prospective Diabetes study, 1998).

5.2.1 Type I diabetes

Type I diabetes is the less common, juvenile-onset form of diabetes which affects the young age group with 0.025% of 0-14 year olds developing the disease every year in the UK (The Diabetes Control and Complications Trial Research Group 2009). This insulin-dependent form of the disease occurs via the autoimmune destruction of pancreatic β-cells and results in defective insulin secretion and attendant insulin deficiency (Gepts, 1965).

5.2.1.1 Genetic and environmental factors in the onset of type I diabetes

Both genetic and environmental factors influence the aetiology of human type I diabetes (Hirschhorn, 2003). Genetic factors play a large role in the onset of type I diabetes; Csorba et al showed that the development of type I diabetes requires coordinated abnormal variations, thus limiting the occurrence of the disease to a small minority of susceptible individuals (Csorba et al., 2010). The genetic nature of type I diabetes has been postulated to involve six stages in development of the disease starting with genetic susceptibility due to the interaction of several genes conferring
the sensitivity and risk for the disease such as aberrant antigen presentation (Gianani and Eisenbarth, 2005). Stages two to four involve the triggering of autoimmunity by contentiously-discussed environmental agents, the destruction of β-cells and the progressive decline in insulin secretion. The final two stages are when patients develop overt diabetes with insulin dependence and symptoms of type I diabetes such as ketoacidosis.

The elevation in the incidence of type I diabetes does not correlate with increases in the frequency of the major risk genes involved in the disease, thus environmental factors also play a large role in the onset of type I diabetes (Knip and Siljander, 2008; Barrett et al., 2009; Patterson et al., 2009). Examples of environmental factors which have been suggested to promote the onset of type I diabetes include infectious agents such as viral infections, and dietary factors such as wheat (Hettiarachchi et al., 2008).

5.2.2 Type II diabetes

Type II diabetes is the prevalent form of the disease with 2.8 million people currently diagnosed in the UK, furthermore the type II diabetes epidemic is escalating with an annual increase in sufferers of 8% (UK Prospective Diabetes study, 1998). Type II diabetes is the insulin-independent form of the disease which is associated with insulin resistance and hyperglycaemia, in addition to the classical phenotype; polyuria, polydipsia, polyphagia, fatigue and weight loss.

Insulin resistance in type II diabetes arises from impaired insulin action in skeletal muscle, liver and adipose tissue, and defective β-cell function (Bonadonna et al., 1990; Tripathy et al., 2004; DeFronzo, 2009; Karastergiou and Mohamed-Ali, 2010). Impaired insulin sensitivity in these tissues results in reduced insulin-dependent glucose uptake which therefore raises plasma glucose concentration (Mari et al., 1994). Reduced insulin receptor sensitivity results in decreased insulin action but
elevated circulating insulin levels culminating in insulin resistance (Alberti et al., 1992). As type II diabetes progresses, β-cell function declines, due to the action of high extracellular glucose load on β-cells, causing impaired insulin secretion (Lencioni et al., 2008). In early hyperglycaemia, the correction of high plasma glucose stimulus can restore β-cell function (Abdul-Ghani et al., 2006). Additionally, the incretin hormones glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) influence insulin secretion and glucose homeostasis postprandially; in type II diabetic patients, a reduction in GIP responsiveness of β-cells and lowered plasma levels of GLP-1 occur (Thorens and Waeber, 1993; Nauck et al., 1993; Elahi et al., 1994; Vilsboll et al., 2002; Kjems et al., 2003).

5.2.2.1 Genetic and environmental factors in the onset of type II diabetes

Type II diabetes, like type I diabetes, is caused by a combination of genetic and environmental factors. Although the latter influences the onset of type II diabetes more than genetic factors, it has been shown that genes affecting insulin secretion are closely associated with increased risk of developing the disease (Sladek et al., 2007; Lyssenko et al., 2007; Saxena et al., 2010). Environmental factors which effect the development of type II diabetes are well-established with diet and nutrition being the predominant causes, although aging, sedentary lifestyle and intrauterine development are also risk factors. The influences of nutrition, obesity and metabolic syndrome on type II diabetes are discussed in section 5.3. Aging has been shown to correlate with insulin-resistance, for example Bluher et al found an 18% increase in the life span of mice which had selective insulin-receptor knockout in adipocytes (Bluher et al., 2003). Physical inactivity, although strongly linked with the obesity risk factor, has been shown to affect the onset of type II diabetes independently of weight gain (Hamilton et al., 2007). Furthermore, studies in rodent models and man observed that low birth-weight elevates the risk of onset of type II diabetes in later life, demonstrating a role for intrauterine nutrient restriction in the predisposition of the disease (Ozanne et al., 2005; Breant et al., 2006).
5.2.2.2 Incretins in type II diabetes

In recent years, the association of incretins, gut-derived hormones, with type II diabetes has been hypothesised. GLP-1 is a potent stimulator of insulin secretion and can thus exert significant effects on the regulation of glucose metabolism (Scrocchi and Drucker, 1998). GLP-1 regulates mature β-cell function as well as β-cell differentiation, proliferation and survival whereas the dysregulation of β-cell growth and function are central in the pathology of type II diabetes (Solcia et al., 1985; Stoffers, 2004). GLP-1 increases postprandial insulin secretion in a dose-dependent manner and reduces plasma glucagon levels, an effect which is preserved in type II diabetes (Nauck et al., 1993). Furthermore low treatment quantities of GLP-1 normalises the β-cell response to glucose, however the concentration-dependent relationship between β-cell responsiveness to glucose and GLP-1 is severely impaired in type II diabetes (Kjems et al., 2003).

5.2.3 Potential Type III diabetes

Interestingly, Alzheimer’s disease has been postulated to represent type III diabetes since impaired insulin signalling is a feature of the disease (de la Monte and Wands, 2008). Although largely confined to the brain, the chronic insulin resistance and insulin deficient state of Alzheimer’s disease coupled with the improvement of cognitive performance following insulin treatment suggests a strong relationship between Alzheimer’s disease and the diabetic state (Steen et al., 2005; Rivera et al., 2005; Craft, 2006; Haan, 2006). Furthermore the increased risk of Alzheimer’s-like dementia in type II diabetic patients may be linked to the chronic hyperglycaemia, peripheral insulin resistance and oxidative stress seen in diabetes (Whitmer, 2007). Therefore Alzheimer’s disease represents a form of diabetes mellitus that selectively affects the brain, although it should be emphasised that type II diabetes alone is not sufficient to cause Alzheimer’s disease.
5.2.4 Diabetic nephropathy

5.2.4.1 Classification of diabetic nephropathy

Hyperglycaemia is associated with the development of diabetic complications such as neuropathy, retinopathy and cardiovascular disease (Laakso, 1999; Gerstein et al., 2005; Fox et al., 2007). Diabetic nephropathy is another major complication in diabetes and a major cause of end-stage renal disease (ESRD), accounting for over a third of patients on kidney dialysis treatment (Ruggenenti and Remuzzi, 2000; Molitch et al., 2004; Gerstein et al., 2005). The criteria for validating mouse models of progressive diabetic nephropathy include a greater than 50% decline in glomerular filtration rate (GFR), a greater than 10-fold increase in albuminuria, a greater than 50% thickening of glomerular basement membrane and advanced mesangial matrix expansion (Brosius, III et al., 2009). In man, diabetic nephropathy is characterised by early renal hypertrophy, glomerular damage and proteinuria with decreased GFR in later stages (Keen H. and Chlouverakis C., 1964; Rasch, 1979; Boot-Handford and Heath, 1981; Rasch, 1984).

Hyperglycaemia results in a high intracellular glucose concentration resulting from an increase in cellular glucose uptake from the glomerular filtrate and directly from the blood at the brush-border and basolateral membrane respectively. Elevated glucose uptake into the proximal tubule activates several pathways, outlined below, related to the production of advanced glycation end-products (AGE’s), cytokines, chemokines, growth factors and reactive oxidative species (ROS), all of which are mediators of renal damage and are associated with the polyol and hexosamine pathways (Figure 5.2). However, studies have yet to ascertain whether the elevated intracellular glucose concentration during hyperglycaemia is a result of glucose from the glomerular filtrate or blood.
Figure 5.2 – The development of diabetic nephropathy in glomerular cells following high extracellular glucose stimulation. Glucose entry into the mesangial cell is mediated by GLUT1, resulting in up-regulated glucose metabolism via the polyol and hexosamine pathway; thus elevated intracellular glucose-6-phosphate (G-6-P) and thus sorbitol, fructose and diacylglycerol (DAG) are produced which activate protein kinase C (PKC) and result in advanced glycation end-product (AGE) and reactive oxygen species (ROS) synthesis. The subsequent stimulation and generation of cell signalling molecules, transcription factors and cytokines, such as vascular endothelial growth factor (VEGF), JAK/STAT and transforming growth factor-β (TGF-β), produces the aberrant cell growth and survival associated with symptoms of diabetic nephropathy such as excess extracellular matrix (ECM) production, proteinuria and hyperfiltration (modified from (Kanwar et al., 2008)).
5.2.4.2 Glomerular signalling pathway in the onset of diabetic nephropathy

The facilitative glucose transporter GLUT1 is expressed on the glomerular cell membrane and GLUT1-mediated glucose entry into glomerular cells is up-regulated during hyperglycaemia; furthermore GLUT1-overexpressing mesangial cells display excessive extracellular matrix (ECM) production (Brosius and Heilig, 2005).

High ambient glucose conditions result in the promotion of the polyol and hexosamine pathway, the latter results in the production of glucose-6-phosphate (G-6-P) which generates pyruvate. G-6-P, in addition to the high intracellular glucose, promotes the polyol pathway; this results in the reduction of glucose to sorbitol, utilising aldose reductase, and the oxidation of sorbitol to fructose, via sorbitol dehydrogenase. The polyol pathway, in addition to elevating cellular fructose levels, depletes the cell of NADPH and thus alters the cellular redox state and also leads to oxidant stress and elevated ROS levels (Xu et al., 2003). Furthermore, sorbitol and G-6-P up-regulate the production of diacylglycerol (DAG) production which is an intermediate in the activation of ECM and protein kinase C (PKC) (Quest et al., 1997;Mason and Wahab, 2003).

Under high ambient glucose conditions, via Amadori rearrangement, dehydration and polymerisation, AGE’s activate PKC, mitogen-activated protein kinase (MAPK) and transcription factors such as NF-κB; these signalling events result in increased activity of growth factors, such as TGF-β which alter the expression of ECM proteins (Jakus and Rietbrock, 2004). Elevated ECM increases the synthesis of type I and IV collagens, reduces proteoglycan expression and ECM polymerisation and expansion (Li and Gobe, 2006; Tan et al., 2007). In addition, AGE’s cause the formation of ROS which also contributes to the pathology of ECM (Thallas-Bonke et al., 2004).

PKC activation, along with ROS’s, AGE’s and DAG, all activate TGF-β signalling (Leask and Abraham, 2004), with TGF-β being the major cytokine responsible for ECM pathophysiology in diabetic nephropathy (Ziyadeh, 2004). Indeed sufferers of diabetic
nephropathy exhibit elevated TGF-β expression and urinary excretion of the cytokine (Wolf, 2006). Other cell signalling molecules, transcription factors and cytokines involved in the development of diabetic nephropathy include VEGF, JAK/STAT, AP1, SP1 and MCP-1 (Brosius et al., 2010).

5.2.4.3 Tubular injury in diabetic nephropathy

The effects of high glucose on mesangial cells outlined above are well-established and there is also evidence for similar pathways being involved in tubular injury in diabetic nephropathy. Mechanisms of tubulointerstitial injury involve increased glycation of structural and regulatory cell proteins and extracellular proteins such as albumin. The response of tubular cells to glycaemic injury include the elaboration of chemokines such as macrophage inflammatory protein 3 (MIP3-α) and cytokines such as transforming growth factor-β (TGF-β) (Qi et al., 2007) and platelet-derived growth factor (PDGF) (Deuther-Conrad et al., 2001) which activate the immune response and initiate pro-fibrotic gene expression respectively. The infiltration of macrophages and their products is involved in the initiation of the pathological changes of human diabetic nephropathy (Furuta et al., 1993; Young et al., 1995; Sassy-Prigent et al., 2000). In tubular cells, hypertrophy and associated basement membrane alterations precede the tubulointerstitial fibrosis which accompanies progressive renal dysfunction (Brito et al., 1998). There is a direct effect of hyperglycaemia on the turnover of tubular basement membrane matrix components such as collagen and fibronectin accumulation (Ziyadeh et al., 1990; Ziyadeh et al., 1991; Phillips et al., 1997; Phillips et al., 1997; Phillips et al., 1997). Furthermore, Phillips et al have shown that proximal tubule cells contribute to renal fibrosis via the production of profibrotic growth factors such as TGF-β1 and cytokines such as PDGF (Phillips et al., 1995; Phillips et al., 1996; Phillips et al., 1997). Thus it has been postulated that, in addition to glucose influx from the basolateral membrane of the proximal tubule cell, elevated glucose uptake from the tubular lumen in diabetes may also be a major player in the onset of diabetic nephropathy.
5.2.5 Current treatment for diabetes

5.2.5.1 Treatment for type I diabetes

The principle treatment for type I diabetes is currently insulin-replacement therapy, administered via subcutaneous injection or an insulin pump, which is effective when used in conjunction with vigilant blood glucose monitoring and dietary management (Cardella, 2005; McIntyre, 2006). Over the last two decades, there have been major advances in the development of new types of insulin and different delivery methods to mimic insulin release by β-cells; more user-friendly modes of glucose monitoring and islet transplantation have resulted in substantial improvements in morbidity and mortality as well as the quality of life of patients with type I diabetes (Benhamou et al., 2009).

5.2.5.2 Treatment for type II diabetes

The worldwide prevalence of type II diabetes has led to the development of many different treatments and therapies for the disease, however the complex nature of type II diabetes means that a combination-drug approach is often required. Diet and exercise are central in the treatment of type II diabetes due to the efficacy of weight loss in reducing hyperglycaemia as well as ameliorating cardiovascular risk factors in this condition (Tinker et al., 1994; Macfarlane and Jeffcoate, 1997). Furthermore, recent studies have shown that gastric bypass surgery in morbidly obese patients strongly improves in glucose homeostasis. Thus this surgical procedure may provide insights into the treatment of diabetes.
5.2.5.2.1 Pharmaceutical treatments of type II diabetes

There are a variety of therapeutic options in the treatment of hyperglycaemia in type II diabetes although most patients require combination therapy to achieve effective glycemic control.

Metformin, a member of the biguanides class of drugs, is one of the most common treatments for type II diabetics presenting with obesity and normal renal function (Inzucchi, 2002). The efficacy of metformin is mediated by its ability to stimulate insulin binding to its receptor and thus the drug increases glucose uptake and utilisation (Johansen, 1999; Fonseca and Kulkarni, 2008). However metformin treatment is strongly associated with gastrointestinal side-effects such as nausea and diarrhea, which occurs in up to 50% of type II diabetics prescribed metformin (Bailey and Turner, 1996).

Another class of drugs in the treatment of type II diabetes is the sulfonylureas which includes glyburide and glipizide. These drugs bind to their receptor on the surface of pancreatic β-cells and facilitate cell membrane depolarisation which stimulates insulin secretion and thus lowers circulating glucose levels (Zimmerman, 1997). However, sulfonylureas cannot be used by type II diabetics with hepatic or renal impairment and are associated with insulin-induced hypoglycaemia and weight gain (Stenman et al., 1993). Meglitinides, the group of type II diabetic drugs including repaglinide and nateglitinide, mirrors the action of sulfonylureas to stimulate the release of insulin from pancreatic β-cells via a different receptor from sulfonylurea (Luna et al., 1999). However these agents exhibit a shorter onset time for action and shorter half life, therefore they display a faster response than sulfonylureas.

The α-glucosidase inhibitors, acarbose and miglitol, target the small intestinal brush-border membrane (BBM) enzyme, α-glycosidase; this enzyme breaks down disaccharides and more complex carbohydrates into monosaccharides (Balfour and McTavish, 1993; Takenaka and Uchiyama, 2001). Therefore the drugs delay intestinal carbohydrate absorption and mitigate postprandial glucose excursions; however side effects of these drugs include malabsorptive symptoms such as diarrhoea (Goke and Herrmann-Rinke, 1998; Mochizuki et al., 2010).
Rosiglitazone and pioglitazone are two members of the thiazolidinediones family of type II diabetic treatments. These pharmacological ligands bind to and activate the nuclear receptor peroxisome-proliferator-activated receptor gamma (PPARγ) which binds to responsive elements in DNA to alter the transcription of genes involved in carbohydrate and lipid metabolism (Mudaliar and Henry, 2001). The main outcome of these drugs is the increase in insulin-stimulated glucose uptake by skeletal muscle cells, resulting in reduced peripheral insulin resistance (Chao et al., 2000). However, side-effects associated with thiazolidinediones include weight gain and oedema, as well as potential hepatic impairment (Kelly et al., 1999).

Another class of drugs are dipeptidyl peptidase-IV (DPP-IV) inhibitors which are based on the ability of DPP-IV to degrade GLP-1. DPP-IV inhibitors suppress the degradation of GLP-1 and extend the activity period for GLP-1 action; with examples including vildagliptin and stialiptin (Weber, 2004; Augeri et al., 2005). DPP-IV inhibitors evoke a reduction in glucose excursion following an oral glucose challenge (Pospisilik et al., 2002) and chronic DPP-IV inhibition can preserve islet function resulting in elevated glucose-mediated insulin response which is accompanied by increased glucose uptake via GLUT2 in β-cells (Reimer et al., 2002). This potential for the preservation and improved function of β-cells implies a role for these inhibitors in the later stages of diabetes.

5.2.5.2.2 Newer treatments for type II diabetes

In type II diabetes, low postprandial GLP-1 levels are found in the small intestine (Toft-Nielsen et al., 2001; Vilsboll et al., 2001; Lugari et al., 2002). Incretin mimetics are a newer class of drugs in the treatment of type II diabetes; these pharmacological agents mirror the ability of GLP-1 to regulate glucose activity except their actions are not solely regulated by the pancreatic GLP-1 receptor. Examples of GLP-1 analogues include Liraglutide and CJC-1131. Another incretin mimetic which is commonly prescribed is Exenatide-4, although this is not a GLP-1 analogue, it shares the ability of GLP-1 for glucose-dependent actions such as
enhancement of insulin secretion, suppression of high glucagon secretion, slowing of gastric emptying and reduction of food intake (Edwards et al., 2001; Kolterman et al., 2003; Holst and Gromada, 2004; Fineman et al., 2004; Buse et al., 2004; DeFronzo et al., 2005). Furthermore, Exenatide-4 is resistant to degradation by DPP-IV and thus the drug has a longer physiological half-life than GLP-1 (Egan and Margolskee, 2008). However, Exenatide-4 is associated with gastrointestinal effects such as nausea, vomiting and diarrhoea (Kolterman et al., 2003; Yoon et al., 2009).

Selective inhibitors of SGLT2 target the high-capacity sodium-dependent glucose transporter at the brush-border of cells in the early region of the proximal tubule. SGLT2 inhibitors have been found to reduce the transport maximum for glucose and thus effectively lower hyperglycaemia in diabetes (Katsuno et al., 2007; Han et al., 2008; Meng et al., 2008). These inhibitors have been further described in section 5.6.2.2.2.

5.2.5.2.3 Drug-combination treatment in type II diabetes

The progressive, polymorphic nature of type II diabetes means that combination oral anti-diabetic therapy is most often prescribed for patients (Inzucchi, 2002); the UK Prospective Diabetes Study (UKPDS) found that 3 years after treatment with only one drug, only 50% patients were adequately controlled whereas after 9 years, only 25% were controlled by monotherapy (Turner et al., 1999).

However, the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study showed that there was an increase in mortality when attempts at normalising hyperglycaemia were made with multiple combinations of the drugs outlined above (Gerstein et al., 2008). Therefore there are still limitations and controversy regarding the effectiveness of current drug treatments for diabetes and the search continues for novel therapy for the disease.
5.3 Metabolic models related to diabetes

The rising prevalence of type II diabetes is strongly related to obesity caused by the consumption of a Western diet, which has become considerably enriched in saturated fat and high-glycaemic index sugars such as high-fructose corn syrup. Furthermore obesity is one of five major risk factors in the development of metabolic syndrome, a multi-faceted disease and precursor to type II diabetes (Figure 5.3).

Figure 5.3 – The complex interplay between the onset of obesity, metabolic syndrome and type II diabetes; including the environmental factors associated with elevated or reduced risk of disease and the common symptoms in both obesity and metabolic syndrome. Red bars — for inhibitory effect and black arrows → for stimulatory effect, HDL – high-density lipoproteins.
5.3.1 Obesity

In 2010, 24.5% of the population in England were classified as obese, with a body mass index (BMI) greater than 30 kg/m$^2$ (The National Obesity Observatory, 2010). The obesity epidemic has developed in the last 20 years and is largely diet-related, although there are many studies showing that inherited factors profoundly influence fat mass (Farooqi and O'Rahilly, 2008).

Excess calorie intake has a marked influence on insulin resistance in adipocytes, β-cells, skeletal muscle and hepatocytes; thus in obesity, both hyperinsulinaemia and insulin resistance are seen. Obesity results in continuously elevated circulating levels of free fatty acid (FFA) levels, and this causes hypertrophy in adipocytes (Cinti et al., 2005). In comparison to lean individuals, macrophages in adipose tissue of obese patients expressed augmented levels of proinflammatory hormones such as tumour-necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Weisberg et al., 2003; Hotamisligil et al., 2008). The inflammatory response of the adipocyte is mediated via several signalling pathways including c-jun-N-terminal kinase (JNK) and cAMP-responsive element binding protein (CREBH) (Hirosumi et al., 2002; Zhang et al., 2002; Qi et al., 2009). Therefore, metabolic stress causes adipocytes to secrete cytokines, which induce macrophage accumulation and stimulates muscle and hepatic insulin resistance (Hotamisligil et al., 2008). Obesity exacerbates insulin resistance in the adipocyte, however weight-loss has been shown to reverse these insulin-resistance effects (Gregor et al., 2009). Insulin-mediated glucose uptake in skeletal muscle is very sensitive to excess lipid exposure, with the induction of insulin resistance occurring within 4 hours of FFA exposure in subjects with no predisposition to type II diabetes (Belfort et al., 2005). In hepatocytes, elevated circulating FFA results in insulin resistance, impaired insulin signalling and the stimulation of hepatic glucose production via gluconeogenesis and glycogenolysis (Roden et al., 2000; Boden et al., 2002). The adaptation of circulating FFA-induced hepatic insulin resistance in obesity involves an increase in insulin secretion and reduced hepatic insulin clearance, which causes peripheral hyperinsulinaemia (Kashyap et al., 2003). Therefore there is complex interplay between the physiological effects of obesity and insulin-
resistance, which strongly associates obesity as an important factor in the development of type II diabetes.

Obesity-induced hypertension has been strongly linked with augmented sodium reabsorption; a shift in renal pressure natriuresis is observed to maintain sodium balance in response to the rise in arterial pressure in obesity (Granger et al., 1994; Hall et al., 1998; Hakam and Hussain, 2005). The increased renal tubular reabsorption is also a result of the elevated intra-renal pressure caused by the excess of visceral adipose tissue in obesity which physically compresses the kidneys (Hall, 1997; Hall et al., 2002).

Obesity-dependent renal damage is likely to be mediated by hormonal changes, for example, levels of adipokines are higher in chronic kidney disease, low-grade inflammation and oxidative stress (Axelsson et al., 2006; Knight and Imig, 2007; Foster et al., 2008). There is a significant relationship between the excretion rate of albumin and obesity, for example, in subjects with the same percentage body fat, those with central fat distribution have an increased likelihood of exhibiting elevated albumin excretion rate (Mulyadi et al., 2001). Insulin resistance in sustained obesity causes hyperinsulinaemia which may result in hyperfiltration and glomerular hypertrophy; interestingly, only weight loss, in the early stages of disease development, has been shown to reduce proteinuria (Adelman, 2002). Furthermore, subjects with a high BMI are at increased risk of developing urolithiasis and recurrent stone formation due to a deficiency in ammonia production and elevation in urinary excretion of stone-forming substances such as calcium and uric acid (Curhan et al., 1998; Siener et al., 2004; Taylor et al., 2005; Lee et al., 2008). Therefore obesity predisposes sufferers to type II diabetes via an effect on insulin resistance, as well as resulting in early diabetic-like conditions such as elevated glomerular filtration rate (GFR) and an activated renin-angiotensin system (RAS) in the kidney.

The onset of insulin resistance and hyperglycaemia occurs within one month of increased consumption of dietary fats; thus dietary fats are closely linked with diabetes, as well as their obvious correlation with obesity (Surwit et al., 1988; Surwit et al., 1997; Marshall et al., 1997; Vessby et al., 2001).
Schutz et al. showed that moderate consumption of dietary fat mainly induces stimulation of fat storage with little or no stimulation of fat oxidation (Schutz et al., 1989). In contrast, ingestion of dietary carbohydrates stimulates insulin release which promotes glucose uptake in insulin-sensitive tissues, inhibits endogenous glucose production and reduces the release of FFA from adipose tissue and thus results in the relative increase of carbohydrate oxidation and relative decrease in lipid oxidation (Flatt et al., 1985; Aarsland et al., 1997).

5.3.2 The treatment of obesity with gastric bypass surgery

Interestingly, bariatric surgery, a treatment for morbid obesity, has also been shown to alleviate many symptoms of type II diabetes. Gastric bypass surgery was developed in the 1950’s with the jejun-ileal bypass, which was superseded in the 1970’s by procedures such as Roux-en-Y gastric bypass (RYGB), gastroplasty (also known as ‘stomach stapling’), and biliopancreatic diversion (BPD). In recent years, the application of laparoscopy to gastric bypass surgery has led to elevated interest and research in the field. Gastric bypass surgery has been associated with an 89% reduction in the relative risk of death, as well as an 82% reduction of cardiovascular events among morbidly obese patients (Christou et al., 2004). Bariatric surgery is the only treatment which has been shown to achieve substantial and durable weight loss and there are suggestions to explain the weight loss following bariatric surgery including: 1) the resulting reduced appetite and increased satiety due to elevations in peptide YY (PYY) and GLP-1 as well as reductions in leptin and ghrelin (Borg et al., 2006; Le Roux et al., 2006); 2) alterations in taste perception with a reduced preference for carbohydrate-rich foods (Brown et al., 1982; Brolin et al., 1994; Borg et al., 2006; Olbers et al., 2006); 3) the restricted intake of food due to the resectioning of the stomach into a small pouch with limited food capacity (Olbers et al., 2003; Borg et al., 2006); 4) nutrient exclusion from the duodenum thus either suppressing proximal small intestinal signalling in the secretion of anti-incretin hormones or delivery immediately to the distal small intestine enhancing the release of incretins (Mingrone and Castagneto-Gissey, 2009); 5) malabsorption of nutrients due to limited absorption surface (Olbers et al., 2003); 6) increased energy expenditure, since fat loss following gastric bypass surgery occurs concomitantly with diet-
induced thermogenesis (Bueter et al., 2010); and 7) aversion effects such as vomiting and steatorrhoea (O'Brien, 2010).

Gastric bypass surgery can be classed as either malabsorptive or restrictive; the former consists of surgery such as jejunoileal bypass and the latter includes sleeve gastroectomy however both these forms of surgery provide limitations with regard to patient safety, invasiveness and reversibility (Organ, Jr. et al., 1980; Daskalakis and Weiner, 2009). RYGB surgery is considered a hybrid between restrictive and malabsorptive gastric bypass and has become one of the most popular forms of surgery. As well as providing effective weight loss, with 60-70% of excess weight lost in the initial 12 months following surgery, RYGB also offers few long-term problems, with mortality rates of below 1% (Flum and Dellinger, 2004; Flum et al., 2009).

5.3.2.1 Gastric bypass surgery in type II diabetics

One of the most startling and fascinating effects of gastric bypass surgery is the resolution of hyperglycaemia in very obese diabetic patients post-surgery, before significant weight loss occurred (Mingrone et al., 1997). Although gastric banding has been shown to improve metabolic syndrome and type II diabetes, these effects are seen 6-12 months after banding, when maximal weight loss has occurred (Parikh et al., 2006; Dixon et al., 2008). At this stage, there is a close correlation between weight loss and reduced circulating glucose (Parikh et al., 2006).

Chai et al observed a significant reduction in hyperglycaemia in type II diabetic Goto-Kakizaki (GK) rats 30 days after RYGB surgery (Chai et al., 2011). In man, changes following gastric bypass surgery, after 6-8 months, have been attributed to improved glucose homeostasis with an increase in muscle insulin receptor density (Houmard et al., 2002; Gray et al., 2003). Furthermore, a reduction in moieties causing insulin-resistance, such as hepatic lipid and long-chain fatty acyl-CoA molecules, also results in the elevation in glucose transport and insulin sensitivity noted following gastric bypass surgery (Friedman et al.,
However, gastric bypass surgery also results in 97–100% improvement and remission of diabetes within 1 month post-operation, before significant weight loss can occur and with allowances for dietary intake taken into consideration (Pories et al., 1987; Mingrone et al., 1997; Rubino et al., 2004; Guidone et al., 2006; Mari et al., 2006; Laferriere et al., 2007; Briatore et al., 2008; Chiellini et al., 2009). Following bariatric surgery, diabetic patients with normal weight also exhibited abolishment of the disease (Mingrone et al., 1999). There are two theories for these effects on diabetes, the foregut and hindgut hypothesis.

### 5.3.2.1.1 Foregut hypothesis

The foregut hypothesis is based on the exclusion of the proximal small intestine from the transit of nutrients, resulting in a secondary alteration in incretin signals with consequent improvements in blood glucose levels observed following RYGB surgery (Pories et al., 1995; Pories and Albrecht, 2001; Clements et al., 2004). This is supported by studies with the duodenal-jejunal bypass, which simulates the bypass portion of RYGB surgery without any reduction in gastric size. The duodenal-jejunal bypass results in decreased food intake, elevated insulin levels and improved hepatic insulin sensitivity due to an increase in intestinal gluconeogenesis (Troy et al., 2008). Using GK rats, Rubino et al noted the abolishment of type II diabetes following stomach-preserving surgery which bypasses the proximal intestine (Rubino, 2008). Furthermore, on implantation of a sleeve inside the duodenum, thus preventing nutrient absorption by the proximal small intestine, a 55% improvement in peripheral insulin resistance was observed (Aguirre et al., 2008).

### 5.3.2.1.2 Hindgut hypothesis

The hindgut hypothesis, developed by Mason, suggests that a more rapid delivery of nutrients to the distal small intestine results in enhanced release of incretins, such as GLP-1 and GIP from ileal enterocytes, which is reported to improve glucose homeostasis (Mason, 1999).
Indeed the elevated levels of GLP-1, a nutrient-stimulated, glucose-lowering, incretin is secreted from L-cells primarily in the ileum and colon, seen following RYGB surgery have been shown to stimulate insulin secretion and thus reduce hyperglycaemia (Mason, 1999;Drucker, 2002;Rubino et al., 2004;Cummings and Overduin, 2007). This theory is supported by the understanding that GLP-1 accentuates glucose-dependent insulin secretion, increasing β-cell mass as well as potentially heightening insulin sensitivity (Zander et al., 2002;Drucker, 2007). Furthermore, studies show that ileal transposition chronically stimulates the terminal ileum with nutrient-rich chyme without affecting the transit of food in the gastrointestinal tract (Koopmans et al., 1984). This procedure is associated with insulin-resistant improvements in glucose tolerance in the early post-operative time period (Patriti et al., 2007).

However, DiGiorgi et al observed a significant incidence of recurrence or worsening of type II diabetes in patients 3 years after RYGB surgery, therefore further studies are necessary to understand the long-term connotations of gastric bypass on diabetes (DiGiorgi et al., 2010).

5.3.3 Metabolic syndrome

The abundance of calorie-rich, low-fibre foods with a high-glycaemic index in the Western diet is the main reason for the obesity epidemic seen in recent years and this condition leads to the onset of metabolic syndrome and diabetes. A diet high in fat, carbohydrates and empty calories has been shown to increase the risk of metabolic syndrome (Ford et al., 2002;Freire et al., 2005;Sonnenberg et al., 2005).

In addition, an increase in the consumption of a high-fibre diet has been shown to be inversely correlated with the onset and progression of metabolic syndrome (McKeown et al., 2004;Sahyoun et al., 2006). Other preventative measures include a low-fat diet which can result in up to 50% reduced risk in the incidence of diabetes (Tuomilehto et al., 2001;Charatan, 2001).
Obesity is a central component of metabolic syndrome and thus the increasing prevalence of obesity is paralleled by similar increases in metabolic syndrome sufferers. The prevalence of metabolic syndrome, in young adults worldwide, is 23% with higher levels seen in aging (Scuteri et al., 2005; Cameron et al., 2007; Zuliani et al., 2009). Metabolic syndrome can be viewed as a cluster of disorders and is defined by the presentation of central obesity as well as two out of the four following: raised blood pressure, raised triglycerides, lowered high-density lipoprotein (HDL) cholesterol and fasting hyperglycaemia (The National Obesity Observatory 2010). This latter parameter is representative of the glucose intolerance seen in metabolic syndrome and, along with the ubiquitous presentation of obesity in metabolic syndrome, links the disease with type II diabetes; thus metabolic syndrome is a well-established precursor to type II diabetes. The fasting hyperglycaemia in metabolic syndrome is due to insulin-resistance in obesity, outlined in section 5.3.1, which predisposes obesity sufferers to develop metabolic syndrome.

A core component of metabolic syndrome is dyslipidaemia which occurs due to obesity and is characterised by low HDL cholesterol and elevated triglycerides, both parameters which are noted in metabolic syndrome (IDF 2001). Furthermore, metabolic syndrome sufferers exhibit a prothrombotic and inflammatory profile synonymous with obesity. Abdominal obesity, especially elevations in visceral fat, are more highly associated with risk of metabolic syndrome (Despres et al., 1987; Despres et al., 2000; Despres et al., 2001; Miyazaki et al., 2002).

The cardiometabolic abnormalities exhibited in patients with metabolic syndrome increases their risk of developing cardiovascular disease; in 2003, 19.2% of adults over 50 years of age who had cardiovascular disease also suffered from metabolic syndrome (Alexander et al., 2003). Metabolic syndrome patients without type II diabetes exhibit a 13.9% prevalence of cardiovascular disease; in comparison 7.5% type II diabetics without metabolic syndrome, suffered from cardiovascular disease (Alexander et al., 2003). Thus a tight correlation exists between cardiovascular disease and the onset of metabolic syndrome.

Genetic determinants of metabolic syndrome have been studied, for example although insulin resistance clusters in families, the probability of inheriting problems with insulin secretion is
low; therefore environmental factors are far more influential in the progression of insulin resistance (Groop et al., 1996; Hong et al., 1997). Another example is hypertension, a risk factor in development of metabolic syndrome, and strongly associated with insulin resistance (Ferrannini et al., 1987); there was stronger hereditability of HDL cholesterol with insulin resistance than triglycerides (Hong et al., 1997).

5.3.4 Pre-diabetes

Pre-diabetes is a common condition characterized by either impaired fasting glucose levels, between 6-7 mM, or defective glucose tolerance, between 8-12 mM after an oral glucose load (Standards of Medical Care in Diabetes 2007). Many pre-diabetics exhibit microvascular hyperglycaemia-associated diseases such as blindness, similar to that seen in diabetes (Jaap et al., 1997). The impaired fasting plasma glucose levels exhibited in pre-diabetes is also a component of metabolic syndrome and thus the common progression occurs from the onset of metabolic syndrome and pre-diabetes to type II diabetes.

5.4 Sweeteners in the Western diet

5.4.1 Fructose

The simple sugar, fructose, is consumed in vast quantities in the Western diet, often in the form of the acutely sweet high-fructose corn syrup (HFCS), also defined as inverted sugars, in many processed foods. In addition, the natural form of the sugar fructose is found in fruits and honey. Much controversy surrounds the use of sweeteners in the Western diet with sugar-sweetened soda identified as the major culprit in elevating the consumption of HFCS. Although studies in man have identified a strong causal link between the intake of sugar-sweetened beverages and metabolic syndrome, the relationship has been attributed to the increased consumption of an energy-rich diet in this population rather than a negative response elicited by the intake of HFCS.
Fructose is absorbed and metabolised by the small intestine where it moves into the portal vein to be metabolised by the liver, into glucose, glycogen, lactate or lipids (Mayes, 1993). In hepatocytes, fructose is rapidly converted into fructose-1-phosphate and triose-phosphates via the sequential action of fructokinase, aldolase B and triokinase; the first two enzymes are not regulated by cellular energy status and therefore fructose is rapidly converted into hepatic triose-phosphates, following ingestion (Heinz et al., 1968). Fructose metabolism occurs independently of insulin and plasma glucose (Tappy et al., 1986), therefore fructose was thought to be a potential, natural glucose-replacement for diabetics. However, increased fructose intake in both man and animal has been shown to result in features of metabolic syndrome such as dyslipidaemia, insulin resistance, impaired glucose homeostasis, excess body fat and raised blood pressure (Zavaroni et al., 1980; Bjorkman et al., 1982; Reaven et al., 1990; Donnelly et al., 1994; Reed et al., 1994; Jurgens et al., 2005).

Hyperlipidaemia is a major consequence of high-fructose feeding with increased dietary fructose rapidly elevating plasma triglyceride levels and lowering very low-density lipoprotein (VLDL) triglycerides (Park et al., 1992). High-fructose fed rats are also associated with the development of hypertension and visceral fat accumulation, as well as exhibiting impaired insulin-mediated suppression of plasma FFA, thus insulin-resistance is likely to occur in adipose tissue (Hwang et al., 1987; Reaven et al., 1996; Faeh et al., 2005). Indeed, Bray et al have extensively described the role of high-fructose feeding in insulin resistance (Bray et al., 2004). Additionally, fructose-fed rats display other changes observed in metabolic syndrome such as NADPH oxidase and RAS activation, both precursors in the development of hyperlipidaemia and insulin resistance (Delbosc et al., 2005; Nakagawa et al., 2006; Hsieh et al., 2006; Nyby et al., 2007; Figlewicz et al., 2009). Glucose also causes a more pronounced increase in circulating leptin and a greater reduction in gherlin levels than fructose and therefore fructose has a less overall satiating effect than glucose (Tappy et al., 2010).

Many human studies support the effects of HFCS consumption on weight gain (DiMeglio and Mattes, 2000; Ludwig et al., 2001; Raben et al., 2002). In particular, epidemiological studies have confirmed the effect of sugar-sweetened, HFCS-enriched beverages on the development
of metabolic syndrome; predominantly elevated weight gain, inflammation, insulin resistance, impaired β-cell function and elevated blood pressure are all strongly linked with increased metabolism of fructose following excess consumption of HFCS-sweetened beverages (Malik et al., 2006; Fung et al., 2009; Malik et al., 2010; Hu and Malik, 2010). Due to the extensive evidence that elevated consumption of HFCS in the Western diet leads to the onset of type II diabetes, artificial sweeteners have been developed in recent years as synthetic sugar substitutes.

5.4.2 Artificial sweeteners

Artificial sweeteners are intensely sweet synthetic compounds which are able to elicit a sweet taste response but have no calorific value. The use and thus consumption of artificial sweeteners is widespread and their most common uses are as a sugar-substitute to prevent tooth decay, as an aid in weight loss in obesity and as a non-nutritive sweetener for diabetics. Examples of artificial sweeteners most commonly used are sucralose, acesulfame k, aspartame and saccharin, all of which are 200-600-fold sweeter than sucrose; these sweeteners are often used in sugar-free soft drinks, low-fat foods and as a sweetener in tea and coffee.

Interestingly studies in man show conflicting data on the consumption of artificial sweeteners. There is evidence that the intake of artificial sweeteners can increase hunger, food intake and body weight compared to control groups maintained on a diet of glucose (Blundell and Hill, 1986; Fowler et al., 2008; Switers et al., 2010). The positive effect of artificial sweeteners was also seen by Bailey et al where chronic consumption of saccharin was found to defer the development of hyperglycaemia and improve glucose homeostasis in insulin resistant ob/ob mice via an insulin-dependent mechanism (Bailey et al., 1997). However, other groups have shown a positive correlation between artificially-sweetened soft drink consumption and the prevalence of metabolic syndrome; this correlation was stronger and more significant than the same relationship with sugar-sweetened soft drinks (Dhingra et al., 2007). Horwitz et al found that plasma insulin, but not plasma glucose, was elevated in
response to the consumption of artificial sweeteners; hyperinsulinaemia may therefore explain the relationship between non-nutritive sweeteners and the onset of metabolic syndrome and type II diabetes (Horwitz et al., 1988; Montonen et al., 2007). Thus further studies are necessary to fully understand the impact of a diet high in artificial sweeteners, regularly consumed as diet soda, on glucose tolerance and the development of associated metabolic diseases.

5.5 Animal models of metabolic dysregulation

There are many different rodent models for type I and II diabetes, as well as for obesity and metabolic syndrome, with each model having both advantages and limitations. The use of these models provides insight into the pathophysiological basis of the disease.

5.5.1 Models of type I diabetes

Table 5.1 identifies some different rodent models of type I diabetes, which can be induced by the damage of pancreatic β-cells, either via chemical or surgical induction, or spontaneously developed in genetically-bred rodents.

There are limitations in animal models of type I diabetes compared with human aetiology of the disease; models do not represent the polygenic heterogeneous nature of type I diabetes in man (Polychronakos, 2004). For example, the non-obese diabetic (NOD) mice and BioBreeding (BB) rat are genetically homogenous, whereas humans are genetically diverse and although the end-point of overt diabetes is the same in rodents and humans, the pathways in the development of the disease are different.
Table 5.1- Table of rodent models of type I diabetes induced by chemicals, surgical treatment or spontaneous genetic models

<table>
<thead>
<tr>
<th>Model</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical</td>
<td></td>
</tr>
<tr>
<td>Streptozotocin (STZ)</td>
<td>(VAVRA et al., 1959)</td>
</tr>
<tr>
<td>Alloxan</td>
<td>(Ridout et al., 1944)</td>
</tr>
<tr>
<td>Vacor</td>
<td>(Taniguchi et al., 1989)</td>
</tr>
<tr>
<td>Dithizone</td>
<td>(KADOTA, 1950)</td>
</tr>
<tr>
<td>8-hydroxyquinolone</td>
<td>(Root M.A. and Chen, 1952)</td>
</tr>
<tr>
<td>Surgical</td>
<td></td>
</tr>
<tr>
<td>Thymectomy</td>
<td>(Stumbles and Penhale, 1993)</td>
</tr>
<tr>
<td>Radiation</td>
<td>(Stumbles et al., 1993)</td>
</tr>
<tr>
<td>Spontaneous</td>
<td></td>
</tr>
<tr>
<td>Non-obese diabetic (NOD) mouse</td>
<td>(Makino et al., 1980)</td>
</tr>
<tr>
<td>BioBreeding (BB) rat</td>
<td>(Nakhooda et al., 1977)</td>
</tr>
<tr>
<td>Long Evans Tokushima Lean (LETL) rat</td>
<td>(Kawano et al., 1991)</td>
</tr>
</tbody>
</table>

5.5.2 Models of metabolic syndrome and type II diabetes

The complex, multifactorial nature of metabolic syndrome means no one single animal model is the most appropriate to use, furthermore there is overlap of phenotype in different rodent models of obesity, metabolic syndrome and type II diabetes. Central obesity and alterations of adipokine secretion, together with a concomitant fat accumulation in different metabolically-active tissues such as liver, muscle and pancreas, build the pathophysiologic basis of the metabolic syndrome (McPherson and Jones, 2003; Unger, 2003; Carr et al., 2004).
5.5.2.1 Rat models of metabolic syndrome

Established models of metabolic syndrome include the spontaneously hypertensive obese (SHROB) rat, with the $fa^k/fa^k$ mutation, which exhibits obesity, hypertension and insulin resistance; however studies on metabolic syndrome-associated tubule reabsorption may be compromised as the model is associated with hypertension-induced progressive renal disease (Koletsky, 1973; Abramowsky et al., 1984; Koletsky et al., 1995). The Wistar Ottawa Karlsburg W (WOKW) rat, a euglycaemic inbred strain, provides a useful model of metabolic syndrome due to the polygenic nature of disorders, such as glucose intolerance and obesity, in these animals, however renal studies are limited and only proteinuria at 64 weeks has been established (Kovacs et al., 2000; Grisk et al., 2007). Furthermore the Nile grass rat has recently been identified as a novel spontaneously occurring disease model of metabolic syndrome which exhibits obesity, hyperglycaemia and hypertension and thus develop diabetes at 11 months of age when fed a standard laboratory chow with renal and hepatic hypertrophy (Noda et al., 2010).

5.5.2.2 Mouse models of metabolic syndrome

There are a myriad of mouse models of metabolic syndrome including the leptin-deficient $Lep^{ob/ob}$ (or ob/ob) and leptin receptor-deficient $LepR^{db/db}$ (or db/db) mouse (Ingalls et al., 1950; Hummel et al., 1966; Coleman and Hummel, 1973). More recently, the polygenic New Zealand obese mouse has been identified as a suitable model of metabolic syndrome which exhibits high plasma glucose and cholesterol as well as hypertension and hypoinsulinaemia (Ortlepp et al., 2000; Joost, 2010). Metabolic syndrome can be induced by high-fat or high-calorie diets in mice, for example, the C57BL/6J mouse fed a high-fat diet develop obesity, hyperinsulinaemia and hyperglycaemia within 1 month and continuation of the diet exacerbates adiposity (Surwit et al., 1988; Surwit et al., 1997; Black et al., 1998).

In view of the polygenic character of this disorder, monogenic models of obesity and diabetes, such as the ob/ob mouse or the obese Zucker ($fa/fa$) rat, do not reflect the human
disease sufficiently. The first description of a ‘high-fat diet’ to induce obesity by a nutritional intervention was in 1959 (Masek and Fabry, 1959). Subsequent studies have revealed that high-fat diets promote hyperglycemia and whole-body insulin resistance. It is generally accepted that high-fat diets can be used to generate a valid rodent model for the metabolic syndrome with insulin resistance and compromised β-cell function (Oakes et al., 1997; Ahren et al., 1999; Lingohr et al., 2002). To adequately induce metabolic syndrome, between 30-60% fat-as-calories diet is necessary although strain and gender affects the outcome of a high-fat diets (Levin et al., 1997; Ghibaudi et al., 2002). In addition, the component of fat in the diet affects the manifestation of metabolic syndrome with lard exhibiting the most pronounced influence on obesity and insulin resistance (Yaspelkis, III et al., 2001; Buettner et al., 2004).

In addition to high-fat feeding studies, metabolic syndrome can also be induced by high-fructose or -sucrose diets, with rats displaying insulin resistance, elevated triglycerides and increased body weight, although not excessive obesity (Daly et al., 1997). Indeed, a high-fat/high-fructose diet, consisting of 45% fructose and 20% lard, results in elevated mesenteric fat and insulin resistance, both strongly associated with metabolic syndrome (Medina-Gomez and Vidal-Puig, 2005).

5.5.2.3 Rodent models of type II diabetes

Type II diabetic rodent models are defined as glucose intolerant and hyperglycaemic with impaired secretion and response of insulin, as well as exhibiting other diabetic-like symptoms for example, retinopathy, nephropathy and weight loss seen in human patients of the disease (Laakso, 1999; Gerstein et al., 2005). Table 5.2 displays some of the myriad of rodent models which represent the metabolic syndrome and type II diabetes. It is worth noting however that there are also transgenic/knock out models of type II diabetes, as well as surgically-induced diabetes following partial pancreatectomy (Bonner-Weir et al., 1983).
Interestingly, the administration of STZ 15 minutes after nicotinamide has been found to induce a rodent model closely resembling type II diabetes (Masiello et al., 1998). STZ induces hyperglycaemia whilst nicotinamide protects β-cells from the cytotoxic effect of streptozotocin (Gunnarsson et al., 1974; Hoorens and Pipeleers, 1999). The Zucker diabetic and GK rat represent two of the most commonly used models of metabolic syndrome and type II diabetes.

Table 5.2 - Table of obese and non-obese rodent models for spontaneous or genetically-derived type II diabetes

<table>
<thead>
<tr>
<th>Model</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-obese rat models</strong></td>
<td></td>
</tr>
<tr>
<td>Goto-kakizaki (GK)</td>
<td>(Goto et al., 1976)</td>
</tr>
<tr>
<td>Cohen diabetic</td>
<td>(Weksler-Zangen et al., 2001)</td>
</tr>
<tr>
<td>Brattleboro</td>
<td>(VALTIN and Schroeter, 1964)</td>
</tr>
<tr>
<td>Torri</td>
<td>(Shafrir, 1992)</td>
</tr>
<tr>
<td><strong>Non-obese mouse models</strong></td>
<td></td>
</tr>
<tr>
<td>Akita C57BL/6</td>
<td>(Mathews et al., 2002)</td>
</tr>
<tr>
<td>Alloxan-susceptible (ALS)/Lt</td>
<td>(Mathews et al., 2004)</td>
</tr>
<tr>
<td>Horio-Niki diabetic</td>
<td>(Horio et al., 2005)</td>
</tr>
<tr>
<td><strong>Obese rat models</strong></td>
<td></td>
</tr>
<tr>
<td>UC Davis type II diabetic</td>
<td>(Cummings et al., 2008)</td>
</tr>
<tr>
<td>Spontaneously hypertensive / NIH-corpulent</td>
<td>(Michaelis et al., 1984)</td>
</tr>
<tr>
<td>Otuska Long Evans Tokushima fatty (OLETF)</td>
<td>(Kawano et al., 1994)</td>
</tr>
<tr>
<td>James C. Russell/LA corpulent</td>
<td>(Koletsky, 1975)</td>
</tr>
<tr>
<td>Zucker diabetic fatty (ZDF)</td>
<td>(Zucker, 1965; Etgen and Oldham, 2000)</td>
</tr>
<tr>
<td><strong>Obese mouse models</strong></td>
<td></td>
</tr>
<tr>
<td>ob/ob</td>
<td>(Shafrir, 1992)</td>
</tr>
<tr>
<td>db/db</td>
<td>(Hummel et al., 1966)</td>
</tr>
<tr>
<td>Kuo Kondo</td>
<td>(Velasquez et al., 1990)</td>
</tr>
<tr>
<td>New Zealand obese</td>
<td>(Ortlepp et al., 2000)</td>
</tr>
<tr>
<td>BTBR ob/ob</td>
<td>(Hudkins et al., 2010)</td>
</tr>
<tr>
<td>M16</td>
<td>(Allan et al., 2004)</td>
</tr>
</tbody>
</table>
5.5.2.3.1 Zucker rat model of obesity and type II diabetes

The obese Zucker fatty rat, produced from mutations in the *fa/fa* leptin receptor, is one of the most well-established models for metabolic syndrome since rats exhibit obesity, insulin-resistance, hyperleptinaemia and mild glucose intolerance with circulating triglycerides 10-fold higher than in the lean Zucker animals (Zucker, 1965; Witztum and Schonfeld, 1979; Pico et al., 2002).

Zucker obese animals exhibit proteinuria at a young age, which is related to increased glomerular size and glomerulosclerosis (Kasiske et al., 1985). Blood pressure is slightly elevated in the obese Zucker fatty rat, however this is likely to be in response to insulin-mediated increases in renal sodium reabsorption (DeFronzo et al., 1976; DeFronzo, 1981). Bickel et al observed significant proximal tubule atrophy in Zucker fatty rats at 6 weeks of age; furthermore sodium-dependent brush-border transporters such as NaPiIIa and NHE3 exhibited a down-regulation in expression which correlated with GFR measurements (Bickel et al., 2002). There is a selectively inbred sub-strain of Zucker fatty rats, called Zucker Diabetic Fatty (ZDF) rats, which develop overt type II diabetes at 7-10 weeks of age with hyperglycaemia and low insulin levels (Etgen et al., 2000). In later stages of the disease, over 40 weeks of age, ZDF rats exhibit signs of diabetic nephropathy such as renal hypertrophy and renal fibronectin, collagen and ECM accumulation (Coimbra et al., 2000; Hoshi et al., 2002).

5.5.2.3.2 Goto-Kakizaki rat model of type II diabetes

The Goto-Kakizaki rat is another well-established model of type II diabetes although, unlike the Zucker rat, it is a non-obese, normotensive model of the disease which is produced by the repeated inbreeding of glucose intolerant non-diabetic Wistar rats; at least 42 generations are necessary (Goto et al., 1976; Abdel-Halim et al., 1993). In the breeding of the GK rat, Goto et al indicated that the development of diabetes is not due to a specific gene mutation but, like metabolic syndrome, influenced by many genes involved in carbohydrate metabolism (Goto...
et al., 1976). Unlike obese models of type II diabetes, the GK rat exhibits no change in serum cholesterol, triacylglycerol or free fatty acids (Nolte et al., 1995). The impairment in the ability of the pancreatic β-cell to release insulin in response to a glucose stimulus was found in association with a down-regulation in β-cell expression of the facilitative glucose transporter, GLUT2 (Ohneda et al., 1993; Abdel-Halim et al., 1993). The defect in insulin secretion in GK rats is likely to be due to low levels of islet cell glycogen and impaired movement of glucose through the glycerol phosphate shuttle (pathway where glycolysis-produced NADH contributes to ATP generation) (Abdel-Halim et al., 1993; Sener et al., 1993).

Ghannat-Pour et al. used genome-wide RNA profiling to identify several glucose-regulated islet genes which were altered in GK rats; for example, serum-glucocorticoid kinase-1 (SGK-1) and GLUT3 were up-regulated whilst GLUT1 was down-regulated (Ghanaat-Pour et al., 2007).

In addition to defects in pancreatic β-cell mass and function, GK rats also exhibit impaired hepatic glucose production and this causes the hepatic insulin resistance which defines type II diabetes (Picarel-Blanchot et al., 1996). In GK rats, skeletal and cardiac muscle and adipocytes exhibit reduced glucose transport which may be due, in the heart, to reduced GLUT4 expression (Villar-Palasi and Farese, 1994; Nolte et al., 1995; Krook et al., 1997; Desrois et al., 2004). Furthermore, skeletal muscle from GK rats also demonstrates defective glucose conversion to glycogen which may contribute to the mild but consistent hyperglycaemia in the GK rat (Villar-Palasi et al., 1994).

GK rats exhibit functional and structural renal alterations such as renal hypertrophy and increased glomerular volume, total mesangial volume and thickening of glomerular and tubular basement membrane (Phillips et al., 2001; Schrijvers et al., 2004). Kohler et al. attributed these changes to increased nuclear transport in GK rats (Kohler et al., 2001). Interestingly, the accompanying albuminuria and reduced creatinine clearance in this type II diabetic model, in the absence of alterations in mitochondrial oxidative phosphorylation, make the GK rat a good experimental model for patients who suffer prolonged type II diabetes but have not developed overt renal disease (Phillips et al., 2001; Schrijvers et al., 2004; Seica et al., 2005).
5.6  Glucose transporters

Plasma glucose homeostasis is influenced by specific transporter proteins which enable the movement of glucose across cell membranes. The expression of glucose transporters in different cell types varies depending on the specific needs of these cells. However membranes are hydrophilic and therefore glucose must traverse the lipid bilayer via facilitative or active transport mechanisms.

5.6.1 Facilitative glucose transporters

The facilitative glucose transporters, GLUT’s, mediate the movement of glucose down its concentration gradient, thus transport is dependent on the glucose concentrations across the membrane. The family of closely related (~40% overall conservation) GLUT proteins contain twelve transmembrane domains which generate internal and external ligand binding sites, N- and C-terminal cytoplasmic domains and a single glycosylation site on one of the extracellular loops associated with glucose transport and are believed to form a hydrophilic tunnel containing a glucose-binding site (Mueckler et al., 1985; Wilson-O'Brien et al., 2010). There are currently fourteen members of the human SLC2A (GLUT) family which are divided up into three classifications (class I, II, III).

5.6.1.1 Class I GLUT transporters

Class I consists of the ‘classical’ transporters and these have been well described; the class is made up of GLUT1-4 and the GLUT3 gene duplicon, GLUT14 (Joost and Thorens, 2001; Joost et al., 2002). GLUT2 is of relevance in this class of GLUT’s due to its ability for high-capacity glucose transport. GLUT2 is a vital component of glucose absorption across the small intestine and kidney brush-border, and its expression in these locations has been noted in metabolic dysregulation such as in diabetes and Fanconi syndrome.
5.6.1.1 GLUT1

GLUT1 was first proposed to be involved in placental glucose transport by Widdas et al but GLUT1 has since been located at the plasma membrane of erythrocytes, hepatocytes and in endothelial cells in the microvasculature of the brain, as well as the BLM of the late S3 segments of proximal tubules (Widdas, 1952; Mueckler et al., 1985; Birnbaum et al., 1986; Widdas, 1988). Interestingly, GLUT1 is also found in adipocytes where it has been shown to detect glucose via its C-terminal domain activating the ERK pathway, independent of any glucose transport or metabolic activity of the transporter (Bandyopadhyay et al., 2000). GLUT1 has been shown to have a $K_T$ for glucose of 5-10 mM using the Xenopus oocyte expression system however the transporter also has substrate-specificity for galactose (Gould et al., 1989; Gould et al., 1991; Nishimura et al., 1993). Furthermore GLUT1-related disorders are based on the role of the transporter in glucose transport across the blood-brain barrier and include infantile seizures and developmental delay, as well as a link between GLUT1 and the early stages of Alzheimer’s disease (Simpson et al., 1994; Klepper et al., 1999).

5.6.1.1.2 GLUT2

GLUT2 is a high-capacity, low-affinity glucose transporter with a $K_T$ of 15-25 mM which can also transport galactose, glucosamine, mannose and fructose with $K_T$ values of 92 mM, 0.8 mM, 125 mM and 76 mM respectively (Johnson et al., 1990; Dominguez et al., 1992; Colville et al., 1993; Uldry et al., 2002). GLUT2 is expressed in various tissues since it is involved in metabolic glucose sensing (described in section 5.6.1.1.2.1); this role in glucose homeostasis is mediated by the ability of GLUT2 to transport glucose bi-directionally and thus it can modulate intracellular glucose concentrations. GLUT2 is highly expressed in pancreatic β-cells and hepatocytes as well as at the BLM of small intestinal and renal epithelial cells; interestingly, in response to high luminal glucose, GLUT2 is also expressed at the BBM at these locations (Fukumoto et al., 1988; Orci et al., 1989; Johnson et al., 1990; Thorens, 1992; Kellett and Helliwell, 2000; Marks et al., 2003; Marks, 2004).
5.6.1.1.2.1 GLUT2 as a glucose sensor

GLUT2 has been proposed to be a glucose sensor as it is primarily responsible for glucose homeostasis through its role in glucose absorption in the intestine, glucose reabsorption in the kidney, glucose sensing in the pancreatic β-cell and glucose uptake and release by the liver (Stolarczyk et al., 2010). In pancreatic β-cells, GLUT2 is coupled with the glucokinase enzyme to form a glucose sensory mechanism; indeed GLUT2 mediates the initial phase of insulin secretion in mice in order to correct plasma glucose fluctuations (Unger, 1991; Guillam et al., 1997). In hepatocytes, GLUT2 can sense glucose and activate a signalling pathway via its large cytoplasmic domain which causes glucose-induced transcription, via a signalling pathway independent of glucose metabolism (Guillemain et al., 2000; Guillemain et al., 2002). In enterocytes, the polar nature of GLUT2 glucose sensing is from the BLM where GLUT2 senses alterations in glycaemia which leads to changes in enterocyte glucose absorption (Le et al., 2007). Furthermore, GLUT2-loop transgenic mice continue to express GLUT2 in renal cells therefore the phenotype of the GLUT2-loop mice may not be caused by defective glucose entry but the inability of the kidney to sense sugars (Stolarczyk et al., 2007). This suggests that the involvement of GLUT2 in glucose detection may also influence renal glucose reabsorption (Stolarczyk et al., 2007).

5.6.1.1.2.2 Genetic regulation of GLUT2

The transcriptional regulators of the GLUT2 gene include HNF-1α, -3β and PDX-1 (Pontoglio et al., 2000; Shen et al., 2001; Parrizas et al., 2001; Freitas et al., 2009). In the rat GLUT2 gene, the peroxisomal-proliferator-response element (PPRE) is functional and promoter activity of the gene was elevated by PPARγ-ligand binding (Kim et al., 2000). Special regions of the GLUT2 promoter have been characterised as containing glucose- and cAMP-responsive element therefore these substrates are enhancers and suppressors, respectively, of GLUT2 gene expression (Rencurel et al., 1997).
Homozygous or compound heterozygous mutations in the \textit{GLUT2} gene cause Fanconi-Bickel syndrome, the rare autosomal-recessive congenital disease which is characterised by glycogen accumulation in the kidney and liver, and nephropathy (Santer \textit{et al.}, 1997; Santer \textit{et al.}, 2002; Taha \textit{et al.}, 2008). GLUT2 mutations can also result in the abolition of the transporter resulting in the inability of patients to tolerate simple sugars in the diet (Santer \textit{et al.}, 2002). One outcome of Fanconi-Bickel syndrome is impaired glucose tolerance with hypoinsulinaemia and severe glycosuria; this is also seen with GLUT2 KO mice where the disease causes a lethal diabetic phenotype following weaning which is likely to be due to the inability to handle excess glucose and thus sugar entry and detection by the pancreas are impaired (Guillam \textit{et al.}, 1997; Guillam \textit{et al.}, 2000). Interestingly, a clinical study with a newborn exhibiting symptoms of Fanconi-Bickel who exhibited severe renal tubular dysfunction, rickets and increased serum galactose levels showed a positive response to a lactose-free diet supplemented with fructose and corn starch; and the disorder was abolished within 6 months (Kentrup \textit{et al.}, 1999). However no mutational hotspots have been identified within the \textit{GLUT2} gene; there are 33 different mutations detected in 49 sufferers of Fanconi-Bickel syndrome, making gene localisation difficult (Santer \textit{et al.}, 2002).

The genetic variation of the \textit{GLUT2} promoter has also been associated with the onset of type II diabetes, for example, the \textit{Val-197-Ile} missense mutation results in abolition of the transport activity of GLUT2 and has been postulated to influence the pathogenesis of type II diabetes (Mueckler \textit{et al.}, 1994). Interestingly, renal damage in patients with GLUT2 deficiency, such as renal hyperfiltration, microalbuminuria, and glomerular mesangial expansion, closely matches diabetic nephropathy (Berry \textit{et al.}, 2005).

The role of GLUT2 in renal and jejunal glucose transport is covered in sections 5.7 and 5.8.
5.6.1.1.3 GLUT3

GLUT3 has been located in sperm, embryo and both dendrites and axons in neurons as well as pancreatic β-cells (Mantych et al., 1992; Sato et al., 1996). The transporter has also been found in lymphocytes, platelets, monocytes and macrophages; thus GLUT3 may have a role in immune and inflammatory cell activation (Estrada et al., 1994; Maratou et al., 2007). The transporter has a high-affinity for glucose with *Xenopus* oocyte data showing a $K_T$ of 1 mM; furthermore GLUT3 has a high-turnover number and therefore can ensure efficient glucose uptake by neurons (Sato et al., 1996; Maher et al., 1996). The GLUT14 gene, *SLC2A14*, is a duplcon of GLUT3 which exhibits 95% homology to GLUT3; it has been located in the testis, of man, as two alternative splice forms (Wu and Freeze, 2002).

5.6.1.1.4 GLUT4

GLUT4 is located in skeletal and cardiac muscle and adipose tissue and has a high-affinity for glucose, with a $K_T$ of 5 mM when expressed in *Xenopus* oocytes (James et al., 1989; Nishimura et al., 1993). Under normal conditions, GLUT4 is expressed at low levels in cell membranes but upon insulin stimulation, GLUT4 translocates to the membrane and, as a result, glucose uptake is elevated (Simpson et al., 2001). The insulin-sensitivity of GLUT4 suggests a role in diabetes; indeed disruption of the *GLUT4* gene, by homologous recombination, results in the development of a type II diabetic-like phenotype including reduced muscle glucose uptake and elevated circulating levels of insulin (Stenbit et al., 1997; Rossetti et al., 1997). Furthermore, in other models of insulin-resistance such as in response to a high-fat diet, GLUT4 is down-regulated in adipose tissue and skeletal muscle and this has been associated with reduced glucose utilization by these tissues (Petersen et al., 1991; Kim et al., 1994).
### 5.6.1.2 Class II and III GLUT transporters

Class II of GLUT transporters consists of GLUT5, GLUT7, GLUT9 and GLUT11, all of which transport fructose as well as glucose, and, with the exception of GLUT5 they all have a $K_T$ of less than 1 mM for glucose (Kane et al., 1997; Manolescu et al., 2005; Scheepers et al., 2005). Class III comprises of the more obscure GLUT6, GLUT8, GLUT10, GLUT12 and GLUT13 (HMIT). This class of the GLUT family contains members sharing an intracellular localisation signal and large extracellular loop 9, which carries their glycosylation site (Scheepers et al., 2001; Uldry et al., 2001; Rogers et al., 2002; Wood et al., 2003). However, GLUT5 is the only transporter to be reported here due to its association with fructose-related metabolic diseases and its location at both the renal and small intestinal brush-border epithelia.

#### 5.6.1.2.1 GLUT5

The rate of glucose transport by GLUT5, when assessed using the Xenopus oocyte expression system, is low and is inhibited by cytochalasin B (Kayano et al., 1990). However using in vivo studies GLUT5 has not been shown to transport glucose (Burant et al., 1992; Rand et al., 1993; Miyamoto et al., 1994). GLUT5 is a major fructose transporter having a $K_T$ of 6 mM for cytochalasin B-sensitive, fructose transport when expressed in Xenopus oocytes (Burant et al., 1992; Mate et al., 2001; Douard and Ferraris, 2008). Interestingly, the expression of GLUT5 in the central nervous system (CNS), where fructose is not used as a metabolic substrate, suggests an important role for GLUT5 in glucose transport despite the low affinity for glucose (Mantych et al., 1993). GLUT5 is also located at the BBM of the small intestine where it is important in absorption of fructose from any sources in the diet, as well as at the BLM of human small intestine, implying a role of GLUT5 in transport from enterocyte to the circulation (Blakemore et al., 1995). In intact small intestine, GLUT5 is so specific for fructose that GLUT5-mediated fructose transport is not inhibited by a 100-fold excess of glucose (Burant et al., 1992). Furthermore, fructose is rapidly cleared from the blood thus
circulating fructose levels are low and fructose transport can be mediated by the facilitative transporter GLUT5. In addition, GLUT5 is abundant in other fructose-utilising tissues such as the testes, erythrocytes, adipose tissue and skeletal muscle, and cells in the S3 segment of proximal tubules (Burant et al., 1992; Miyamoto et al., 1992; Rand et al., 1993e; Corpe et al., 2002).

Differences have been documented for GLUT5 tissue distribution in the rat and mouse (Rand et al., 1993). However Inukai et al found that rat GLUT5 mRNA exhibits tissue distribution very similar to that in man with the jejunum presenting the highest expression of the transporter (Inukai et al., 1993). In this location, levels of GLUT5 mRNA and protein are elevated in response to fructose-enriched, but not starch-enriched, diets (Inukai et al., 1993). In rats, immediately after weaning, intestinal fructose transport and GLUT5 mRNA expression, is up-regulated; these changes occur irrespective of dietary intake of fructose (Rand et al., 1993; Castello et al., 1995; Shu et al., 1997). Barone et al used a GLUT5 KO mouse to show that the transporter mediates fructose metabolism and physiological effects mediated by high-fructose diets, such as hypertension (Barone et al., 2009); these are elaborated on in section 5.4.

GLUT5 KO mice present with a 75% reduction in fructose absorption across the jejunum and a 90% decline in serum fructose levels (Barone et al., 2009). These GLUT5-null mice also exhibit normal blood pressure and weight gain in response to high-fructose feeding, although dietary glucose handling was unaffected in this KO model (Barone et al., 2009). Indeed Mesonero et al showed that Caco-2 cells, incubated with fructose, resulted in 3-fold higher GLUT5 protein and mRNA expression when compared with incubation with the same concentration of glucose (Mesonero et al., 1995). Furthermore GLUT5 protein and mRNA, and GLUT5-mediated fructose uptake, was elevated in Caco-2 cells by forskolin, the adenylate cyclase activator which elevates intracellular cAMP (Mahraoui et al., 1994). In addition, fructose has been shown to be involved in a positive regulatory loop with gastric leptin; fructose triggers the release of gastric leptin which up-regulates GLUT5 levels and modulates metabolic function in the liver, this offers a likely explanation of the damaging effects of fructose on lipidaemia as outlined in section 5.4.
5.6.1.3 Inhibitors of GLUT-mediated transport

Both cytochalasin B, a major metabolite of the fungus *Helminthosporium mastoideum*, and phloretin are potent inhibitors of the GLUT family, with some exceptions (Ebstensen and Plagemann, 1972; Graff et al., 1973). For example, cytochalasin B and phloretin bind to GLUT5 in a different location to the fructose-binding site, and therefore GLUT5-mediated fructose transport is unaffected by these inhibitors (Ebstensen *et al.*, 1972; Doege *et al.*, 2001). The anti-retroviral therapies, indinavir and fasentin, inhibit glucose uptake of cells which preferentially express GLUT1 and GLUT4, as does the flavonoid apigenin (Ebstensen *et al.*, 1972; Murata *et al.*, 2002; Wood *et al.*, 2008; Melstrom *et al.*, 2008).

5.6.2 Active glucose transporters

Secondary active transporters are energy-requiring symporters which move specific nutrient substrates against their concentration gradient. The energy required for this transport is harnessed from electrochemical gradient-coupling of nutrient transport to the translocation of specific ions.

5.6.2.1 SGLT family

There are currently 6 members of the sodium-dependent glucose transporter (SGLT) family: SGLT1, SGLT2 and SGLT3 (SAAT-pSGLT2) have been well-characterised whereas SGLT4, SGLT5 and SGLT6 still need to be fully characterised (Wright *et al.*, 2004; Wright and Turk, 2004; Wright *et al.*, 2007). Interestingly SGLT4, SGLT5 and SGLT6 have been located in the kidney, as well as other tissues, where glucose and galactose are known to be substrates (Nishimura and Naito, 2005).
SGLT transporters comprise twelve transmembrane segments and a leucine zipper domain on one extracellular loop; furthermore SGLT1 and SGLT2 have 60% identity homology with closely-related amino acid sequences (Wright et al., 1994; Turk et al., 1994). SGLT1 and SGLT2, mediate reversible glucose transport across epithelial membranes by using free energy from an electrochemical Na\(^+\)-gradient into a glucose concentration gradient across the membrane (Hummel et al., 2011).

The sodium gradient is maintained by the Na\(^+\)/K\(^+\)-ATPase at the basolateral membrane of small intestinal and renal epithelial cells; the pump exports three sodium (Na\(^+\)) from the cell to maintain a sodium gradient across the BBM and transports two potassium (K\(^+\)) ions into the cell (Gloor, 1997; Scheiner-Bobis, 2002; Faller, 2008).

5.6.2.1.1 SGLT1

SGLT1 co-transport two Na\(^+\) ions into the cell with one glucose molecule and has a high-affinity for glucose and galactose with a K\(_T\) of 0.2 – 0.5 mM; however, SGLT1 has a low-capacity for glucose transport (Hirayama et al., 1996; Brown, 2000; Wright, 2001). SGLT1 has been located at the BBM of absorptive enterocytes, but is also expressed in lower abundance in proximal straight tubule cells, as well as intracerebral capillaries, mammary glands, liver and alveolar cells in the lung (Hwang et al., 1991; Takata et al., 1991; Cramer et al., 1992; Takata et al., 1992; Elfeber et al., 2004; Zhao et al., 2005; Sabino-Silva et al., 2010). A single missense mutation in the SGLT1 gene causes a rare glucose/galactose malabsorption condition; this autosomal recessive disorder results in severe watery diarrhoea and dehydration in the newborn period which can be treated by replacing glucose and galactose in the diet with fructose (Turk et al., 1991; Carbonnel et al., 1995; Martin et al., 1997).

Interestingly, a wide aqueous cavity in SGLT1 structure was found within the substrate-binding conformation (Choe et al., 2010). Rabbit SGLT1 expressed in Xenopus oocytes has been shown to co-transport 424 water molecules across epithelia for every 2 Na\(^+\) ions and 1 glucose molecule; furthermore this water transport is not osmotic (Zeuthen et al., 2001).
phlorizin-sensitive, isotonic water transport has been postulated to serve the role of directly co-transporting water across epithelia and to generate the osmotic driving force for other pathways such as aquaporin-mediated water transport (Loo et al., 1999; Zeuthen et al., 2001). Water transport via SGLT1 is gated with no release of water molecules from the transporter until the glucose molecule is released (Watanabe et al., 2010). Recent studies have shown that SGLT1 is able to co-transport a more modest 70 molecules of water per 1 molecule of galactose (Watanabe et al., 2010).

5.6.2.1.2 SGLT2

SGLT2 co-transportSGLT2s one glucose molecule with one Na\(^+\) ion and is highly specific for glucose, but not galactose, and has a lower affinity than SGLT1 with a K\(_T\) of 2-10 mM (Wright, 2001). Furthermore SGLT2 has a higher capacity for glucose transport than SGLT1 and is predominantly expressed in the kidney, at the BBM of proximal convoluted tubule cells (Wells et al., 1992; Kanai et al., 1994; Brown, 2000). A missense mutation in the SGLT2 gene, of which there are approximately 21 different gene mutation sites, causes the autosomal recessive condition familial renal glycosuria; this benign genetic defect of renal glucose reabsorption causes persistent glycosuria, with glucose excretion of up to 160 g/day, in the absence of hyperglycaemia, or any other clinical phenotype (Santer et al., 2003; Francis et al., 2004; Scholl-Burgi et al., 2004). Interestingly this disorder does not affect renal transport of other sugars and the uptake and utilisation of glucose by other tissues is normal (Carbonnel et al., 1995).

5.6.2.1.3 SGLT3

SGLT3 is found in the small intestine, kidney and skeletal muscle with a K\(_T\) of 2 mM for glucose however rather than being a sugar transporter like SGLT1 and SGLT2, SGLT3 is a glucose-activated Na\(^+\)-channel found in the plasma membrane of cholinergic neurons (Zhao and Keating, 2007). Interestingly, in enteric neurons, glucose sensing by SGLT3 triggers
specific phlorizin-sensitive, Na\(^+\)-dependent depolarisation of the membrane potential. However, unlike the enterocyte BBM, the glucose-induced inward currents in the cells are not accompanied by glucose transport (Diez-Sampedro et al., 2003). Recently, Bianchi et al identified that the mutation of SGLT3 from glutamate to glutamine, in position 457, changes the sugar sensor into a glucose transporter with the same stoichiometry as SGLT1 (Bianchi and Diez-Sampedro, 2010).

5.6.2.2 SGLT inhibitors

5.6.2.2.1 Phlorizin

Phlorizin, a β-glucopyranoside first isolated from the root bark of apple trees, is a potent competitive and non-competitive inhibitor of SGLT1 and SGLT2 with no effect on the GLUT family of glucose transporters (Brazy and Dennis, 1978; Bell et al., 1990; Petersen et al., 1991). The administration of phlorizin to diabetic rats results in the development of glycosuria accompanied by the normalisation of blood glucose levels (Rossetti et al., 1987; Kahn et al., 1991; Zhao et al., 2004; Masumoto et al., 2009). Phlorizin treatment has also been shown to prevent proteinuria, hyperfiltration and kidney hypertrophy exhibited by type I diabetic rats (Malatiali et al., 2008). Freitas et al compared the efficacy of phlorizin with insulin treatment and found the SGLT inhibitor to be useful for reducing blood glucose levels in type II diabetes; although both treatments significantly reduced the plasma glucose levels in diabetic rats, phlorizin had no effect on body weight or plasma insulin levels whereas insulin treatment increased both parameters (Freitas et al., 2008). However phlorizin is not selective for renal SGLT transporters but also targets SGLT1 in the small intestine (Pajor et al., 1992; Tyagi et al., 2007). Phlorizin is rapidly degraded by β-glucosidases in the gastrointestinal tract which results in low bioavailability as well as the production of phloretin, a GLUT inhibitor (Miyamoto et al., 1994; Crespy et al., 2001). However Masumoto et al found a 3-fold rise in plasma phlorizin levels in STZ-induced diabetic mice fed a diet containing 0.5% phlorizin, for 14 days, when compared to 0.1 % phlorizin, suggesting the inhibitor may be absorbed across the enterocyte (Masumoto et al., 2009).
5.6.2.2 SGLT2 inhibitors in type II diabetes treatment

Despite the multitude of type II diabetic drugs currently available (outlined in 5.2.5.2) these treatments have their limitations, therefore SGLT2 inhibitors present a novel and potentially effective method in the treatment of type II diabetes. Subsequently alternatives for phlorizin have been studied for their use in managing glucose reabsorption in the kidney by targeting SGLT2, without exerting an effect on SGLT1-mediated glucose transport. The first example of this was the oral pro-drug T-1095, which is metabolised into T-1095A in the liver and therefore T-1095A, but not T-1095, was detected in plasma following oral administration of T-1095 (Tsujihara et al., 1999; Arakawa et al., 2001). T-1095A exerts a hypoglycaemic effect in animal models of diabetes, such as the ZDF rat, db/db mice and STZ-induced type I diabetic animals, with concomitant glycosuria (Nawano et al., 2000; Oku et al., 2000; Arakawa et al., 2001). Furthermore, T-1095 prevented the age-related decrease in plasma insulin levels and loss of pancreatic insulin seen in db/db mice, as well as improving symptoms of diabetic nephropathy such as urinary albumin excretion (Arakawa et al., 2001).

5.6.2.2.1 Potential treatment of diabetes by SGLT2 inhibitors

Subsequently there are currently over 21 SGLT2 inhibitors being developed for treatment in diabetes, with examples including the C-aryl glucoside, dapagliflozin and the O-glucoside, sergliflozin (Katsuno et al., 2007; Han et al., 2008; Meng et al., 2008).

Dapagliflozin is a selective inhibitor of SGLT2 and reduces plasma glucose levels between 50—60%, within 2 hours of a single oral dose in STZ-induced type I diabetic rats; dapagliflozin exerts a dose-dependent decrease in glycaemia with a concomitant increase in glycosuria (Han et al., 2008; Meng et al., 2008; Komoroski et al., 2009). Furthermore, this SGLT2 inhibitor does not influence body weight or glucose transport in other tissues, nor does it induce hypoglycaemia (Han et al., 2008).
Sergliflozin is the pro-drug for the potent and selective SGLT2 inhibitor sergliflozin-A; like dapagliflozin, it exhibits a dose-dependent decrease in glycaemia with an increase in glycosuria (Katsuno et al., 2007; Hussey et al., 2010). Despite the inhibition of renal glucose reabsorption, sergliflozin has no effect on GLUT1 activity at the BLM of proximal tubules, nor does it cause hypoglycaemia (Katsuno et al., 2007). Interestingly, when used at supraphysiological doses, it causes transient osmotic diuresis and a reduction in body weight (Katsuno et al., 2007; Hussey et al., 2010).

The safety and efficacy of SGLT2 inhibitors as a treatment for diabetes requires further clinical testing. However, in the rare genetic disease, Familial Renal Glycosuria (FRG), sufferers exhibit extensive glycosuria with no other complications or adverse effects (Santer et al., 2003). This implicates the use of SGLT2 inhibitors as a powerful and safe tool in the treatment of diabetes. However, Ly et al have recently developed the Sweet Pee mouse model which carries a nonsense mutation in the SGLT2 gene, resulting in the loss of protein function (Ly et al., 2011). On induction of diabetes with STZ, these mice display glycosuria with accompanying euglycaemia however no change in insulin sensitivity is observed. Furthermore, although there are no changes in GFR in the Sweet Pee mouse, the diabetic animals develop multiple urinary tract infections and kidneys from older animals exhibit massive white blood cell infiltration; furthermore, an earlier age of mortality was noted in type I diabetic Sweet Pee mice compared with diabetic wild-type mice (Ly et al., 2011). Therefore the long-term effects of SGLT2 inhibition in diabetes requires further investigation to characterise the efficacy of SGLT2 inhibitors as treatment in the disease.
5.7 Glucose handling in the kidney

The kidneys are vital for glucose homeostasis due to their role in the filtration and reabsorption of glucose; thus it is important to understand the regulation of these processes in order to understand the pathology of diseases in glucose transport. Studies show that the kidneys are able to generate glucose and potentially act as a storage pool for glucose, thus the control of glucose handling by the kidney plays a major role in maintaining whole body glucose status.

5.7.1 Renal glucose filtration and reabsorption

5.7.1.1 Glucose filtration

Under normal conditions, when glycaemia is approximately 5.5 mM, since 180 L of plasma is filtered by the kidneys per day, 180 g of glucose is filtered by the kidney over this time period. Circulating glucose moves from the glomerular capillaries into the Bowman’s capsule, together with low molecular weight constituents such as amino acids and electrolytes. The filtrate moves through the proximal tubule, along the loop of Henle, into the distal convoluted tubule and through a series of collecting ducts to form urine for excretion. As plasma glucose levels increase, the amount of glucose in the filtrate increases in a linear manner, assuming no change in glomerular filtration rate (GFR). The reabsorption of glucose is saturable and increases in a linear manner until the maximal reabsorptive capacity is exceeded, after which the glucose is excreted in increasing amounts (Deetjen and Boylan, 1968; Zelikovic and Przekwas, 1995; Hall et al., 1996).
5.7.1.2 Glucose reabsorption

Normally, over 99% of glucose filtered from the plasma is reabsorbed along the nephron, with very low glucose excretion in the range of 0.03 to 0.3 g per day (Wright, 2001). The glucose transporters SGLT1 and SGLT2, and GLUT1 and GLUT2, are vital for glucose reabsorption by the kidney (Figure 5.4).

Free-flow micropuncture studies in the rat show that 90% of the filtered glucose is reabsorbed in the early proximal convoluted tubule, S1/S2 segment, by SGLT2 at the BBM of the proximal tubule cell, the remaining fraction of glucose in the filtrate is absorbed in the late proximal straight tubule, S3 segment (Cramer et al., 1992; Brown, 2000; Lee et al., 2007; Chen et al., 2008). This is reinforced by studies using the isolated perfused tubule technique; it has been shown that the maximal rate of active glucose transport decreases considerably from the proximal convoluted tubule to the proximal straight tubule. This reduced rate of uptake is accompanied by increased affinity of transporters at the BBM for glucose (Barfuss and Schafer, 1981). Thus in the early proximal tubule a low-affinity, high-capacity glucose transport system has been established which is attributed to SGLT2 whereas SGLT1, a high-affinity but low-capacity glucose transport system was identified in the later proximal tubule, which reabsorbs most of the remaining glucose from the tubular fluid (Turner and Silverman, 1977; Turner and Moran, 1982; Turner and Moran, 1982). Sodium ions co-transported with glucose by SGLT1 (2 Na⁺) and SGLT2 (1 Na⁺) are pumped out of the proximal tubule cell via the basolateral Na⁺/K⁺-ATPase, which is responsible for maintaining the sodium electrochemical potential gradient at the BBM, for SGLT-mediated glucose transport.
**Figure 5.4** – Schematic diagram of the specific regions of the proximal tubule associated with glucose reabsorption from tubular fluid, across the cell, via the sodium gradient maintained by the basolateral Na⁺/K⁺-ATPase. A: cell diagram for glucose transporters involved in glucose reabsorption across the early S1/S2 region of the proximal tubule, where the luminal glucose load is high following glucose filtration by the glomerulus. B: cell diagram from glucose transporters involved in glucose reabsorption across the late S3 region of the proximal tubule, where the luminal glucose load is considerably reduced following glucose reabsorption in the early proximal convoluted tubule (Figure adapted from (Marks, 2004)). ■ Glucose molecule  ● sodium ion  ● potassium ion.
A recent study by Hummel et al indicates that SGLT1 contributes a greater proportion of glucose transport than originally believed, with a $K_T$ for glucose of 1.8 mM compared to a value of 4.9 mM for SGLT2 (Hummel et al., 2011). As well as confirming the 2:1 Na$^+$-glucose coupling for SGLT1 and 1:1 Na$^+$-glucose coupling for SGLT2, the study also found that the $K_T$ of SGLT1 for galactose transport was 6.1 mM whereas galactose is a poor substrate for SGLT2 with a $K_T$ of more than 100 mM (Hummel et al., 2011). Interestingly, the contribution of SGLT1 to glucose reabsorption has been shown in SGLT2 KO mice which, despite the concomitant 40% reduction in SGLT1 mRNA and protein, still reabsorb 30-40% glucose from the glomerular filtrate (Vallon et al., 2010). In the Sweet Pee mouse model with loss of SGLT2 protein function, mice exhibited a significant elevation in SGLT2 mRNA in the kidney with no change in SGLT1, GLUT1 or GLUT2 mRNA (Ly et al., 2011). However it is worth noting that, although in vitro studies with dapagliflozin show complete inhibition of SGLT2 activity, in vivo the drug only blocks glucose reabsorption by 50% (Meng et al., 2008;Komoroski et al., 2009). Although dapagliflozin is still an effective drug in the reduction of hyperglycaemia and treatment of type II diabetes, this data provides evidence for a larger role of renal SGLT1 in glucose reabsorption then has hitherto been recognised (Hummel et al., 2011).

Glucose efflux from the proximal tubule cell across the BLM is driven by the outwardly-directed glucose gradient, mediated through the facilitative glucose transporters GLUT1 and GLUT2, like SGLT transporters, there is an axial distribution of GLUT transporters along the proximal tubule. The high-affinity GLUT1 is found at the BLM of proximal convoluted and straight tubule cells whereas the low-affinity GLUT2 is confined to the proximal convoluted tubule BLM (Thorens et al., 1990;Cramer et al., 1992;Dominguez et al., 1992).
5.7.1.3 Renal glucose production and storage

In humans, glucose is released into the circulation by both the liver and kidney which, as the only tissues containing significant amounts of the enzyme glucose-6-phosphatase, are the only two organs able to perform gluconeogenesis (Ekberg et al., 1999). The renal contribution to systemic glucose is between 20-42% following ingestion of a meal (Stumvoll et al., 1995). Glucose production by the kidney is not mediated by glycogenolysis as, under normal conditions, the kidney stores low levels of glycogen (Biava et al., 1966). Thus the kidney relies on gluconeogenesis, from alanine, lactate or glutamine, to release glucose into the circulation (Stumvoll et al., 1998; Cersosimo et al., 1999; Meyer et al., 2002). Gluconeogenesis is up-regulated in response to adrenalin; the hormone elevates renal uptake of precursors lactate and alanine, and increases renal gluconeogenic efficiency (Meyer et al., 2003). Interestingly, Meyer et al. showed that, unlike the down-regulation seen in the liver, renal gluconeogenesis is up-regulated by two-fold in the postprandial state (Meyer et al., 1999).

5.7.2 Regulation of renal glucose handling

There are many factors which influence renal glucose handling including the diabetic disease state, feeding, fasting as well as severe inflammation and hepatocyte nuclear factors.

5.7.2.1 Renal glucose filtration, production and storage in diabetes

5.7.2.1.1 Filtration and tubuloglomerular feedback

One of the earliest indicators of altered kidney function in diabetes is an elevation in GFR; GFR and tubular function are closely interlinked via the tubular composition of the tubular fluid (O'Donnell et al., 1988). However, the diabetes-mediated effects on GFR are unrelated to renal tubule glucose transport or plasma osmolality (Skott et al.,
Diabetic hyperglycaemic elevates the tubular glucose load and, provided the transport maximum for glucose has not been exceeded, this leads to elevated proximal tubule reabsorption. The fractional reabsorption of fluid and electrolytes in the proximal tubule is elevated in response to STZ-induced diabetes; furthermore in long-term type I diabetic patients, the rise in reabsorption occurs irrespective of the presence of diabetic nephropathy (Skott et al., 1989; Bank and Aynedjian, 1990; Vallon et al., 1995; Vallon et al., 1999). These changes result in glomerular hyperfiltration and oxidative stress, and culminate in inactivated tubuloglomerular feedback mechanisms (Tucker et al., 1978; Tucker and Blantz, 1978; Skott et al., 1989).

5.7.2.1.2 Renal glucose production and release

One of the main factors causing fasting hyperglycaemia in type II diabetic patients is the elevated release of glucose by the kidney and liver (Bearn et al., 1951; Dinneen et al., 1992). Although there is a rise in both renal and hepatic gluconeogenesis; the kidney contributes a greater amount to the whole body glucose pool than the liver, with renal glucose release in type II diabetic patients elevated by nearly 300% (Meyer et al., 1998). The increase in renal gluconeogenesis is also seen in obese patients who undergo prolonged fasting (Owen et al., 1969). Postprandially, type II diabetes in man also leads to elevated renal glucose release, which is not unexpected with regards to the reduced postprandial increase in insulin release and the effects of insulin on renal glucose release (Meyer et al., 2004). Diabetic Zucker rats, an established model of type II diabetes, showed a high rate of renal gluconeogenesis which correlated with the increased activity of key gluconeogenic enzymes (Eid et al., 2006).

5.7.2.1.3 Renal glucose storage

Raised renal glucose uptake and gluconeogenesis in diabetes increases glucose levels in tubular cells and provides a likely explanation for the elevated glycogen levels found in both type I and II diabetes (Rasch, 1984; Williams and Airey, 2002; Barmi-
Ezzine et al., 2003; Ohta et al., 2007). The accumulation of glycogen, seen in human and rodent models of diabetes, has been proposed to be a result of impaired AMPK-mediated reduction by glycogen synthase and can result in tubular cell apoptosis (Bamri-Ezzine et al., 2003; Londono et al., 2004; Cammisotto et al., 2008).

Thus in addition to the contribution of renal glucose handling to the hyperglycaemia of diabetes, knowledge of changes in renal glucose handling has increased out understanding of the pathology of diabetes-induced renal complications.

5.7.2.1.4 Renal glucose reabsorption

In diabetic patients, glycosuria does not occur at plasma glucose concentrations that would normally produce glycosuria in non-diabetics (Mogensen, 1971). This tolerance is likely to be due to increased glucose reabsorption in diabetes implying a raised transport maximum for glucose.

Uncontrolled diabetes, by increasing blood and interstitial glucose concentrations, influences the glucose gradient across the proximal tubule cell via impairment of glucose efflux from epithelial cells and thus glucose reabsorption. To this end, increased gene expression of renal glucose transporters could be viewed as being important in the maintenance of glucose reabsorption in diabetes (Dominguez et al., 1992; Dominguez et al., 1994a; Dominguez et al., 1994b).

The increase in renal glucose reabsorption during hyperglycaemia implies an augmentation of glucose movement across both the BBM and BLM of the proximal tubule cell. Bank et al hypothesised that the higher rate of glucose reabsorption seen in diabetes is a result of elevated sodium-dependent glucose uptake from the luminal fluid with basolateral glucose transport increasing concomitantly to maintain steady-state transcellular glucose efflux (Bank et al., 1990).
5.7.2.1.4.1 Renal glucose transporters involved in glucose reabsorption

There are a plethora of changes in glucose transporters in proximal tubule cells in response to diabetes. An elevation in renal GLUT2 expression has been reported in diabetes while GLUT1 is suppressed (Dominguez et al., 1994a; Chin et al., 1997; Kamran et al., 1997; Vestri et al., 2001). Marks et al. found augmented GLUT2 protein levels at the BBM of proximal tubule cells prepared from STZ-induced diabetic rats, along with elevated GLUT5 protein, which was matched by augmented GLUT-mediated glucose transport activity; however, no change in GLUT1 expression at the BBM was noted (Marks et al., 2003). Interestingly, the study also reported that GLUT2 at the proximal tubule BBM, and GLUT-mediated glucose uptake was down-regulated by an overnight fast, whereas GLUT5 was unaffected. These data suggested a relationship between glycaemic status and GLUT2 expression (Marks et al., 2003). Indeed, Goestemeyer et al. used nicotinamide to modulate the extent of hyperglycaemia in STZ-induced diabetes and showed that GLUT2 expression at the BBM exhibited a strong positive correlation with plasma glucose (Goestemeyer et al., 2007). The relationship between plasma glucose level and GLUT2 protein expression in type I diabetes was also seen by Freitas et al. The group found that both acute and short-term insulin treatment induced an early elevation in the already raised levels of GLUT2 in the renal cortex, seen in type I diabetes (Freitas et al., 2005). Despite this early increase in GLUT2 being transitory, acute insulin treatment may exacerbate diabetes-induced renal dysfunction by increasing intracellular glucose concentrations (Freitas et al., 2005). Interestingly, the normalisation of renal GLUT2 expression in the proximal tubule cell seen with long-term insulin treatment, is also seen following the treatment of control rats with the structurally similar hormone, insulin-like growth factor 1, IGF-1 (Asada et al., 1997).

Type II diabetic patients and Zucker diabetic rats exhibit tubular overexpression of GLUT2, although the contribution of GLUT-mediated glucose transport in these models has not yet been established (Kamran et al., 1997; Rahmoune et al., 2005). Interestingly, Kamran et al. postulated that an interaction between tubular glucose levels with SGLT at the BBM is necessary for GLUT2 activation (Kamran et al., 1997). Thus it is possible that SGLT signals to GLUT2 via elevating intracellular...
glucose concentration; a mechanism which has been identified to regulate GLUT2 expression at the enterocyte BBM (see section 5.8.2.3) (Kellett and Helliwell, 2000). In agreement with this is the down-regulation in renal expression of GLUT2 in response to phlorizin treatment of type I diabetic rats, suggesting interplay between the regulation of renal SGLT’s and GLUT2 expression (Freitas et al., 2007).

In contrast to GLUT2, the effects of diabetes on SGLT-mediated glucose transport at the proximal tubule BBM are less clear. STZ-induced type I diabetes of 2-7 weeks duration has been shown to have no effect on SGLT1 expression or SGLT-mediated glucose transport at the proximal tubule BBM; however an increase in SGLT1 expression was evident after 8-week diabetes and also in 12-week alloxan-induced diabetes (Vestri et al., 2001; Marks et al., 2003; Vidotti et al., 2008). Furthermore in studies using cultured proximal tubule cells, SGLT-mediated glucose uptake was found to be inhibited by high glucose levels; this is a likely consequence of high-glucose-mediated PKC activation in proximal tubule cells, which has been shown to inhibit rodent but not human SGLT1 (Ohta et al., 1990; Hediger and Rhoads, 1994; Ishii et al., 1998). Additionally, a high glucose concentration in tubular fluid induces oxidative stress which inhibits SGLT activity in renal proximal tubule cells (Han et al., 2005).

Albertoni Borghese et al found a decrease in SGLT2 protein and α-methylglucose uptake in short-term 3 and 7 day, but not 2 week, type I diabetic animals (Albertoni Borghese et al., 2009). Although the early elevation in glucose transport may mediate protection of renal proximal tubules against glucose-induced cell damage, this contradicts data showing an increase in SGLT2 mRNA in alloxan-induced type I diabetes which is normalised after insulin treatment (Vestri et al., 2001; Freitas et al., 2008). Both SGLT1 and SGLT2 are up-regulated in proximal tubule cells from type II diabetic patients and Zucker diabetic rats (Rahmoune et al., 2005; Tabatabai et al., 2009). Increased sodium-dependent renal glucose transport was noted in experimental hypertension (Ikari et al., 2001); this raises the possibility that the transport response to diabetes may be a side-effect of the disease and not a direct effect of raised plasma glucose levels.
5.7.2.1.4.2  Involvement of PKC in glucose transporter expression

Five of the twelve known protein kinase C (PKC) isoforms, PKC-α, -βI, -βII, -δ and -ε, are detected in the proximal tubule; PKC-α and -βI are both expressed at high levels at the BBM of the S2/S3 segment of the proximal tubule (Dong et al., 1991; Pfaff et al., 1999; Kang et al., 1999) where, interestingly, PKC-α, -βI and -ε are up-regulated in proximal tubule cells from STZ-induced type I diabetic rats (Kang et al., 1999; Hsieh et al., 2006). In 1988, Hise et al suggested a role for PKC in the control of nutrient transport across the proximal tubule BBM (Hise and Mehta, 1988). An elevation in PKC activity at the proximal tubule BBM in rats was seen during growth accompanied by a decrease in cytoplasmic PKC activity, implying a movement of the enzyme from an intracellular to BBM location; furthermore PKC activity at the proximal tubule BBM has been shown to be elevated in STZ-induced type I diabetes (Hise et al., 1988; Hise and Mehta, 1988). PKC activity has been found to affect proline transport and the Na⁺/H⁺-exchanger at the proximal tubule BBM, as well as glucose transport (Weinman et al., 1989; Pahlavan et al., 1993; Zelikovic et al., 1995; Goestemeyer et al., 2007). Type I diabetes is associated with raised cytoplasmic levels of calcium (Ca²⁺) in proximal tubule cells due to the increased expression of L-type Ca²⁺ channels at the proximal tubule BBM which may be due to high luminal glucose concentrations (Marcinkowski et al., 1997; Park et al., 2001).

Goestemeyer et al have comprehensively shown the relationship between glucose transport and PKC-βI at the proximal tubule BBM (Goestemeyer et al., 2007). In response to either the stimulation of PKC by phorbol myristate acetate (PMA), or the release of Ca²⁺ from intracellular stores, via thapsigargin, the rate of GLUT-mediated glucose transport, at the proximal tubule BBM, was elevated whereas no effect was seen on SGLT-mediated glucose transport. Furthermore, the study shows that PKC-βI was the only PKC isoform to be significantly up-regulated at the proximal tubule BBM in response to STZ-induced type I diabetes, an effect which was reversed by reducing blood glucose levels to normal by overnight fasting or administration of nicotinamide. Thus, PKC-βI showed a strong positive correlation with both plasma glucose levels and GLUT2 expression at the proximal tubule BBM. Therefore, in diabetes-induced hyperglycaemia, elevated tubular fluid glucose concentrations might
result in recruitment of the high-capacity GLUT2 to the proximal tubule BBM, in a PKC-βI-dependent manner, leading to increased glucose entry into the cell (Figure 5.5) (Goestemeyer et al., 2007).

**Figure 5.5** – Schematic diagram of the sugar transporters involved in glucose reabsorption across the proximal tubule cell during diabetes. The high plasma glucose concentration, occurring in diabetes, results in an elevated tubular fluid glucose load, thus SGLT-mediated glucose transport is saturated and GLUT2 is recruited to the proximal tubule BBM, likely due to PKC-βI-mediated signalling. (Figure adapted from (Marks et al., 2003; Goestemeyer et al., 2007)). □ Glucose molecule  ● sodium ion  ● potassium ion.
5.7.2.2 Other factors affecting renal glucose transport

Many other factors apart from hyperglycaemia and diabetes have been shown to influence expression of GLUT2 and SGLT in the kidney cortex, some of which are outlined below. Renal denervation, to cause sympathetic hypoactivity, induced a reduction in renal sympathetic nervous activity which caused a decrease in renal cortical GLUT2 expression (Schaan et al., 2005). Conversely, the same study observed an elevation in GLUT2 expression in the renal cortex in spontaneously hypertensive rats, a model to assess sympathetic hyperactivity.

Another disease model which influences expression of GLUT2 is the X-linked renal tubular disorder, Dent’s disease; mice lacking the gene for the chloride channel ClC5, developed glycosuria as a result of depressed renal GLUT2 expression (Souza-Menezes et al., 2007).

Renal expression of GLUT2 can also be modulated by severe inflammation with a reduction in transporter expression after the injection of lipopolysaccharides (LPS), an effect which is abolished by glucocorticoid treatment (Schmidt et al., 2007). Indeed transcription of the serum and glucocorticoid-inducible kinase 1 (SGK1) is stimulated by both high glucose concentrations and diabetes (Lang et al., 2000; Saad et al., 2005). SGK1 stimulates proximal tubule glucose reabsorption in type I diabetic mice via an increase in SGLT1 activity (Ackermann et al., 2009).

PKC signaling strongly influences SGLT activity, which is likely to be mediated by the PKC-binding site in rabbit renal SGLT1 (Chalumeau et al., 1999). The vasoconstrictor angiotensin II inhibits SGLT1 and SGLT2 expression and glucose transport activity via a PKC-MAPK-cPLA2 signal cascade whereas adenosine binding to its receptor causes a PKC-dependent stimulation of sodium-dependent glucose transport in opossum kidney (OK) cells (Coulson et al., 1991; Han et al., 2004).

The transcription factor hepatocyte nuclear factor 1α (HNF1α) has been shown to influence levels of glucose transporters in the proximal tubule; HNF1α KO mice exhibit impaired proximal tubule reabsorption of glucose (Pontoglio et al., 1996).
Freitas et al found that HNF1α and 3β transcription factors were important in the modulation of renal GLUT2 gene expression (Freitas et al., 2009). The group noted that increases in GLUT2 mRNA in diabetes were mirrored by elevations in HNF1α and 3β whereas the reduction in GLUT2 gene expression in response to insulin therapy resulted in a down-regulation of the HNF transcription factors. Interestingly, HNF1α has also been shown to affect renal SGLT2 by binding DNA that encodes the sodium-dependent glucose transporter (Freitas et al., 2008). In diabetes, increased levels of both SGLT2 and HNF1α mRNA expression has been attributed to raised binding of nuclear proteins to the HNF1α consensus site; this response is likely to be mediated by plasma glucose since modulation of glycaemia by either phlorizin or insulin, resulted in normalised expression of SGLT2 and HNF1α mRNA (Freitas et al., 2008).

5.8 Glucose absorption across the small intestine

There are many similarities between glucose reabsorption across the proximal tubule and glucose absorption in the small intestine, for example the same GLUT and SGLT glucose transporter families mediate glucose uptake in both tissues. However in contrast to the kidney, intestinal glucose transport is influenced by dietary carbohydrate intake; indeed, dietary sugars provide a large proportion of the daily energy requirement of the body and therefore contribute strongly to glucose homeostasis. Intestinal glucose absorption has been studied to a greater degree then renal glucose reabsorption. Furthermore, the regulation of glucose absorption in the small intestine is important as dysregulation can occur in metabolic diseases such as diabetes.

The majority of studies on glucose transport in the small intestine are centred on the jejunum; this region is the principal site of absorption of carbohydrate digestion products. Furthermore, the jejunum is also involved in the terminal stages of digestion, for example dissacharidases such as maltase are present at the BBM of the jejunum (Ferraris and Diamond, 1992; Jang et al., 2000).
5.8.1 Before a meal

Figure 5.6 shows the classical model of glucose absorption across the enterocyte. Before a meal, when luminal glucose concentration is low, the sodium-dependent glucose transporter SGLT1 found at the jejunal BBM is responsible for glucose uptake (Kellett and Brot-Laroche, 2005). The high-affinity SGLT1 scavenges glucose from the lumen and cotransports sugar, against its concentration gradient, into the enterocyte with two Na⁺. Glucose exit from the enterocyte, across the BLM, is mediated by the facilitative glucose transporter GLUT2 (Thorens et al., 1990; Cheeseman, 1993). Under conditions of low luminal glucose concentrations, GLUT2 is also present at the jejunal BBM but in very low levels, furthermore, GLUT2 expression is abolished following an overnight fast (Gouyon et al., 2003). The intrinsic activity of GLUT2 at the jejunal BBM, under basal conditions, is low and therefore any glucose transported by the facilitative glucose transporter is rapidly utilised via the enterocyte glycolytic pathway (Levine et al., 1982).

5.8.2 Following a meal

There is an elevation in glucose uptake following either short-term luminal exposure to glucose or the feeding of a high-carbohydrate diet. Early studies by Windmueller & Spaeth showed that the jejunum of fed rats absorbed 97% of glucose perfused through the lumen (Windmueller and Spaeth, 1980). Transport studies showed that an increase in luminal glucose in vivo for 30 minutes caused an enhanced SGLT1-mediated glucose uptake, implying a dynamic response of the jejunum to manage dietary carbohydrates (Sharp et al., 1996). Karasov & Debnam found a 60% increase in carrier-mediated glucose absorption in the jejunum following exposure to luminal glucose, an effect not seen in other segments of the small intestine (Karasov and Debnam, 1987). Furthermore high-carbohydrate feeding over several days has been shown to stimulate intestinal glucose uptake (Figure 5.6) (Diamond et al., 1984; Solberg and Diamond, 1987).
**Figure 5.6** – Schematic diagram of the sugar transporters involved in glucose absorption across the enterocyte before and after a meal. A: Before a meal, where little or no dietary carbohydrate is present in the jejunal lumen, SGLT1 scavenges glucose from the lumen into the cell whilst GLUT2 and PKC-βII are expressed at very low levels at the BBM. At the BLM, GLUT2 balances glucose levels between the enterocyte and the blood. B: After a meal, raised luminal carbohydrates lead to increased glucose concentration at the BBM. This saturates SGLT1 and results in trafficking of GLUT2 to the BBM; in this position, GLUT2 transports the high load of glucose from the lumen into the cell (Figure adapted from (Kellett and Brot-Laroche, 2005)).

- Glucose molecule
- Carbohydrate molecule
- Sodium ion
- Potassium ion
- BBM hydrolase.
5.8.2.1 Breakdown of carbohydrates at the enterocyte BBM

Before they can be absorbed by the enterocyte, dietary carbohydrates are broken down by the action of luminal followed by membrane-bound hydrolytic enzymes, such as the intestinal BBM dissacharidases, isomaltase and sucrase, into the monosaccharides D-glucose, D-galactose and D-fructose (Goda et al., 1999; Kishi et al., 1999). Dietary fructose has been shown to modulate activity of dissacharidases; Goda et al found that ingestion of a high-starch diet elevates sucrase and lactase activity in the lower jejunum (Goda and Takase, 1994). Early studies showed that, following feeding, glucose concentration in the jejunal lumen reaches 50 mM in the rat and 75 mM in humans (Borgstrom et al., 1957; Murakami et al., 1977). However more recent studies by Ferraris et al have found more conservative concentrations of free glucose in the jejunal lumen of the rat, with ranges from 16-28 mM with peak postprandial concentrations reaching 48 mM (Ferraris et al., 1990).

5.8.2.2 Sodium-dependent glucose absorption into the enterocyte

The $K_T$ for the phlorizin-sensitive component of intestinal glucose transport, indicative of SGLT1-mediated transport, has been reported to be 4 mM measured in vitro (Debnam et al., 1988). However, in vivo studies have found the value to be 20 mM; thus SGLT1-mediated glucose transport becomes saturated at levels lower than the postprandial glucose concentration in the lumen (Debnam et al., 1988). At these concentrations, a diffusive component of glucose transport has been identified which, unlike SGLT-mediated transport, is non-saturable, phlorizin-insensitive and non-electrogenic, and was originally believed to represent paracellular movement (Kimmich, 1973; Murer and Hopfer, 1974; Debnam and Levin, 1975; Pappenheimer and Reiss, 1987; Pappenheimer, 1993). However the localisation of the facilitative glucose transporter GLUT2 and the elucidation that it was present at the intestinal BBM of STZ-induced type I diabetic rats led to the understanding that the diffusive component of glucose transport was GLUT2-mediated (Chowrimootoo et al., 1992; Corpe et al., 1996; Kellett, 2001).
5.8.2.3 GLUT2-mediated glucose absorption and its regulation

Following perfusion of jejunal loops with 75 mM glucose, GLUT2-mediated glucose absorption reaches a steady-state within 15 minutes; in fact, GLUT2 trafficking to and from the jejunal BBM can occur with a half-life time of 3.5 minutes (Kellett and Helliwell, 2000; Helliwell et al., 2003). Furthermore an elevation in GLUT2 transporter levels, at the jejunal BBM was seen with increasing luminal glucose concentrations (Kellett and Helliwell, 2000). At high glucose concentrations, approximately 75% of absorption was GLUT2-mediated (Kellett et al., 2008). Interestingly, the inhibition of SGLT1, with phlorizin, has been shown to down-regulate jejunal GLUT2-mediated transport (Kellett and Helliwell, 2000). As the luminal glucose concentration decreases, GLUT2 is trafficked away from the BBM to restore basal low levels of the transporter (Kellett and Brot-Laroche, 2005).

The insertion of GLUT2 into the BBM is regulated not only in the short-term, by high luminal glucose, but also by long-term dietary changes. Wild type mice receiving a complex diet of low-glycaemic index carbohydrates were found to have reduced GLUT2 expression at the BBM (Gouyon et al., 2003). In the same study, mice given a diet that was rich in high-glycaemic index carbohydrates were shown to have increased expression of GLUT2 at the intestinal BBM and augmented plasma glucose levels, thus indicating the role of the facilitative transporter in glucose absorption from lumen to circulation.

5.8.2.4 PKC-βII and calcium signalling

The influence of SGLT-mediated glucose transport on expression of GLUT2 at the jejunal BBM has been well-established; studies have shown that PKC-βII modulates this effect.

PKC-βII is regulated by SGLT-mediated glucose transport and the recruitment of GLUT2 to the jejunal BBM correlates with PKC-βII activation (Helliwell et al., 2000; Kellett and Helliwell, 2000). PKC-βII is activated by increased levels of intracellular Ca\(^{2+}\), which cross the BBM via Ca\(_{\text{v}1.3}\), the L-type calcium channel at the jejunal BBM (Morgan et al.,
In addition to PKC-βII activation, Ca$^{2+}$ absorption depolarises the BBM causing the recruitment of GLUT2 to the membrane where it up-regulates glucose absorption (Hug and Sarre, 1993; Morgan et al., 2007). Thus after a meal, the large transepithelial gradient causes Ca$^{2+}$ transport across the BBM, via Ca$_v$.1.3, leading to elevated glucose absorption. In addition, high cellular Ca$^{2+}$ levels activate the myosin light chain kinase (MLCK); MLCK mediates phosphorylation of the regulatory light chain (RLC$_{20}$) of myosin II within the perijunctional actomysin ring and results in cytoskeletal rearrangement (Turner et al., 1999; Berglund et al., 2001; Clayburgh et al., 2004). The movements in the cytoskeleton assists the translocation of GLUT2 from intracellular vesicles to the intestinal BBM (Figure 5.7) (Mace et al., 2007).

**Figure 5.7** – Original model of the regulation of GLUT2 recruitment to the enterocyte BBM: SGLT1-mediated glucose transport at the enterocyte BBM causes membrane depolarisation; as a result, the calcium channel Ca$_v$.1.3 is activated and an elevation in uni-directional calcium influx occurs. The rise in intracellular calcium stimulates the myosin light chain kinase (MLCK) which stimulates rearrangement of the terminal web and cytoskeleton; these events are known to stimulate the movement of GLUT2-containing vesicles to the enterocyte BBM via PKC-II activation (Figure adapted from (Morgan et al., 2007; Mace et al., 2007)).
5.8.3 Sweet taste receptors in the enterocyte

The absorption of Ca²⁺ across the BBM reaches maximal levels at 20 mM glucose, when GLUT2 levels are unchanged at the BBM, whereas at glucose levels above 20 mM, GLUT2-mediated absorption is unaffected by increasing luminal calcium levels (Morgan et al., 2003; Mace et al., 2007). Therefore, although luminal calcium is a necessary signal, there is a second downstream signal required for GLUT2 insertion at the jejunal BBM which operates at higher glucose concentrations than those influencing Ca²⁺ movement.

Studies to elucidate the second signal required to recruit GLUT2 to the jejunal BBM implicate sweet taste receptors, members of the G-protein-coupled receptor family. These taste sensors T1R2 and T1R3 form a sweet taste heterodimer T1R2/3, that detects simple sugars, sweet proteins and artificial sweeteners (Nelson et al., 2001; Li, 2009). The glucose concentration range of the second pathway for GLUT2 expression at the enterocyte BBM matches that for T1R2/3 activation in response to simple sugars (Li et al., 2002; Mace et al., 2007). Furthermore, studies indicate the presence of a luminal sugar sensor located at the BBM which is able to initiate a signalling pathway, independent of glucose metabolism, to cause enhanced SGLT1 expression in the small intestine (Dyer et al., 2003).

The components of the sweet taste heterodimer, and its major signalling molecule, the gustducin α-subunit, have been located along the length of the mouse and rat small intestine (Hofer et al., 1999; Dyer et al., 2005). However there remains some controversy over the location of sweet taste receptors in the small intestine; Mace et al identified the clear distribution of receptors on the enterocytes, brush cells and Paneth cells whereas Margolskee et al noted expression solely on enteroendocrine cells (Mace et al., 2007; Margolskee et al., 2007). These discrepancies may be due to the differing artificial sweeteners used to elicit a response and the varying antibodies used to study localisation. Margolskee et al found that both sugars and non-metabolisible artificial sweeteners promoted SGLT1 expression and caused a cAMP-dependent rise in glucose absorption across the BBM which was absent in T1R3 or Ga-gustducin KO mice (Dyer et al., 2003; Margolskee et al., 2007). Stearns et al found that both fructose and glucose were able to stimulate SGLT1 expression, despite the
transporter being unable to transport fructose (Stearns et al., 2010). Furthermore this effect was also seen using artificial sweetener saccharin, with the response occurring after 3 hours, similar to the time needed for SGLT1 synthesis (Stearns et al., 2010).

In contrast, up-regulation of GLUT2 at the jejunal BBM, at low glucose concentration required only 15 minutes exposure to 1 mM sucralose; this was similar to that seen following a high luminal glucose load (Mace et al., 2007). Within this short time frame, SGLT1 expression was unaffected by exposure to the artificial sweetener (Mace et al., 2009; Stearns et al., 2010). The artificial sweeteners saccharin and acesulfame K are also able to increase GLUT2 recruitment to the BBM, as well as GLUT2-mediated glucose absorption, in parallel with elevated levels of cellular calcium (Li et al., 2002; Mace et al., 2007).

Although the effects of T1R2/3 activation on SGLT1 need further studies, the effect on GLUT2 recruitment to the jejunal BBM has already been hypothesised. Mace et al propose that the activation of T1R2/3 by high glucose concentrations or low artificial sweetener plus low glucose concentration, results in the conversion of GDP to GTP and thus the dissociation and release of α-gustducin from its G-protein heterodimer and into the cytoplasm (Figure 5.8) (Mace et al., 2009). The remaining BBM-bound βγ-subunits of the G-protein activate PLCβ2, resulting in movement of inactive PLCβ2 to the BBM; this results in the IP3-dependent production of DAG, a messenger molecule which activates PKC-βII (Stehno-Bittel et al., 1995; Huang et al., 1999; Rhee, 2001). Although intracellular calcium is able to promote PKC-βII insertion at the BBM, the generation of DAG, via PLCβ2 activation, results in complete activation of PKC-βII which stimulates GLUT2 insertion at the jejunal BBM (Helliwell et al., 2000; Helliwell et al., 2000; Wright et al., 2003; Helliwell et al., 2003).

Another consequence of T1R2/3 activation is the release of cAMP. Cytosolic α-gustducin, released from the G-protein heterodimer, activates adenyl cyclase causing an elevation in cAMP (Gilbertson et al., 2000; Bezencon et al., 2007). This may explain the longer term cAMP-mediated synthesis of SGLT1 (Stearns et al., 2010). Thus it is likely that the rapid induction of GLUT2 in response to stimulation by artificial sweeteners is PLCβ2-mediated, unlike the longer-term alterations in SGLT1 synthesis which are cAMP-mediated.
Figure 5.8 – The signalling cascade activated by the stimulation of the sweet taste heterodimer; on binding of simple sugars or artificial sweeteners to T1R2/3, the gustducin heterodimer converts GDP to GTP and the α-gustducin subunit is released into the cytoplasm whereas the βγ-subunits remain membrane-bound. The dissociation of the gustducin heterodimer activates phospholipase β2 (PLCβ2) which stimulates the activation of PKC-βII at the enterocyte BBM (Figure adapted from (Stehno-Bittel et al., 1995; Rhee, 2001; Mace et al., 2009))
5.8.4 Revised model of glucose absorption across the enterocyte

Therefore the original model for regulation of glucose absorption across the enterocyte BBM has been updated and is shown in Figure 5.9. In addition to causing cytoskeletal rearrangement, which results in the release of GLUT2 from the terminal web, Ca\(^{2+}\) accumulation within the enterocyte also stimulates a common cellular pool of PKC-βII to translocate to the BBM. In this location, PKC-βII needs to be activated; sweet taste receptor agonists such as artificial sweeteners or simple sugars, at critical concentrations are able to activate T1R2/3. This causes an α-gustducin-mediated signalling pathway resulting in PLCβ2-dependent DAG generation, which phosphorylates PKC-βII. In this active state, the protein kinase isoform is able to recruit GLUT2 from cellular vesicles to the BBM. This signalling pathway enables the enterocyte to rapidly modulate the process of glucose absorption in response to changes in dietary carbohydrate load.

The implications of the involvement of sweet taste receptors are vast, especially in light of the widespread use of artificial sweeteners and HFCS in the western diet, outlined in section 5.4.1 and 5.4.2. The consumption of a high-fructose meal results, within minutes, in the trafficking of GLUT2 to the enterocyte BBM and is a far more effective stimulant than sucrose or glucose (Gouyon et al., 2003). Thus the rapid elevation in a high-capacity process for glucose absorption, following stimulation of the intestinal sweet taste receptor heterodimer, provides an explanation for the excess weight gain and onset of type II diabetes noted in response to consumption of HFCS and artificial sweeteners (Stellman and Garfinkel, 1986; Fowler et al., 2008; Swithers et al., 2010; Bocarsly et al., 2010; Hu et al., 2010).
**Figure 5.9** – Revised model of the regulation of GLUT2 recruitment to the enterocyte BBM, mediated by sweet taste receptors and intracellular calcium. Diagram on the left shows the recruitment of PKC-βII to the enterocyte BBM via calcium influx into the cell, however the activation of PKC-βII requires a secondary stimulus from sweet taste receptor activation. Diagram on the right shows, on stimulation, the PLCβ2-dependent activation of PKC-βII causes the recruitment of GLUT2 to the enterocyte BBM (Figure adapted from (Mace et al., 2009)).
5.8.5 Glucose transport at the small intestine in diabetes

Abnormal regulation of intestinal glucose transporters occurs in response to metabolic diseases such as insulin-dependent and non-insulin-independent diabetes, as well as insulin-resistant models such as obesity and metabolic syndrome. These conditions can result in elevated glucose delivery to the blood which will exacerbate hyperglycaemic conditions.

Both type I and type II diabetes increase the rate of glucose absorption; this correlates with the increased activity of the disaccharidases and α-glucosidases as well as hyperplasia and hypertrophy of the intestinal epithelium (Stenling et al., 1984; Debnam et al., 1990; Zoubi et al., 1995; Fujita et al., 1998; Adachi et al., 2003). Thus the small intestine adapts to diabetes via increased hydrolysis of carbohydrates, as well as elevated recruitment of brush-border glucose carriers, which has been shown to occur in the enterocytes in the midvillus-to-tip region (Fedorak et al., 1991). In fact, the trafficking of glucose transporters has been shown in both type I diabetes and hyperglycaemia induced by IV glucose infusion (Maenz et al., 1986).

BBM hyperpolarisation, as a result of decreased Na\(^+\) permeability, occurs in early type I diabetes and increases the driving force for SGLT1-mediated glucose transport (Debnam et al., 1988; Sharp et al., 1997). In agreement with this data, up-regulation of SGLT1 mRNA, protein and capacity for glucose transport has been established at the enterocyte BBM in type I diabetes (Debnam et al., 1990; Fedorak et al., 1991; Ferraris et al., 1993; Burant et al., 1994; Sharp et al., 1997). A possible signal for this up-regulation has been presented by Wong et al, who suggested that a down-regulation of the RAS in type I diabetes reduces the inhibitory effect of angiotensin II on SGLT1, culminating in an increase in SGLT-mediated glucose transport (Wong et al., 2009).
The elevated SGLT1 expression is paralleled by augmented GLUT2 levels at both the BBM and BLM in type I diabetes and hyperglycaemia induced by IV infusion of glucose (Csaky and Fischer, 1981; Corpe et al., 1996; Le et al., 2007; Wong et al., 2009). However, prolonged type I diabetes is associated with decreased GLUT2 expression at the BLM (Burant et al., 1994; Wong et al., 2009). In addition, expression of both GLUT1 and GLUT5 at the BBM are up-regulated in diabetes, with levels of GLUT5 at the BLM also elevated (Burant et al., 1994; Boyer et al., 1996; Corpe et al., 1996). Corpe et al found a reduction in intestinal fructose uptake measured in vivo in type I diabetes (Corpe et al., 1996); this discrepancy between GLUT5 expression and fructose uptake studies during diabetes requires further investigation.

In contrast to type I diabetes, far fewer studies have been performed on insulin-independent diabetes. However, it is known that type II diabetic patients exhibit increased expression of SGLT1 and GLUT5 transporters at the enterocyte BBM and up-regulated GLUT2 mRNA expression (Dyer et al., 2002).

In response to insulin secretion following a meal, the insulin receptor at the enterocyte BLM is activated; this signals the internalisation GLUT2 from both the BBM and BLM of the enterocyte and thus limits glucose absorption from the diet (Buts et al., 1997; Tobin et al., 2008). However, in diabetes, this protection mechanism to prevent excess glucose absorption following a carbohydrate-rich meal is overridden by elevated expression of GLUT2 at the BBM, resulting in hyperglycaemia. Insulin resistance, induced via high-fructose feeding, results in the loss of control of GLUT2 trafficking and therefore GLUT2 is permanently expressed at the BBM, an effect which was unchanged by overnight fasting or IV infusion of insulin (Tobin et al., 2008).
5.8.5.1 Other regulators of intestinal glucose absorption

In addition to the effects of hyperglycaemia on glucose absorption specific regulators of glucose transporters at the BBM and BLM of the enterocyte have been postulated.

5.8.5.1.1 Flavonoids

The flavonoid quercetin has been shown to abolish GLUT2 overexpression at the BBM and non-competitively inhibit GLUT2-mediated glucose and fructose transport (Kwon et al., 2007). Additionally, the administration of quercetin and glucose to Zucker diabetic rats significantly blunted postprandial hyperglycaemia (Song et al., 2002). Thus quercetin could represent a therapeutic method for reducing sugar absorption in metabolic syndrome and type II diabetes (Mooradian and Thurman, 1999).

5.8.5.1.2 Stages of development

During the first 24-30 days of age, until the completion of weaning, a low abundance of GLUT5 has been noted in rat enterocytes; however if fed a high-fructose diet, animals show increased GLUT5-mediated fructose absorption at a younger age (Davidson et al., 1992; Castello et al., 1995; David et al., 1995; Shu et al., 1997; Reimer et al., 1997). In contrast, SGLT1 levels at the BBM remain constant from birth with no early postnatal developmental changes (Buddington and Diamond, 1989; Shu et al., 1997; Jiang et al., 2001). Intestinal GLUT2 mRNA is expressed on day 16 after conception and expression remains constant in the neonatal and adult rat with the transporter induced by both luminal glucose and fructose (Cui et al., 2003).
5.8.5.1.3 Stress

Environmental stress on rats has been found to reduce GLUT2-mediated glucose absorption across the enterocyte BBM, without influencing SGLT1 expression or SGLT-mediated glucose transport (Shepherd et al., 2004). In addition, the technique employed to study glucose absorption can influence GLUT2-mediated glucose transport. Helliwell et al found that high-perfusion rates during experimentation can alter GLUT2-mediated glucose absorption (Helliwell and Kellett, 2002). This may be an explanation of why the GLUT2 component of glucose uptake is more readily detected using in vivo rather than in vitro techniques. Interestingly, water avoidance stress, inflicted by placing rats on a platform surrounded by water for 1 hour a day, resulted in elevated GLUT2 abundance and glucose transport across the enterocyte BBM (Boudry et al., 2007). However, in this model of psychological stress, SGLT-dependent glucose absorption at the enterocyte BBM was down-regulated (Boudry et al., 2007).

Chronic starvation in rats caused an increase in SGLT-mediated glucose absorption across the BBM, to enable more efficient uptake of glucose from low levels of glucose within the lumen (Habold et al., 2005). Upon refeeding, GLUT2 levels at the BBM increased rapidly to enable efficient glucose absorption from higher levels within the lumen.

5.8.5.1.4 AMPK

Under metabolic stress, such as energy depletion in long-term starvation, the AMP-activated protein kinase (AMPK) is activated by AMP-binding, as well as via ATP-independent signalling pathways (Stein et al., 2000; Kemp et al., 2003). The activation of AMPK resulted in an elevation in net glucose transport, across the jejunum, which was GLUT2-mediated (Walker et al., 2003). A recent study by Sopjani et al showed that AMPK-stimulated Na⁺-coupled glucose transport occurred in Xenopus oocytes and elevated SGLT1 expression at the BBM of Caco-2 cells (Sopjani et al., 2010). The augmentation of SGLT1-mediated glucose transport
occurred despite the low cellular ATP levels available to drive the basolateral $\text{Na}^+$/K$^+$-ATPase (Woollhead et al., 2007; Vadasz et al., 2008).

Interestingly, the activation of AMPK also maintains glucose homeostasis in many tissues; therefore, AMPK activators, such as amino imidazole carboxamide ribonucloetide (AICAR), have been proposed as effective treatment in metabolic disorders. Indeed PPARγ activators rosiglitazone and pioglitazone, which activate AMPK by elevating the intracellular AMP:ATP ratio, are current drugs in the treatment of type II diabetes (section 5.2.5.2.1) (Fryer et al., 2002; Saha et al., 2004).

5.8.5.1.5 RELMβ

Resistin-like molecule β (RELMβ) is released from adipocytes to impair insulin signalling via MAPK activation and influences lipidaemia and inflammation; thus the regulation of RELMβ has been indicated in obesity, metabolic syndrome and type II diabetes (Steppan et al., 2001; Kushiyama et al., 2005; Kusminski et al., 2005). RELMβ is also expressed in the small intestine where it effects goblet cell secretion and is strongly influenced by nutrient intake (Gerstmayer et al., 2003). In response to a high-carbohydrate diet, RELMβ expression is down-regulated whereas RELMβ expression is up-regulated by insulin treatment (Fujio et al., 2008). Interestingly, RELMβ is also up-regulated in high-fat fed or type II diabetic rat models (Shojima et al., 2005).

Krimi et al reported that oral administration of RELMβ resulted in hyperglycaemia caused by augmented GLUT2-mediated glucose absorption across the jejunal BBM; interestingly, the accompanying elevation in GLUT2 expression at the BBM was associated with PKC-βII activation and AMPK phsophorylation (Krimi et al., 2009). In contrast, the study also showed a reduction in SGLT1 abundance and SGLT-mediated glucose transport at the jejunal BBM in response to luminal exposure to RELMβ (Krimi et al., 2009). Thus RELMβ in the lumen of the small intestine elevates glucose absorption, whereas high-glucose in the lumen down-regulates intestinal expression of RELMβ.
Incretins are gut-derived hormones which have been found to exhibit regulation of glucose absorption. Nutrient intake and delivery are the primary stimulators of incretin secretion, with glucose being one of the most potent stimulators in the release of the enteric hormones GLP-1 and GIP (Roberge and Brubaker, 1991; Elliott et al., 1993; Rocca and Brubaker, 1999; Holst, 2007; Tolhurst et al., 2009).

In response to an oral glucose load, GIP and GLP-1 levels are elevated but not in response to plasma glucose stimulus, therefore there is a luminal sensor for glucose which triggers incretin secretion (Unger et al., 1968). SGLT1-dependent glucose transport stimulates the release of GLP-1 and GIP (Cheeseman, 1997).

GLP-2, another incretin, promotes SGLT1 expression at the small intestinal BBM within an hour (Cheeseman, 1997). Circulating GLP-2 also increases GLUT2-mediated transport at the BLM (Cheeseman and Tsang, 1996; Cheeseman and O'Neill, 1998). Au et al showed that levels of GLUT2 at the jejunal BBM are augmented in response to circulating GLP-2 (Au et al., 2002). GLP-1 and GLP-2 release from L-cells in the ileum is promoted by GIP, which is released from K cells, promoted by luminal glucose in the duodenum and jejunum (Crouzoulon and Korieh, 1991; Inukai et al., 1995; Limb et al., 1997). Therefore GLP-2 release may require the presence of glucose in the intestinal lumen and thus elevated GLUT2-mediated glucose absorption occurs in response to secretion of the incretin.

In addition, the effect of incretins on glucose absorption across the BBM may be mediated by sweet taste signalling, which has already been described in association with jejunal glucose transport (section 5.8.3). Jang et al found that the taste signalling elements involved in sweet taste, such as T1R2, T1R3 and PLC-β2, are co-expressed with GLP-1 in duodenal cells (Jang et al., 2007). Furthermore, the study also showed that α-gustducin KO mice display impaired GLP-1 secretion in response to luminal glucose with reduced α-gustducin expression causing a decrease in glucose-mediated GLP-1 secretion; interestingly, the artificial sweetener sucralose has been shown to
induce GLP-1 secretion from L-cells in a concentration-dependent manner (Jang et al., 2007). Thus the regulation of GLUT2 and SGLT1 expression involves a complex feedback pathway with incretin secretion to modulate glucose absorption across the enterocyte.

5.8.5.1.6.2 Leptin

Following food consumption, leptin is released from both adipocytes and the stomach, into the circulation and small intestine respectively (Zhang et al., 1994; Ahima and Flier, 2000; Cammisotto et al., 2007). The leptin receptor (Ob-R) is expressed at both the BBM and the BLM of enterocytes in the small intestine and, on binding with luminal leptin, SGLT-1 mediated absorption is rapidly inhibited (Hoggard et al., 1997; Lostao et al., 1998; Bado et al., 1998). This response has been suggested to delay the entry of sugars into the enterocyte, thus enabling the cell to process glucose (Barrenetxe et al., 2004). In contrast, *in vitro* studies by Sakar *et al* showed that luminal leptin, acting on the jejunal BBM, elevates the transport activity of GLUT2 and GLUT5, via Ob-R-coupled activation of PKC-βIII (Sakar *et al*., 2009). Furthermore, the group found that consumption of a high-fructose diet triggers the release of gastric leptin which up-regulates GLUT2 and GLUT5-mediated fructose absorption (Sakar *et al*., 2009). Thus further studies are necessary to elucidate the physiological relevance of postprandial leptin release on elevated GLUT-mediated absorption and down-regulated SGLT-mediated transport across the enterocyte.
5.9 Aims of thesis

Diabetic nephropathy occurs as a consequence of hyperglycaemia-induced renal cell damage with tubular injury in the disease postulated to be the result of elevated GLUT-mediated glucose entry into proximal tubule cells, in a manner similar to that seen in mesangial cells (Brosius and Heilig, 2005). Previous studies have established that in type I diabetes PKC-βI-dependent GLUT2 recruitment to the proximal tubule BBM occurs as a direct result of hyperglycaemia, resulting in up-regulated glucose transport across this membrane (Marks et al., 2003; Goestemeyer et al., 2007). However type II diabetes is the prevalent form of the disease in which sufferers develop insulin-resistance; therefore type II diabetes presents an interesting model to characterise the factors modulating glucose reabsorption across the proximal tubule cell in the presence of a basolateral insulin stimulus. The development of type II diabetes is strongly linked to the current epidemic of obesity and metabolic syndrome; consequently, it was important to characterise glucose transport across the proximal tubule BBM in rodent models of metabolic dysregulation. In addition, the novel modulation of renal glucose transport across the BBM, by sweet taste receptors, was studied using recently characterised mediators of jejunal glucose transport as a model, with the aim of identifying targets in the treatment for diabetes.
6 Materials and Methods
6.1 Short-term intravenous (IV) infusions

Male Sprague-Dawley rats (250-300 g) were anaesthetised with 60 mg.kg$^{-1}$ intraperitoneal (IP) pentobarbitone sodium (Pentoject, Animalcare Ltd, York, UK) and maintained at 37$^\circ$C with a thermostatically-maintained heat blanket (Harvard Apparatus Ltd, Kent, UK). A catheter was placed in the bladder and the jugular vein was cannulated for administration of anaesthetic and infusate. The femoral artery was cannulated for blood sample removal, which was assessed for plasma glucose concentration. Animals were infused with saline (154 mM NaCl), at a rate of 5.5 ml.hr$^{-1}$, for 1 hour after cannulations. After the ‘rest’ period, animals were infused with saline or saline with 0.08 M mannitol, 1.09 M mannitol, 1.1 M glucose or 1.37 mM saccharin for 1 or 2 hours. 2 hour infusions were begun with a 3 ml.min$^{-1}$ bolus into the jugular vein. Blood glucose concentration was measured every 15 minutes with Accucheck Active (Roche Diagnostics, West Sussex, UK) and urine was collected every 15 minutes to assess urine flow rate and glucose concentration. At the end of the experiment, 2 ml blood was collected from the femoral cannulae, the kidneys were harvested, sliced into 2 mm sections and the cortex was dissected away for brush border membrane (BBM) vesicle preparation. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986.

6.2 Animal models

6.2.1 Insulin-dependent diabetes mellitus (IDDM)

Type I diabetes was induced in male Sprague-Dawley rats (250-300 g) by administering a single tail vein injection of 55 mg.kg$^{-1}$ streptozotocin (STZ) (Sigma, Dorset, UK) under halothane anaesthesia (2% halothane in 100% oxygen). STZ was prepared in 0.05 M citrate buffer (pH 4.5) and control animals were administered with citrate buffer only. Animals were allowed ad libitum access to a standard rat chow (diet RM1, SDS Ltd, Witham, Essex, UK) and water, until time of experimentation (between 2-30 days). For all experimental procedures, animals were terminally
anaesthetised with 60 mg.kg\(^{-1}\) IP pentobarbitone sodium (Pentoject, Animalcare Ltd, York, UK) before cardiac puncture, urine collection and removal of kidneys for BBM vesicle preparation and mRNA expression. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986.

### 6.2.2 Non insulin-dependent diabetes mellitus (NIDDM)

Type II diabetes was studied in the non-obese, non-hypertensive Goto-Kakizaki (GK) rat (Goto et al., 1976). The model was developed spontaneously by the repeated inbreeding of glucose-intolerant Wistar rats, over several generations (Charles River, USA). Rats exhibit fasting hyperglycaemia, hepatic and peripheral insulin-resistance and polyuria.

Lean Wistar rats and type II diabetic GK rats were used at 8-9 weeks of age and allowed *ad libitum* access to a standard rat chow (diet RM1, SDS Ltd, Witham, Essex, UK) and water, until time of experimentation. For all experimental procedures, GK animals were terminally anaesthetised with 60 mg.kg\(^{-1}\) IP pentobarbitone sodium (Pentoject, Animalcare Ltd, York, UK) before cardiac puncture, urine collection, removal of kidneys and small intestine segments for BBM vesicle preparation. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986.

### 6.2.3 High-fat diet

A lipid-rich chow was used as a model of high-fat diet-associated insulin-resistance. Wistar rats were allowed *ad libitum* access to a 60% fat-as-calories chow (Research Diets, New Brunswick, USA), over a 5-week period. Control Wistar animals were allowed *ad libitum* access to a standard rat chow (Lillico, Betchworth, UK) and water, over a 5-week period. Animals were housed in cages of 4 and fasted overnight, prior to cull; they were terminally anaesthetised with CO\(_2\) and decapitated before cardiac puncture, urine collection, and removal of kidneys for BBM vesicle preparation. All
procedures were carried out at the Royal Veterinary College with the assistance of Dr. M. Cleasby, in accordance with the Animals (Scientific Procedures) Act 1986.

### 6.2.4 Junk-food diet

Wistar rats were allowed *ad libitum* access to water and a balanced standard rat chow diet (RM3, SDS Ltd, Betchworth, Surrey, UK) in the case of control animals or chow and junk-food in the case of experimental animals (Rothwell and Stock, 1979; Bayol *et al.*, 2005). The junk-food diet consisted of an *ad libitum* choice of palatable, processed foods with a high fat and/or high sugar content including muffins, jam doughnuts, biscuits, cheese, marshmallows, potato crisps and chocolate bars (nutritional information, Table 6.1). Chow-fed animals consumed 17.5 kcal (73.2 kJ) per day whereas the junk-food animals consumed 198 kcal (830.6 kJ).

**Table 6.1 - Nutritional Information for chow and junk-food diet given to animals over an 8 week period: components of the diet are expressed as a percentage of the total diet offered to rats**

<table>
<thead>
<tr>
<th></th>
<th>Chow</th>
<th>Junk-food</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein (%)</strong></td>
<td>77.4</td>
<td>4.4</td>
</tr>
<tr>
<td><strong>Carbohydrates (%)</strong></td>
<td>3.7</td>
<td>35.9</td>
</tr>
<tr>
<td><strong>Of which sugars (%)</strong></td>
<td>2.1</td>
<td>15.4</td>
</tr>
<tr>
<td><strong>Fat (%)</strong></td>
<td>0.8</td>
<td>19.6</td>
</tr>
<tr>
<td><strong>Of which saturates (%)</strong></td>
<td>15.7</td>
<td>4.6</td>
</tr>
<tr>
<td><strong>Fibre (%)</strong></td>
<td>0.2</td>
<td>1.5</td>
</tr>
<tr>
<td><strong>Sodium (%)</strong></td>
<td>0</td>
<td>18.6</td>
</tr>
</tbody>
</table>

Animals were housed in cages of 3. Daily food intake of both chow and junk-food was measured, as well as weekly body weight measurements, for the 8 week experimental period. Unfasted rats were terminally anaesthetised with 60 mg.kg⁻¹ IP pentobarbitone sodium (Pentoject, Animalcare Ltd, York, UK) before cardiac
puncture, urine collection, removal of kidneys for BBM vesicle preparation. All procedures were carried out at the Royal Veterinary College with the assistance of Dr. S. Wildman, in accordance with the Animals (Scientific Procedures) Act 1986.

6.2.5 Roux-en-Y Gastric Bypass (RYGB) surgery

Gastric bypass surgery was studied using the RYGB model in control Wistar rats, performed by Dr. M Bueter at Imperial College London. RYGB surgery was performed by transecting the stomach close to the junction with the oesophagus to create a small gastric pouch which was then anastomosed to a loop of jejunum distal to the pylorus in an end-to-side fashion. A small bowel anastomosis was performed between the biliopancreatic (duodenum) and alimentary (jejunum) limbs to create a common channel. The sham operation consisted of a laparotomy on the stomach wall and jejunum which was subsequently closed. Both gentamicin and carprofen were administered preoperatively as IP for the treatment of post-operative infection and pain relief (Bueter et al., 2010).

Animals were maintained for 60 days with *ad libitum* access to chow and fasted overnight, prior to cull. Rats were terminally anaesthetised with CO₂ before cardiac puncture, urine collection, and removal of kidneys and small intestine for BBM vesicle preparation and analysis of mRNA expression. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986.

6.3 LLC-PK₁ cell culture

Porcine renal epithelial LLC-PK₁ cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (FBS), 4 mM glutamine, 0.1 mg.ml⁻¹ streptomycin sulfate and 100 U.ml⁻¹ penicillin G. Cells were maintained at 37°C in a 5% CO₂ atmosphere and, at confluence, washed with ice-cold phsophate-
buffered saline (PBS) and harvested with RIPA buffer (25 mM Tris HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS, protease inhibitors, pH 7.4). For harvesting, 1 ml of RIPA buffer, per 75 cm$^2$ flask containing 5 x 10$^6$ cells, was added to cells for 5 minutes on ice. Cells were then scraped from the flask and centrifuged for 10 minutes at 13 000 rpm to pellet cell debris.

6.4 Membrane preparation

6.4.1 Whole membrane preparation

Whole membrane was prepared from the kidney cortex and jejunal mucosa, from male Sprague-Dawley rats and C57BL/6 mice. Tissue was homogenised three times for 20 seconds at setting 5, with an Ultra Turrax homogeniser (Janke & Kunkel, IKA-Labortechnik, Germany) in buffer (50 mM mannitol, 2 mM HEPES, protease inhibitors, pH 7.4) used at 10 ml per 1 g tissue. Homogenate was centrifuged for 15 minutes at 3500 rpm and the supernatant fraction was then centrifuged for 30 minutes at 10 500 rpm and resuspended in ~ 500 μl buffer using a 21-gauge needle and syringe. All procedures were carried out at 4°C.

6.4.2 Renal BBM vesicle preparation

BBM vesicles were prepared using a double Mg$^+$ chelation protocol, previously described by Biber et al (Biber et al., 1981). Renal cortical fragments were pooled and homogenised for 2 minutes at setting 5, with an Ultra Turrax homogeniser (Janke & Kunkel) in 30 ml R1 buffer (300 mM mannitol, 12 mM Tris HCl, 5 mM EGTA and protease inhibitors, pH 7.4). Deionised water (42 ml) was added to the homogenate and stirred, on ice, for 15 minutes with 12 mM MgCl$_2$ before centrifugation for 15 minutes at 4500 rpm. The supernatant fraction was centrifuged for 30 minutes at 16 000 rpm and the pellet was re-suspended in 20 ml R2 buffer (150 mM mannitol, 6 mM Tris HCl, 2.5 mM EGTA and protease inhibitors, pH 7.4) with a hand-held Teflon homogeniser. The re-suspensions were stirred for 15 minutes with 12 mM MgCl$_2$ and centrifuged at the low- and high-speed spins outlined above. The resulting
pellet was re-suspended in 20 ml R3 buffer (300 mM mannitol, 12 mM Tris HCl, 2.5 mM EGTA and protease inhibitors, pH 7.4) with a hand-held Teflon homogeniser. The solution was centrifuged for 30 minutes at 16 000 rpm and the pellet re-suspended in ~ 500 μl R3 buffer using a 21-gauge needle and syringe. All procedures were carried out at 4°C.

6.4.3 Small intestinal BBM vesicle preparation

Intestinal segments were opened longitudinally and the mucosa was scraped off the small intestine walls using glass slides. Mucosa was suspended in homogenising buffer (50 mM mannitol, 2 mM HEPES and protease inhibitors, pH 7.4) and homogenised three times for 20 seconds at setting 5, with an Ultra Turrax homogeniser (Janke & Kunkel). The homogenate was then stirred, on ice, for 20 minutes with 10 mM MgCl₂ before centrifugation for 10 minutes at 6000 rpm. The supernatant fraction was centrifuged for 30 minutes at 18 000 rpm and the pellet was suspended in re-suspension buffer (300 mM mannitol, 20 mM HEPES, 0.1 mM MgSO₄ and protease inhibitors, pH 7.4) by passing six-times through a 21-gauge needle. The re-suspension was centrifuged for 15 minutes at 8500 rpm and the resulting supernatant was centrifuged at 18 000 rpm for 30 minutes. The purified BBM pellet was finally re-suspended in resuspension buffer using a 21-gauge needle and syringe. All procedures were carried out at 4°C.

The purity of BBM vesicles was assessed using the alkaline-phosphatase assay (section 6.7) for BBM enrichment and Bradford assay (section 6.6) for protein concentration.
6.5 Renal glucose uptake studies

6.5.1 Validation of BBM vesicles for glucose uptake studies

To ensure the BBM vesicles were not ‘leaky’ and were able to transport glucose effectively, various methods were employed to validate the glucose uptake capacity of the vesicles.

6.5.1.1 Intravesicular volume

One such method is the assessment of the intravesicular space of the BBM vesicles. This can be established after vesicles have been exposed to 100 μM glucose and uptake buffer for 20 minutes. At this point, the vesicles have reached equilibrium and the volume accumulated within the vesicle can be measured using the equation below:

\[
\text{Glucose uptake (pmol.mg protein}^{-1}) = \frac{[\text{Glucose}] (\mu M)}{}
\]

6.5.1.2 Time-dependent overshoot

To verify the ability of BBM vesicles to accumulate glucose, sodium-dependent glucose transport was determined at 100 μM glucose concentration, with or without phlorizin. An early peak in glucose uptake has been previously shown (Marks et al., 2003), where there is a rapid influx in Na\(^+\) ions into the BBM vesicles. This SGLT-mediated Na\(^+\) uptake co-transports glucose into the vesicles and is blocked by phlorizin.

Glucose uptakes were performed on freshly-made renal BBM vesicles, brought up to room temperature (20°C) for 10 minutes. Uptake buffer (200 mM NaSCN, 12 mM Tris HCl, pH 7.4), with or without 1 mM phlorizin (Sigma, Dorset, UK) was prepared to 100 μM with \(^3\text{[H]}\) and cold glucose. Glucose uptake was measured at different time points from 3 seconds to 20 minutes, at glucose concentration of 100 μM, by adding
20 μl of uptake buffer to 20 μl BBM vesicles. Glucose uptake at each time point was terminated with 2 ml ice-cold stop solution (0.5 mM phlorizin in 154 mM saline). The solution was then filtered, under vacuum, using 0.45 μm nitrocellulose filters (Sartorius, Germany), then washed with 2 ml stop solution three more times. Scintillation counting was performed on the filters with the addition of 2.5 ml Ultimo Gold (Perkin-Elmer, Cambridge, UK) scintillant, using a Beckman liquid scintillation counter LS2000 (Beckman-Coulter Research, Buckinghamshire, UK).

6.5.2 SGLT- and GLUT-mediated glucose uptake

SGLT-mediated glucose uptake was measured at lower glucose concentrations (10 μM to 958 μM), whilst, GLUT-mediated glucose uptake was assessed at higher glucose concentration (20 mM). The accumulation of these concentrations of glucose, by BBM vesicles, was measured in the presence and absence of phloridzin.

Glucose uptakes were performed as described in section 6.5.1.2, except that uptake buffer was prepared containing 3[H]-glucose with cold glucose concentrations ranging from 30 μM to 20 mM, and uptake was measured at 4 seconds only. Glucose uptake was terminated with 2 ml ice-cold stop solution and counted, and calculated, as previously described.

6.5.3 Glucose uptake calculations

Disintegrations per minute (DPM) of 3[H] in the samples represented the accumulation of glucose by the BBM vesicles, whereas DPM of 3[H] in the background represented non-specific retention of glucose on the filters in the absence of BBM vesicles (DPM\textsubscript{background}). DPM of 3[H] in the initial uptake buffer, with glucose, was also measured with scintillant (DPM\textsubscript{initial counts}).

The counts were converted to uptake (pmol.mg protein\textsuperscript{-1}) using the equation below, with glucose concentration measured in μM and by doubling the protein concentration
(mg.ml\(^{-1}\)), from section 6.6, to allow for the 1:1 dilution of BBM vesicles in uptake buffer.

\[
\frac{[\text{DPM}_{\text{samples}} - \text{DPM}_{\text{background}}] \times [\text{glucose}]}{\text{DPM}_{\text{initial counts}} \times \left\{ [\text{protein}] \right\}}
\]

The kinetic parameters for SGLT-mediated glucose transport between 30 μM to 960 μM, \(V_{\text{max}}\) (maximum transport capacity) and \(K_T\) (glucose concentration at half \(V_{\text{max}}\)), was calculated using the Lineweaver-Burk plot.

### 6.6 Determination of protein concentration

Protein concentration of whole membrane, LLC-PK\(_1\) cells and BBM vesicle preparations was measured using the Bradford assay (Bradford, 1976). This assay is based on the absorbance shift of acidic Coomassie Brilliant blue G-250 dye (BDH Chemicals Ltd, Poole, UK) from 465 nm, when unbound, to 595 nm, when bound as protein-dye complex. The increase in absorbance, at 595 nm, is proportional to the amount of bound dye and therefore the concentration of protein in the sample.

\[
\text{protein + NaOH} \quad \xrightarrow{\text{Coomassie Brilliant Blue (brown)}} \quad \text{bound protein-dye complex (blue)}
\]

Homogenates and BBM vesicles were diluted 1:10 in 0.1 M NaOH and 100 μl of stock was measured with 4.5 ml Bradford reagent (0.01% (w/v) Coomassie Brilliant Blue G-250, 5% (w/v) ethanol and 10% orthophosphoric acid). Bovine serum albumin (BSA) was used as a protein standard with a concentration range from 15 - 90 mg.ml\(^{-1}\), increasing at 15 mg.ml\(^{-1}\) increments, in 0.1 M NaOH; 4.5 ml Bradford reagent was also added to measure standards.

Duplicates of samples and standards were measured, in 3 ml cuvettes, at 595 nm with a Beckman Du 650 spectrophotometer (Beckman-Coulter Research, Buckinghamshire, UK).
6.7 Determination of alkaline phosphatase

There are many different markers to validate BBM vesicle preparations, including assaying for sucrase activity, or change in Na\(^+\)/K\(^+\) - ATPase activity (BLM marker). The enzyme alkaline phsophatase was used in these experiments, as a BBM marker, to assess the enrichment of the BBM vesicles, compared to the original homogenate. The alkaline phosphatase assay is based on the conversion of p-nitrophenol phosphate substrate, by alkaline phosphatase, into free p-nitrophenol and phosphate. The free p-nitrophenol can be measured spectrophotometrically at 400 nm wavelength (Forstner et al., 1968).

\[
\text{p-nitrophenol phosphate} \xrightarrow{\text{alkaline phosphatase}} \text{p-nitrophenol + phosphate (yellow)}
\]

Homogenate samples were diluted 1:5 and BBM vesicle at 1:50, in dH\(_2\)O, and 100 µl stock was used in the assay. 4-nitrophenol was used as a standard and prepared, in dH\(_2\)O, at dilutions from 50 nM to 300 nM, increasing in 50 nM increments. The substrate was prepared in alkaline buffer (50 µM glycine, 5 mM MgCl\(_2\) and 1 mM zinc acetate, pH 9.4) and 500 µl was incubated, with the samples and standards, for 15 minutes at 37°C. The reaction was terminated with 2.5 ml 1 mM NaOH.

Duplicates of samples and standards were measured, in 3 ml cuvettes, at 400 nm with a Beckman Du 650 spectrophotometer (Beckman-Coulter Research, Buckinghamshire, UK).
6.7.1 BBM vesicle enrichment of alkaline phosphatase

The purity of BBM vesicles was evaluated by assessing the relative specific activity of the membrane marker alkaline phosphatase, using the equation below:

$$\left( \frac{\text{BBM vesicle alkaline phosphatase activity}}{\text{BBM vesicle [protein] (mg.ml}^{-1})} \right) \times 10 \left( \frac{\text{Homogenate [protein]}}{\text{Homogenate alkaline phosphatase activity (nmol)}} \right)$$

6.8 Determination of plasma and urine glucose concentration

6.8.1 Plasma

Blood from cardiac puncture, or collected via femoral artery cannulation, was placed into heparinised tubes, containing 7500 kallikrain inactivator units (KIU) of the broad range protease inhibitor Aprotinin. After centrifugation at 8000 rpm for 15 minutes, the resulting supernatant (plasma) was used in the glucose oxidase assay. Plasma from diabetic or glucose-infused rats was diluted 1:10, in dH$_2$O, for the assay, for readings to remain within the standard curve.

6.8.2 Urine

Urine from diabetic animals or glucose-infused rats was diluted 1:100, in dH$_2$O, for the glucose oxidase assay, for readings to remain within the standard curve.

6.8.3 Glucose oxidase assay

In the assay, glucose is oxidised, by glucose oxidase, to produce hydrogen peroxide which, in the presence of peroxidase, oxidises dianisidine, to produce a chromogenic product (oxidised o-dianisidine), which can be measured spectrophotometrically at 450 nm.
Glucose + H₂O + O₂ → gluconic acid + H₂O₂

Glucose oxidase + o-dianisidine (colourless)

H₂O₂ + o-dianisidine → oxidised o-dianisidine (brown)

Glucose oxidase reagent (GOR) was prepared with 44 mg glucose oxidase, 1.76 ml peroxidise (0.53 mg.ml⁻¹ stock) and 2.8 ml dianisidine (2.5 mg.ml⁻¹ stock) in 176 ml H₂O.

Glucose standards (25 μl) were used at 5, 10 and 15 mM, with 500 μl dH₂O and 4 ml GOR. Samples (25 μl) were also prepared in 500 μl dH₂O with 4 ml GOR and incubated for 1 hour at room temperature to allow glucose to oxidise.

Duplicates of samples and standards were measured at 450 nm with a Beckman Du 650 spectrophotometer (Beckman-Coulter Research, Buckinghamshire, UK).

6.9 Insulin assay

Plasma insulin concentrations were established using a rat insulin sandwich ELISA kit (Millipore, Watford, UK). In short, standards (0.2, 0.5, 1, 2, 5 and 10 ng.ml⁻¹) or plasma (10 μl) was incubated in ELISA wells pre-coated with mouse anti-rat insulin antibody, after which insulin in the plasma samples was captured using biotinylated polyclonal antibody (80 μl) and incubating the ELISA for 2 hours. Horseradish peroxidise (HRP)-linked secondary antibody (100 μl) binds to the immobilised biotinylated antibody and this complex was quantified by monitoring HRP activity in the presence of the substrate tetramethylbenzidine (TMB); the increase in absorbance at 450 nm, corrected from absorbance at 590 nm, after the acidification of TMB was directly proportional to the amount of captured insulin in the plasma samples. Absorbance of the duplicates of samples and standards were measured with a Beckman Du 650 spectrophotometer (Beckman-Coulter Research, Buckinghamshire, UK).
For the high-fat rat model, an intra-peritoneal insulin sensitivity test (IPIST) was performed (by Dr. Cleasby) on plasma, using a rat insulin ELISA kit (Crystal Chem Inc, Illinois, USA) and analysed statistically with a 2-way ANOVA/Holm-Sidak $t$ test, for multiple comparisons.

### 6.10 Osmolality measurements

Osmolality was measured, using freezing-point depression osmometry, with a micro-osmometer model 3M0 (Advanced Instruments Inc, Norwood, Massachusetts, US). A 20 μl sample of plasma or urine was frozen and the freezing point is depressed in direct relation to the amount of solute in solution and displayed in mOsM.

### 6.11 Protein analysis

#### 6.11.1 Dot blots

Whole membrane preparations (50 μg) were solubilised in Laemmli sample buffer (10% SDS, glycerol, Tris HCl (pH 6.8), bromophenol blue and dithiothreitol (DTT)). Proteins were directly applied to dampened Sequi-blot PVDF nitrocellulose membrane (Bio-Rad, Hemel Hempstead, UK) and left for 10 minutes to adhere. Membranes were then blocked in 6% milk-PBS-T (PBS with 0.1% Tween 20) and incubated in primary antibody, anti-T1R2 and –T1R3 (described in Table 6.2) on a shaker overnight at 4°C.
6.11.2 Western blotting

BBM vesicles, LLC-PK₁ cells and whole membrane preparations (20 – 50 μg) were solubilised in Laemmli sample buffer (10% SDS, glycerol, Tris HCl (pH 6.8), bromophenol blue and dithiothreitol (DTT)). Samples were loaded onto 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gels with kaleidoscope pre-stained standards (Bio-Rad, Hemel Hempstead, UK) as a molecular weight marker. Gels were electrophoresed, in running buffer (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS-running buffer (Sigma, Dorset, UK)) for approximately 1 hour, or until the dye-front reached the bottom of the gel. Proteins were transferred onto Sequi-blot PVDF nitrocellulose membrane (Bio-Rad) at 100 – 130 mA for 1 ½ hours. Membranes were blocked in 6% milk-PBS-T (PBS with 0.1% -Tween 20) and incubated in primary antibody on a shaker. Antibodies were used as described in Table 6.2 and were incubated overnight at 4°C, with the exception of anti-mouse β-actin antibody which was incubated at room temperature for 1 hour.

Blots were rinsed in PBS-T (1 x 15 minutes, 3 x 5 minutes) and incubated with the appropriate horseradish peroxidise-linked secondary antibody, outlined in Table 6.3 for 1 hour on a shaker at room temperature. After repeat rinses as above, the filters were visualised using ECL, prepared at a 1:1 ratio of ECL I (0.25 mM luminol (3-aminophthalic acid hydrazide), 0.04 mM p-coumaric acid, 100 mM Tris HCl, 200 mM NaCl, pH 8.8) and ECL II (30% H₂O₂). Proteins were detected with a Fluor-S MultiImager system (BioRad, Hertfordshire, UK); all values were calculated relative to β-actin and % BBM expression was displayed as a ratio of control average.
**Table 6.2** - Table of the primary antibodies used to detect proteins analysed in western blotting, including the species that the antibody was raised in and the dilution that the antibody was used for incubation

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species raised in</th>
<th>Concentration used at</th>
<th>Epitope antibody raised against</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT2</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Rat C-terminal</td>
<td>AbDSerotec</td>
</tr>
<tr>
<td>GLUT5</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Rat C-terminal (cytoplasmic domain)</td>
<td>Alpha Diagnostic International</td>
</tr>
<tr>
<td>SGLT1</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Human N-terminal</td>
<td>Gift from G. Kellett (University of York)</td>
</tr>
<tr>
<td>PKC-βI</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Human C-terminal</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>PKC-βII</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Human C-terminal</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>T1R2</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Mouse N-terminal (extracellular domain)</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>T1R3</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Mouse N-terminal (extracellular domain)</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>β-actin</td>
<td>Mouse</td>
<td>1:5000</td>
<td><em>Xenopus laevis</em> C-terminal</td>
<td>Abcam</td>
</tr>
</tbody>
</table>

**Table 6.3** - Table of the secondary antibodies used to bind to and detect primary antibodies (outlined in Table 6.2) analysed in western blotting, including the species that the antibody was raised in and the dilution that the antibody was used for incubation

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species raised in</th>
<th>Concentration used at</th>
<th>Species antibody raised against</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse-HRP</td>
<td>Goat</td>
<td>1:5000</td>
<td>Mouse</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti-rabbit-HRP</td>
<td>Donkey</td>
<td>1:1000</td>
<td>Rabbit</td>
<td>GE Healthcare</td>
</tr>
</tbody>
</table>
6.12 Real time PCR

6.12.1 cDNA preparation

The mRNA expression profile of glucose transporters and sweet taste receptors was assessed with RT-PCR in kidney and small intestine tissues. Mucosal scrapes from duodenum, jejunum and ileum, and kidney cortex were placed in RNALater (Ambion, Texas, USA), snap frozen in liquid N\textsubscript{2} and stored at -70°C. RNA was extracted from tissue, using Trizol, according to manufacturer’s instructions (Invitrogen, Paisley, UK).

Extracted RNA samples were prepared in load buffer (Sigma, Dorset, UK) and loaded on a 1.2% agarose gel, containing 0.1% ethidium bromide, and run at 120 V to ensure RNA quality. RNA concentration was determined using nanodrop (Bio-Rad Laboratories Ltd. Hemel Hempstead, UK) and RNA was diluted to 1 µg.ul\textsuperscript{-1}. RNA was reverse transcribed with 0.5 µg of oligo-dT 12-18 primer and a First Strand cDNA synthesis kit (Superscript II RNase H-reverse transcriptase; Life Technologies, Paisley, UK).

Transcript levels were measured on a Chromo4\textsuperscript{TM} Real-Time Detector (Bio-Rad Laboratories Ltd. Hemel Hempstead, UK), using QuantiTech SYBR Green PCR kit (Invitrogen, Paisley, UK), with β-actin used as the house-keeping gene. All primers were run at 95°C for 15 minutes, 95°C for 15 seconds, 60°C for 20 seconds, 72°C for 40 seconds and cycled 42 times.

6.12.2 Primers

Gene expression was measured, using specific intron-spanning primers designed from the published sequence of the gene of interest (Table 6.4). Primers were tested with cDNA and QuantiTech SYBR Green PCR kit (Invitrogen, Paisley, UK) and amplified using Chromo4\textsuperscript{TM} Real-Time Detector (Bio-Rad Laboratories Ltd. Hemel Hempstead,
UK). PCR products were run on an agarose gel (as in section 6.12.1) to check there was no primer dimerisation.

Table 6.4 – Table of intron-spanning primers designed for use in mRNA expression analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>GenBank accession number</th>
<th>Forward position</th>
<th>Reverse position</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT2</td>
<td>NM_012879.2</td>
<td>1101-1116</td>
<td>1420-1405</td>
<td>319</td>
</tr>
<tr>
<td>GLUT5</td>
<td>NM_031741.1</td>
<td>921-939</td>
<td>1280-1264</td>
<td>359</td>
</tr>
<tr>
<td>SGLT1</td>
<td>NM_013033.2</td>
<td>1871-1886</td>
<td>2198-2183</td>
<td>327</td>
</tr>
<tr>
<td>SGLT2</td>
<td>NM_022590.2</td>
<td>838-857</td>
<td>1088-1069</td>
<td>250</td>
</tr>
<tr>
<td>T1R1</td>
<td>NM_053305.1</td>
<td>1315-1334</td>
<td>1473-1454</td>
<td>158</td>
</tr>
<tr>
<td>T1R2</td>
<td>XM_00107479.1</td>
<td>3759-3778</td>
<td>3923-3904</td>
<td>164</td>
</tr>
<tr>
<td>T1R3</td>
<td>NM_130818.1</td>
<td>2107-2126</td>
<td>2327-2308</td>
<td>220</td>
</tr>
<tr>
<td>β-actin</td>
<td>NM_031144</td>
<td>937-955</td>
<td>1223-1208</td>
<td>286</td>
</tr>
</tbody>
</table>

6.13 Statistics

Values were expressed as mean ± S.E.M.; n values represent numbers per experimental group. Differences between groups were tested by Student’s unpaired t test and using one-way analysis of variance (ANOVA) with post-hoc comparisons performed using Dunnett’s Multiple comparisons test with P considered significant at < 0.05.
7 Acute regulation of GLUT2 at the proximal tubule brush-border
7.1 Introduction

The ‘classical’ model for intestinal glucose absorption is that glucose, the major product of dietary carbohydrate digestion, traverses the brush border membrane (BBM) of enterocytes via the sodium-dependent transporter SGLT1, and exits through the basolateral membrane (BLM) using the facilitative transporter GLUT2. At low plasma glucose concentrations, SGLT1 is the primary route of glucose absorption at the BBM, however this is not the case at higher concentrations.

Brush-border expression of GLUT2 in the intestine is dependent on glucose transport by SGLT1 and its insertion into the BBM is regulated by a short-term high glucose stimulus in the lumen and long-term dietary changes. Before a meal, any glucose in the lumen is scavenged by the high affinity SGLT1 and transported into the cell against a concentration gradient. However, after feeding there is a high luminal glucose concentration which saturates SGLT1 (Sharp et al., 1996) and stimulates PKC-βII, via the T1R family and calcium (Ca^{2+}), to move to the enterocyte BBM (Morgan et al., 2007;Mace et al., 2007). In this position, PKC-βII causes GLUT2 recruitment from intracellular vesicles to the BBM and this insertion occurs within 15 minutes of high glucose signal. This enables GLUT2 to shuttle glucose from the lumen, along its concentration gradient, and reduce the luminal glucose concentration (Kellett and Helliwell, 2000). As luminal concentration decreases, both PKC-βII and GLUT2 are inactivated and trafficked away from the BBM which restores resting low levels of the transporter (Helliwell et al., 2000).

A high-carbohydrate diet causes an increase in the number of high affinity ‘glucose-protectable’ phlorizin binding sites, suggesting an increase in phlorizin-sensitive SGLT1 transporters at the enterocyte BBM (Ferraris et al., 1992;Burant et al., 1994). It has also been shown that brush-border GLUT2 expression is reduced in mice receiving a complex diet of low-glycaemic index carbohydrates (Kellett and Brot-Laroche, 2005). In the same study, mice given a diet rich in high-glycaemic index carbohydrates were shown to have increased GLUT2 and augmented plasma glucose levels.
There are striking similarities between the reabsorption of glucose across the proximal tubule cell and glucose absorption in the jejunal enterocyte. However there are far fewer studies on the molecular mechanisms affecting the recruitment of GLUT2 to the BBM in proximal tubule cells, in response to luminal glucose stimulus.

Dominguez et al found that both GLUT1 and GLUT2 coexist in the early proximal tubule whereas only the high affinity GLUT1 was found in the late proximal tubule (Dominguez et al., 1994a). The group also established that 2-4 weeks streptozotocin (STZ)-induced diabetes caused a decrease in GLUT1 protein and mRNA expression in the proximal tubule. The opposite was seen for GLUT2 where there was a marked increase in proximal tubule mRNA and protein expression, which was accompanied by an increase in $^3$H-deoxyglucose (DOG) uptake. Up-regulation of renal GLUT2 in type I diabetes was also seen by Chin et al, where a 5-fold increase in mRNA levels occurred within 1-3 months of the disease, and continued to rise after 6 months of diabetes (Chin et al., 1997).

The expression of both SGLT1 and SGLT2 has been extensively demonstrated in the proximal tubule (Kanai et al., 1994;Wallner et al., 2001;Wright, 2001;Balen et al., 2008), however, how the expression of sodium dependent glucose transporters change in STZ-induced diabetes is more disputed. Yasuda et al showed that diabetes caused a decrease in SGLT-mediated glucose uptake in renal cortex brush-border membranes, which was attributed to reduced SGLT expression (Yasuda et al., 1990). After normalisation of blood glucose with insulin-treatment or starvation, glucose uptake returned to control levels therefore they postulated that SGLT-mediated glucose uptake is regulated by blood glucose concentrations per se (Yasuda et al., 1990). Although a rapid down-regulation of SGLT2 has been observed in response to early diabetes (Albertoni Borghese et al., 2009), Dominguez et al found no change in sodium-dependent glucose transporter protein or mRNA in 2-4 week type I diabetes, with or without insulin treatment (Dominguez et al., 1994a). The discrepancies in SGLT data in renal proximal tubules during diabetes may be due to different methods of induction of the disease, with both alloxan and streptozotocin used, as well as different time points of diabetes studied (Albertoni Borghese et al., 2009). Furthermore, different molecular species of SGLT, possibly depending on the glycosylation or phosphorylation state of the transporter may result in the differences
seen in the data during diabetes (Stearns et al., 2009). Also, the presence of two different SGLT proteins in the proximal tubule, as oppose to only SGLT1 in the jejunum, may explain why early studies showed such conflicting results.

In contrast, studies show agreement that the inhibition of SGLT2 causes normalisation of the increased blood glucose seen in type I diabetes, suggesting a major role for the transporter in this disease (Adachi et al., 2000; Oku et al., 2000; Oku et al., 2000). In the SGLT2 KO mouse, renal SGLT1 expression is also down-regulated and this could be a mechanism of limiting the increase in glucose reabsorption at high luminal glucose concentrations (Vallon et al., 2010).

During STZ-induced diabetes, increases in blood glucose levels, tubular glucose uptake, more specifically GLUT-mediated transport, and GLUT2 protein expression at the BBM have all been noted; however, overnight fasting of type I diabetic rats revokes all these effects (Marks et al., 2003). Thus it has been hypothesised that hyperglycaemia stimulates GLUT2 expression, and these elevated levels of GLUT2 are responsible for the increased glucose uptake found in diabetes. Additionally, this group established that GLUT2 is not detected at the BBM following overnight fasting of diabetic rats, suggesting that GLUT2 is rapidly translocated away from the membrane in response to normalisation of blood glucose levels (Marks et al., 2003).

Freitas et al have also demonstrated the expression of renal GLUT2 in response to hyperglycaemia, using insulin and phlorizin as modulators of blood glucose levels. Despite opposing effects on plasma insulin and urinary glucose levels, both insulin and phlorizin reversed the increased renal GLUT2 mRNA and protein expression present in diabetes (Freitas et al., 2007). This reversal of diabetes-induced GLUT2 expression occurred over a 6-day period of treatment with insulin, suggesting the glucose transporter is removed from the renal BBM at a slower rate than seen after overnight fasting (Marks et al., 2003; Freitas et al., 2007), in response to a more moderate normalisation of plasma glucose levels.

The link between PKC-βII signalling and GLUT2 translocation at the BBM in enterocytes, presented the possibility of a similar PKC-dependent effect on renal expression of GLUT2. This theory was tested by exposing renal proximal tubule cells
to thapsigargin, which promotes the release of intracellular stores of calcium, and PMA, a PKC activator (Goestemeyer et al., 2007). In these experiments, GLUT-mediated transport at the BBM was increased by thapsigargin and PMA. Western blotting analysis revealed the expression of PKC isoforms -α, -βI, -βII, -δ and -ε, although only -βI was affected by STZ-induced diabetes. This study also demonstrated a positive correlation (r = 0.718) between plasma glucose levels and GLUT2 protein levels at the BBM. Positive correlations (r = 0.779) between plasma glucose levels and PKC-βI, and between PKC-βI and GLUT2 protein levels (r = 0.648) were also noted (Goestemeyer et al., 2007).

Thus, BBM expression of both jejunal and renal GLUT2 is up-regulated by high luminal glucose, and down-regulated by the normalisation of luminal glucose. Also, in both tissues the different isoforms of PKC-β are involved in recruitment of the transporter to the BBM; PKC-βII in the small intestine and PKC-βI in the kidney. It is known that jejunal brush-border GLUT2 responds to a glucose stimulus within 15 minutes whereas 2-4 weeks hyperglycaemia causes recruitment of renal GLUT2 and overnight fasting abolishes this GLUT2 insertion. However, despite similarities between GLUT2 expression in the jejunum and proximal tubule, no studies have yet been undertaken to assess the time required for renal GLUT2 insertion at proximal tubule BBM.

### 7.2 Aims of chapter

The aim of this study was therefore to establish the period of time needed for GLUT2 expression at the proximal tubule BBM in response to changes in plasma, and therefore tubular luminal, glucose levels. This was done by assessing GLUT2 movement to the proximal tubule BBM in response to acute and chronic hyperglycaemia. The effect of an acute increase in tubular glucose concentration on glucose transport was studied to evaluate whether short term GLUT2 insertion was mediated by PKC-βI, as has previously been shown for a 2-4 week period of diabetes.
7.3 Results

7.3.1 Acute hyperglycaemia: time course in vivo infusion studies

7.3.1.1 Effect of infusion studies on plasma glucose

Within 15 minutes of 1.1 M glucose infusion, there was a significant increase in plasma glucose from 7.1 ± 0.41 mM at start of infusion, to 14.6 ± 1.4 mM after 15 minutes (Figure 7.1). This elevation of plasma glucose continued for up to 60 minutes, where plasma glucose reached a value of 21.7 ± 2.49 mM. As expected, when infused with saline or the osmotic control, 0.08 M mannitol, rats showed no change in glycaemia throughout the 1 hour infusion (7.3 ± 0.12 mM and 7.06 ± 0.34 mM, respectively) (Figure 7.1).

Similar glycaemic effects were seen in the 2 hour infusion studies. In these experiments, a 3 ml bolus preceded the constant infusion protocol. Both saline and mannitol infusions did not affect normal plasma glucose levels, 5.39 ± 0.2 mM and 6.13 ± 0.54 mM respectively, during the 2 hour infusion period. The glucose-infused animals exhibited a significant elevation in plasma glucose levels within the initial 15 minutes of the experiment, increasing from a pre-bolus value of 5.8 ± 0.3 mM to 23 ± 2.6 mM. Hyperglycaemia continued over the experimental period, reaching values of 32.2 ± 0.3 mM after 2 hours (Figure 7.2). Table 7.1 shows that these effects on plasma glucose had no effect on body or kidney weight between those rats infused with saline, mannitol or glucose. To correct for the effect that infusing glucose would have on plasma osmolality, 0.08 M mannitol was used as a control infusate. This concentration of mannitol maintained plasma osmolality at 306 ± 2 mOsM, which was within the range of that occurring with the other infusates, saline and glucose (Table 7.2).
**Figure 7.1** – Effect of 1 hour 1.1 M glucose infusion (dotted line) on plasma glucose concentrations compared with 154 mM saline (broken line) and 0.08 M mannitol (solid line) control infusions. Values are expressed as mean ± SEM, n=4-6, ***p<0.005, ****p<0.001 compared with both controls at each time point.

**Figure 7.2** – Effect of 2 hour infusion of 1.1 M glucose (dotted line), with initial bolus, on plasma glucose concentrations, compared with 154 mM saline (broken line) and 0.08 M mannitol (solid line) control infusions. Values are expressed as mean ± SEM, n=4-6, ****p<0.001 compared with both controls at each time point.
Table 7.1 – Body and kidney weights and renal BBM vesicle enrichment of alkaline phosphatase marker enzyme, in rats infused with 154 mM saline, 1.1 M glucose, 0.08 M and 1.09 M mannitol. Values are expressed as mean ± SEM for 1 and 2 hour time period, n = 3-6, no significant change between infusions, p>0.5.

<table>
<thead>
<tr>
<th></th>
<th>154 mM Saline</th>
<th>0.08 M Mannitol</th>
<th>1.1 M Glucose</th>
<th>1.09 M Mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>291.4 ± 21.8</td>
<td>255.6 ± 3.07</td>
<td>301.6 ± 23</td>
<td>263.7 ± 12.7</td>
</tr>
<tr>
<td><strong>Kidney weight (g)</strong></td>
<td>1.04 ± 0.05</td>
<td>0.99 ± 0.03</td>
<td>1.06 ± 0.08</td>
<td>1.02 ± 0.09</td>
</tr>
<tr>
<td><strong>BBMV enrichment</strong></td>
<td>6.02 ± 0.29</td>
<td>5.82 ± 0.44</td>
<td>6.12 ± 0.36</td>
<td>6.19 ± 0.37</td>
</tr>
</tbody>
</table>

Table 7.2 – Infusate osmolality and its effect on plasma osmolality for 154 mM saline, 0.08 M mannitol, 1.1 M glucose and 1.09 M mannitol. Values are expressed as mean ± SEM, n=4-6, ****p<0.001 compared with saline infused control

<table>
<thead>
<tr>
<th></th>
<th>154 mM Saline</th>
<th>0.08 M Mannitol</th>
<th>1.1 M Glucose</th>
<th>1.09 M Mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Infusate osmolality</strong></td>
<td>312 ± 7 mOsM</td>
<td>388 ± 10 mOsM</td>
<td>1706 ± 26 mOsM</td>
<td>1675 ± 18 mOsM</td>
</tr>
</tbody>
</table>
| **Plasma osmolality**    | 310 ± 5 mOsM  | 306 ± 2 mOsM    | 319 ± 4 mOsM  | 617 ± 9 mOsM   | ****
7.3.1.2 Glycosuria in acute hyperglycaemia

The infusion of glucose caused a significant, albeit expected, effect on glucose concentration in the urine in the 1 hour infusion experiments, increasing from 0.53 ± 0.03 mM pre-infusion to 27 ± 3.21 mM in the initial 30 minutes. This further increased to 136 ± 5.13 mM at the 1 hour end-point compared with both saline and mannitol infusions where there were negligible levels of glucose in the urine, 0.55 ± 0.05 mM and 0.53 ± 0.09 mM, respectively.

Glycosuria was significantly higher in the 2 hour glucose infusion with bolus group, with an increase from 0.157 ± 0.037 mM pre-infusion, to 306 ± 6 mM after 30 minutes. After 2 hours infusion, the glucose concentration in the urine had dropped to 191 ± 3 mM, suggesting the initial peak was due to the bolus administered. In both control infusions, there was no glycosuria with glucose concentrations in the urine similar to those seen after 1 hour infusion.

7.3.1.3 Effect of acute hyperglycaemia on GLUT2 expression at the proximal tubule BBM

Despite the hyperglycaemic and glycosuric effects of the glucose infusions described above, there was no effect on renal GLUT2 expression in BBM vesicles prepared from both the 1 and 2 hour infused animals (Figure 7.3). There was also no significant effect on expression of the GLUT2 effector, PKC-βI or the sodium-dependent transporter, SGLT1 (Figure 7.3). This lack of response to short-term (1 and 2 hour) hyperglycaemia, suggests that, unlike the jejunum, a longer period of high glucose stimuli is required to stimulate GLUT2 movement to the proximal tubule BBM.
Figure 7.3 – Effect of acute hyperglycaemia, induced by I.V. infusion of 1.1 M glucose (slatted bars) for 1 and 2 hours, on GLUT2 (1), PKC-βI (2) and SGLT1 (3) expression at the proximal tubule BBM (expressed as control ratio %) compared with expression in animals infused with 154 mM saline control (open bars) and 0.08 M mannitol as osmotic control for glucose (closed bars). Values are expressed as mean ± SEM, n=4-6, no significant change between infusions p>0.5.

1 – GLUT2

![GLUT2 graph]

2 – PKC-βI

![PKC-βI graph]

3 – SGLT1

![SGLT1 graph]
7.3.1.4 Effect of plasma osmolality on renal GLUT2 expression

As an osmotic control for the short-term infusion studies, 1.09 M mannitol was also used as an infusate since its osmolality is similar to 1.1 M glucose (1675 ± 18 mOsM compared to 1706 ± 26 mOsM, (Table 7.2). The 1.09 M mannitol infusion had a dramatic effect on GLUT2 expression at the proximal tubule BBM with a 2.2-fold increase in expression compared with the saline infused control animals (Figure 7.4). This increased pattern of expression was also seen in the GLUT2 effector, PKC-βI (Figure 7.4). In contrast, 1.09 M mannitol infusion was without effect on the BBM expression of SGLT1 (Figure 7.4). It was noted that animals infused with 1.09 M mannitol had significantly augmented plasma osmolality in comparison to the saline, 0.08 M mannitol and glucose infused animals (Table 7.2).
**Figure 7.4** – Effect of 1.09 M mannitol infusion (closed bars) on the expression of GLUT2 (1), PKC-βI (2) and SGLT1 (3) at the proximal tubule BBM (expressed as control ratio %) compared with expression in 154 mM saline-infused animals (open bars). Values are expressed as mean ± SEM, n=4-6, ***p<0.005, ****p<0.001 compared with saline controls, (3) no significant change between infusions p>0.5.

1 – GLUT2

2 – PKC-βI

3 – SGLT1
7.3.2 Chronic hyperglycaemia: type I diabetes induced with streptozotocin

The infusion experiments above established that a longer time-course of hyperglycaemia was required to stimulate GLUT2 expression at the proximal tubule BBM. To study this, hyperglycaemia was induced using the STZ-induced type I diabetic model.

7.3.2.1 Effects of short-term STZ-induced diabetes

STZ treatment rapidly induced glycosuria with urine glucose levels at 5.75 ± 0.06 mM within 5 hours of tail-vein injections, although interestingly there was no effect on glycaemia at this stage of diabetes. However, 2 days after STZ, unfasted plasma glucose levels were significantly increased (35.7 ± 3.36 mM) compared with the citrate buffer controls (10.26 ± 0.88 mM). These animals also exhibited glycosuria, with urine glucose concentrations reaching 316 ± 19 mM. However, despite these responses to STZ, no changes in GLUT2, PKC-βI or SGLT1 expression at the proximal tubule BBM were observed 2 days after STZ (Figure 7.5).

7.3.2.2 Effect of 7-day STZ-induced diabetes on GLUT2 expression at the proximal tubule BBM

GLUT2 was studied in STZ-induced type I diabetes at further time points, with the aim to elucidate the maximum time for the hyperglycaemic stimulus to induce GLUT2 expression at the proximal tubule BBM. At 7 days hyperglycaemia, GLUT2 expression at the proximal tubule BBM was significantly increased compared with that of the citrate buffer-injected controls, and from expression seen at 2-6 days STZ (Figure 7.5). This expression of GLUT2 at the proximal tubule BBM (744% increase) was mirrored by an increase in PKC-βI (496% increase), but there was no change in SGLT1 expression, a result similar to that previously shown at 2-4 weeks diabetes (Marks et al., 2003). Table 7.3 shows that there was a 23% increase in
kidney weight in 7-day STZ diabetic animals compared with those from control animals; however no difference in BBM enrichment was detected in membranes prepared from control and 7-day STZ diabetic animals. There was also a decrease in body weight in the 7-day STZ diabetic rats compared to the controls, although this did not reach statistical significance (Table 7.3).

Table 7.3 – Body and kidney weights and renal BBM vesicle enrichment of alkaline phosphatase marker enzyme in rats with 7-day STZ-induced type I diabetes. Values are expressed as mean ± SEM, n = 3-6, *p<0.05 compared with control.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>268.5 ± 11.3</td>
<td>244.4 ± 7.1</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>0.95 ± 0.11</td>
<td>1.17 ± 0.04*</td>
</tr>
<tr>
<td>BBMV enrichment</td>
<td>5.86 ± 0.31</td>
<td>5.79 ± 0.22</td>
</tr>
</tbody>
</table>
Figure 7.5 – Effects of 2 – 7 days of STZ-induced diabetes on GLUT2 (green bars), PKC-βI (pink bars) and SGLT1 (grey bars) expression at the proximal tubule BBM.

1) Western blots 2) quantification of western blots (expressed as control ratio %) standardised to β-actin. Values are expressed as mean ± SEM, n=3-6, ****p<0.001 compared with citrate buffer vehicle controls by ANOVA.

1

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>7 day STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT2</td>
<td><img src="image1" alt="GLUT2 control" /></td>
<td><img src="image2" alt="GLUT2 7 day STZ" /></td>
</tr>
<tr>
<td>PKC-βI</td>
<td><img src="image3" alt="PKC-βI control" /></td>
<td><img src="image4" alt="PKC-βI 7 day STZ" /></td>
</tr>
<tr>
<td>SGLT1</td>
<td><img src="image5" alt="SGLT1 control" /></td>
<td><img src="image6" alt="SGLT1 7 day STZ" /></td>
</tr>
<tr>
<td>Actin</td>
<td><img src="image7" alt="Actin control" /></td>
<td><img src="image8" alt="Actin 7 day STZ" /></td>
</tr>
</tbody>
</table>

2

![Graph showing % BBM expression over time](image9)
7.3.3 Effect of STZ-induced diabetes on GLUT2 intracellular pool in proximal tubule cells

GLUT2 mRNA in the renal cortex was studied to assess possible changes in the intracellular pool of GLUT2 in the early stages of STZ-induced hyperglycaemia. It was important to ascertain whether hyperglycaemia induced an increase in intracellular GLUT2 or whether a greater proportion of the available intracellular pool was shuttled to the proximal tubule BBM.

The baseline level of renal GLUT2 mRNA was significantly increased by 2- and 3-fold after 10 and 30 days STZ-induced diabetes, respectively (Figure 7.6), suggesting raised levels of intracellular GLUT2 in renal cortical cells in response to hyperglycaemia seen in type I diabetes.

**Figure 7.6** – Effects of 10- and 30-day STZ-induced type I diabetes on GLUT2 mRNA expression in renal cortex. Values are expressed as mean ± SEM, n=9-12, *p<0.05, ****p<0.001 compared with control by ANOVA.
7.4 Discussion

The aims of this study were to establish the time period for GLUT2 expression at the proximal tubule BBM in response to IV glucose infusion that will increase plasma glucose concentration and therefore proximal tubule fluid. The methods to study the response to luminal glucose in the small intestine, for example, in vivo jejunal loops (Kellett and Helliwell, 2000) cannot be utilised in the kidney without the use of microperfusion studies. The latter technique was previously used by Bank et al using in vivo reperfusion studies, where each tubule was perfused in the presence or absence of glucose (Bank et al., 1971). Sodium absorption was assessed in the proximal tubule in response to increased luminal glucose concentrations and the same procedure could be applied to study the amount of glucose reabsorption occurring in the early and late proximal convoluted tubules. As this technique was not available for the present study, glucose was infused into the circulation in vivo and the assumption made that all the glucose in the blood was freely filtered at the glomerulus into the proximal tubule lumen.

The data obtained from the short-term glucose infusions highlights the difference between the regulation of renal GLUT2 compared with that previously noted at the small intestinal BBM. Glucose transport in the jejunum needs to rapidly respond to ingested carbohydrates; therefore, within 15 minutes of a glucose stimulus, when jejunal luminal glucose concentrations have been estimated to be between 50 to 300 mM (Ferraris et al., 1990; Pappenheimer, 1993), GLUT2 is recruited to the brush-border to absorb the bulk of glucose from the luminal fluid (Kellett and Helliwell, 2000).

In contrast to the jejunum, the GLUT2 response at the renal BBM was unaffected by short-term (1 and 2 hour) glucose stimuli, despite blood, and therefore tubular fluid glucose concentrations, exceeding 30 mM. Glycosuria under these conditions was noted throughout the infusion period, suggesting that the renal glucose absorptive mechanism was saturated. Furthermore, there was no change in the expression of SGLT1 or the renal GLUT2 activator, PKC-βI, in response to 1 or 2 hour glucose infusion. This implies that there was no up-regulation of upstream signals to GLUT2.
in response to short-term hyperglycaemia. There are several possible explanations for the absence of renal GLUT2 recruitment to the proximal tubule BBM following a short-term glucose stimulus. The renal BBM is not usually exposed to dramatically and dynamically changing glucose concentrations compared to those present at the jejunal BBM where glucose levels may approach 50 mM after a meal (Ferraris et al., 1990); therefore renal glucose transporters may not have the necessary signalling pathways to allow rapid shuttling of GLUT2 in response to high glucose concentrations. It has been shown that jejunal GLUT2 responds to SGLT1-mediated PKC-βII activation, as well as complex calcium (Ca\(^{2+}\)) and sweet taste receptor-mediated PKC-βII activation (Morgan et al., 2007; Mace et al., 2007). However, data on the control of GLUT2 shuttling to the renal brush-border is limited; currently it is known that altered expression of PKC-βI at the BBM correlates with that of GLUT2 in 2-4 week type I diabetes (Goestemeyer et al., 2007), although the presence of a secondary signalling pathway, and the involvement of SGLT, in GLUT2 expression at the renal BBM has not been established in this model of diabetes. However the studies presented here only indicate the expression of glucose transporter at the proximal tubule BBM and do not offer an indication of the mechanism of recruitment from intracellular to membrane location. In the jejunum, GLUT2 has been established to translocate from intracellular vesicles to the BBM, thus to adequately assess the shuttling of GLUT2 to the proximal tubule BBM, immunofluorescence during hyperglycaemia would be necessary (Kellett and Helliwell, 2000).

STZ-induced type I diabetes was used as a model of hyperglycaemia to study the effect of prolonged exposure to glucose on GLUT2 expression at the proximal tubule BBM. The data shows that, despite a significant increase in plasma glucose levels in type I diabetic animals, GLUT2, and its activator, PKC-βI, were not recruited to the BBM until 7 days after the induction of diabetes. This suggests that, rather than a dose-dependent response to tubular glucose levels, there may be an ‘on/off’-switch mechanism acting at 6 - 7 days, which is responsible for inducing GLUT2, via PKC-βI, to the proximal tubule BBM. Expression of both GLUT2 and PKC-βI correlates positively with plasma glucose concentrations, as previously seen in 2-4 week studies of STZ-induced diabetes (Goestemeyer et al., 2007). Since previous studies using the STZ model have shown an increase in GLUT-mediated glucose uptake (Marks et al., 2003) and increased renal glycogen storage (Khandelwal et al., 1979), the up-
regulation of GLUT2 expression implies an increased glucose uptake by the proximal tubule cell. It is worth noting that GLUT2 expression at the renal BBM is abolished after normalisation of glycaemia by overnight fasting (Marks et al., 2003), and therefore the transporter has the capacity to shuttle away from the BBM at a more rapid rate than it can be inserted. Also, GLUT2 mRNA is increased in short-term diabetes (10-day STZ) which implies that there is an increased intracellular pool of the transporter within proximal tubule cells readily available for shuttling to the proximal tubule BBM. However, mRNA was prepared from the renal cortex of diabetic rats and thus, in addition to proximal tubule cell mRNA, the preparation will include a proportion of distal tubules, fibroblasts and dendritic cells (Kaissling and Le Hir, 1994). The absence of any change in SGLT1 expression, in response to hyperglycaemia of 7-day duration, suggests that, unlike GLUT2, this transporter is unaffected by blood glucose levels. The cytotoxic effect of STZ is mediated by it’s action as an alkylating agent on pancreatic β-cells to abolish insulin secretion; the drug is transported into the pancreas via GLUT2 due to a strong molecular similarity to glucose. Interestingly, STZ accumulates in non-pancreatic locations including the liver and kidney and, in the latter, is responsible for renal toxicity and glycosuria (Hall-Craggs et al., 1982). Therefore, in addition to rendering the rats diabetic, the drug is likely to exhibit a damaging effect on GLUT2 at the proximal tubule; this may offer an explanation for the absence of a rapid GLUT2 response to STZ, between 2 and 7 days.

Recent interest on the use of SGLT2 inhibitors as a treatment for hyperglycaemia in diabetes implies a potential relationship between SGLT-mediated increase in intracellular glucose and GLUT2 recruitment to the renal BBM. The inhibitors act by blocking the high capacity SGLT2 transporter in the early proximal tubule, which causes increased glycosuria in diabetes as a result of lowered transport maximum (T_m) for glucose reabsorption leading to restoration of glycaemia (Bays, 2009). As GLUT2 moves glucose along its concentration gradient, if the transporter was able to move independently of SGLT2, it would be recruited to the BBM of the diabetic proximal tubule, in response to the high tubular glucose concentration and transport glucose into the proximal tubule cell. This does not appear to be the case as the inhibitor’s efficacy has been extensively studied and is currently in clinical trial stages (Adachi et
al., 2000; Fujimori et al., 2009; Patel and Fonseca, 2010); this suggests that GLUT2 may require a SGLT-mediated signal for its recruitment to the proximal tubule BBM.

In fact, the presence of GLUT2 at the BBM exacerbates hyperglycaemia and might be seen as an ‘unwanted feature’ in diabetes; in the absence of GLUT2 at the renal BBM, SGLT-mediated uptake would become saturated at much lower tubular glucose concentrations. Although the enrichment of BBM vesicles was assessed using the membrane marker enzyme, alkaline phosphatase, BLM contamination in the preparation is still possible. To adequately assess the BLM component of the vesicles preparation, the presence of the Na\(^+\)-K\(^+\)-ATPase could be used as a marker enzyme. GLUT2 has been previously identified at the proximal tubule BBM by confocal imaging and immunohistochemistry, thus it is likely, in the experiments presented here, that GLUT2 expression in the vesicle preparation is from the BBM (Marks, 2004). Therefore it is possible that the long period required for brush-border insertion of the facilitative glucose transporter, GLUT2, is a mechanism of protection from hyperglycaemia, and a physiological short-term attempt at maintaining glucose homeostasis.

However, there are limitations with using hyperglycaemia to simulate high tubular glucose levels for the study of GLUT2 expression. When the BBM and BLM are exposed to high levels of glucose, the facilitative transporter could cause bi-directional glucose flux. In studies using LLC-PK\(_1\) proximal tubule cells, the rate of GLUT-mediated glucose uptake from the BLM was higher than that at the BBM (Miller et al., 1992). This suggests that the BLM has a higher capacity for facilitative glucose transport, a likely result of a high GLUT2 expression. Therefore in hyperglycaemic conditions there is a large load of glucose moving from the blood into the proximal tubule cell, via the BLM, thereby raising intracellular glucose levels. Thus, despite the data presented here showing an increase in GLUT2 protein and mRNA in response to 7 day STZ-induced diabetes, the net glucose flux across the BBM will not necessarily be in favour of a luminal to intracellular direction.

Furthermore, the data observed, following 1.09 M mannitol infusion, shows that GLUT2 is capable of rapid expression at the proximal tubule BBM, via PKC-\(\beta\)I activation, in the absence of hyperglycaemia. This highlights an alternative, non-
genomic mechanism for PKC-βI-mediated GLUT2 activation in proximal tubules; high osmotic pressure from the mannitol infusate caused a rapid (within 1 hour) and sustained (maintained at 2 hour) increase in GLUT2 and PKC-βI recruitment to the BBM. Although the infusate osmolality of 1.09 M mannitol matched that of 1.1 M glucose, previous studies have shown that glucose is absorbed and stored by tissues in the body, for example, skeletal muscle (Dimitrakoudis et al., 1992), adipocytes (Fonseca-Alaniz et al., 2008) and the liver (Fery et al., 2001); therefore the resulting plasma osmolality from glucose infusions was within the normal physiological range as the saline control (Table 7.2). However the plasma osmolality after 1.09 M mannitol infusion was 2-fold higher than with 1.1 M glucose, this phenomenon of mannitol raising plasma osmolality has been reported with high-dose mannitol therapy. Mannitol treatment is commonly administered after cardiovascular surgery or to patients at risk of acute renal failure, where side-effects include acute expansion of extracellular fluid volume, hyperosmolality, hyponatremia, and intracellular dehydration in the brain (Visweswaran et al., 1997). The substantial dehydration which occurred in the rats in the study presented here is also likely to result in the rapid shrinkage of proximal tubule cells, accompanied by the activation and release of intracellular acid hydrolases and necrosis (Janigan and Santamaria, 1961). Therefore the expression of GLUT2 noted at the proximal tubule BBM following the high mannitol infusion may be a consequence of the breakdown of cytoplasmic vacuoles as oppose to a direct response of intact proximal tubule cell GLUT2.

Jejunual GLUT2 has been shown to respond to ill-defined stress, for example, strong inhibition of expression of the transporter was seen in animals whilst building works near the building that housed rats used in the study; the reduced levels of GLUT2 were normalised rapidly after this particular stress was removed (Shepherd et al., 2004). However, no change was noted in jejunal SGLT1 expression, under the same stress conditions. These results are in keeping with the pronounced changes in renal GLUT2, but not SGLT1, in response to plasma osmotic stress in the mannitol infusion studies observed in this chapter.

In the kidney, mannitol has been shown to have many different effects on tubular transport, including the up-regulation of the water channel aquaporin in the proximal tubule cell (Bouley et al., 2009). Mannitol inhibits proximal tubular isosmotic fluid
reabsorption because of the presence of the non-reabsorbable solute in the tubular fluid. This causes proximal tubular fluid sodium concentration to decrease below the plasma concentration and creates a concentration gradient that limits net sodium reabsorption. This theory agrees with clinical data that patients suffer hyponatremia after mannitol treatment (Perez-Perez et al., 2002). Although no link between restricted sodium reabsorption and increased glucose transporter expression has been previously elucidated, studies on glucose metabolism in other tissues suggests that the rapid response of GLUT transporters, to infused 1.09 M mannitol, may be stress-induced; for example, Kuzuya et al showed that mannitol-induced hyperosmolarity stimulated glucose uptake and glycogen synthesis in both adipose tissue and the diaphragm (Kuzuya T et al., 1965). Furthermore, it has also been shown that fibroblasts exposed to 600 mM sorbitol for 30 minutes had a 2.6-fold increase in GLUT-mediated glucose transport (Sakoda et al., 2000). A similar phenomenon occurs in 3T3L1 adipocytes; Chen et al showed that sorbitol-induced osmotic shock caused an increase in GLUT4 translocation to the plasma membrane which was mirrored by an increase in glucose uptake (Chen et al., 1997). However, this GLUT2 response to mannitol is activated by a hyperglycaemic-independent, rapid stress-response signalling pathway to increase renal glucose reabsorption during times of osmotic stress.

Previous studies have shown an increase in GLUT3 protein and mRNA in the pituitary gland in response to 3-day water deprivation studies, an effect which is remedied by rehydration (Koehler-Stec et al., 2000). Although Koehler-Stec et al made no plasma osmolality measurements during dehydration, it has been established that 48 hour water deprivation causes a significant increase in plasma osmolality (Gottlieb et al., 2006). The data presented in this chapter shows a similar mediation of GLUT expression in response to normalised osmolality as the use of 0.08 M mannitol, a more accurate control for the plasma osmotic effect of glucose, did not influence GLUT2 levels at the proximal tubule BBM.
7.5 Conclusions

In summary, the results presented here show that, unlike GLUT2 in the jejunum, levels of GLUT2 at the renal BBM are not influenced by short-term hyperglycaemia. A longer-term STZ-induced hyperglycaemic period of 7 days is necessary to induce expression at the proximal tubule BBM. The expression of GLUT2 into the proximal tubule BBM correlates with PKC-βI expression, suggesting that the signals to recruit GLUT2 also require a 7-day hyperglycaemic period for activation. Compared with previous studies in type I diabetes, where renal GLUT2 is rapidly abolished after overnight fasting, present data indicates that the mechanism of increased GLUT2 expression at the renal BBM is different from its removal from the membrane. Furthermore, the data also shows that renal PKC-βI and GLUT2 may be activated via a separate, rapid-response signalling pathway to increase glucose reabsorption during osmotic stress.
8 Renal glucose transport adaptations in rodent models associated with metabolic syndrome
8.1 Introduction

The experiments described in Chapter 7 demonstrated that renal GLUT2 expression at the proximal tubule BBM, via PKC-βI, requires a 7-day streptozotocin (STZ)-induced hyperglycaemic stimuli, although osmotic shock results in a far more rapid response of GLUT2. Studies presented here, and in the literature (Dominguez et al., 1994a; Dominguez et al., 1994b; Chin et al., 1997; Marks et al., 2003; Goestemeyer et al., 2007; Albertoni Borghese et al., 2009) have been performed to understand proximal tubular glucose handling in type I diabetes however less is understood in the insulin-independent form of the disease, type II diabetes. Studies from the small intestine imply that a basolateral insulin stimulus is responsible for the postprandial recruitment of GLUT2 to the enterocyte BBM however similar studies have yet to be undertaken to assess the effect of insulin on proximal tubular GLUT2 (Tobin et al., 2008). Renal glucose handling in response to the metabolic models associated with the onset of type II diabetes, such as obesity, have also been assessed in this chapter. In addition, further understanding of glucose reabsorption in obesity and diabetes led to the study in response to palliative Roux-en-Y (RYGB) gastric bypass surgery.

8.1.1 Type II diabetes – the Goto-Kakizaki model

Currently, type II diabetes is the prevalent form of the disease with over 285 million sufferers worldwide and predictions for more than 438 million by 2030 (Diabetes UK report 2010). Unlike type I diabetes which can be induced in control animals using a single IV injection of alloxan or streptozotocin (Dunn et al., 1944; Rakieten et al., 1963), type II diabetes can be studied in genetically, experimentally and nutritionally altered animal models (Table 5.2). However, due to the complex nature of the disease, some models are related to an obese phenotype with leptin deficiency or resistance, for example, Zucker Diabetic Fatty (ZDF) rats (Pick et al., 1998), whereas other models also have an association with hypertension, for example the ZSF1 rat (Tofovic et al., 2001). The Goto-Kakizaki (GK) rat model was considered most appropriate for the studies reported in this thesis due to its normotensive, non-obese
phenotype; this makes the GK rat one of the best characterised models for studying complications in type II diabetes. The GK rat was developed in 1976 by Goto et al., by the selective inbreeding of non-diabetic Wistar rats with glucose intolerance used as a selective index over many generations (Goto et al., 1976). As a result, GK rats carry a susceptibility locus for diabetes (known as Niddm1), which confers defective insulin secretion (Fakhrai-Rad et al., 2000). GK rats exhibit moderate and stable non-ketotic hyperglycaemia of around 15 mM displayed at 12 weeks of age, although glucose intolerance is displayed as early as 2 weeks of age with impaired insulin secretion (Adachi et al., 2003).

GK rats do not exhibit progressive renal disease, characterised by glomerulosclerosis, proteinuria or end-stage renal disease (ESRD); however, at 8-10 weeks of age they display thickening of the glomerular basement membrane, mild mesangial matrix expansion and glomerular hypertrophy, which results in both increased total mesangial volume and glomerular capillary luminal volume (Yogihashi et al, 1978;Phillips et al., 2001). The introduction of a further factor, such as hypertension, is necessary for the GK rat to become a model for renal disease; for example, Janssen et al showed that deoxycorticosterone salt (DOCA)-induced hypertension in GK rats caused an increase in renal damage with progressive and marked podocyte injury and proteinuria, as well as further tubulointerstitial damage (Janssen et al., 2003).

Therefore, since prolonged hyperglycaemia in the GK rat is not associated with changes in renal function, it is an appropriate model for the early pre-clinical phase of human diabetic nephropathy (Osterby et al., 1975). Early studies in diabetes showed an increase in carbohydrate digestion and glucose absorption in the small intestine, suggesting an insulin-dependent consequence of the disease (Stenling et al., 1984;Debnam et al., 1990;Fujita et al., 1998). Hyperglycaemia was believed to be the cause of the increased glucose absorption rate in type I and II diabetes, as a reversal was noted in response to insulin treatment (Caspary, 1973;Csaky et al., 1981;Thomson, 1983). Fujita et al found an increase in SGLT-mediated glucose absorption in response to post-prandial glucose in type II diabetic Otsuka Long Evans Tokushima Fatty (OLETF) rats with an increase in SGLT1 mRNA (Fujita et al., 1998). Additionally, STZ-induced type I diabetic rats exhibited up-regulated active glucose uptake, whereas Ferraris et al saw no change in
glucose absorption in STZ-induced type I diabetic mice (Debnam et al., 1990; Ferraris et al., 1993). However, a complete understanding of glucose absorption and the transporters responsible, in different regions of the small intestine is still unresolved.

8.1.2 Obesity and metabolic syndrome

The imbalance between energy intake (the consumption of processed foods with high-energy and low-fibre content) and energy expenditure (reduction in physical activity due to sedentary lifestyle) are strongly correlated with the prognosis for type II diabetes (Olefsky, 2001; Knowler et al., 2009). Specifically, an increase in the consumption of saturated and trans-saturated fats has been shown to adversely influence glucose metabolism and insulin resistance, whereas a low-glycaemic index diet, high in fibre can reduce the risk of type II diabetes (Hu et al., 2001; Hu et al., 2001). However, the onset of type II diabetes is also linked with genetic mutations and most variants are associated with pancreatic β-cell function (De Silva and Frayling, 2010). The junk-food-induced development of diabetes is more likely in OLETF rats, possessing the diabetogenic gene ODB-1, than the control rats lacking the ODB-1 gene (Ishida et al., 1996; De Silva et al., 2010). Also, the excess adiposity in OLETF rats fed a cafeteria diet, compared with those fed a chow diet, results in increased risk of type II diabetes (Okauchi et al., 1995); thus suggesting that both genetic risk factors and feeding-induced obesity together result in the high incidence of type II diabetes.

As well as being a cause of type II diabetes, central obesity (classified as weight circumference ≥ 94cm for men and ≥ 80cm for women by the 2006 IDF) is the most dominant risk-factor for developing metabolic syndrome (Lorenzo et al., 2003). Both hypertension (classified as systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg by the 2006 IDF) and fasting hyperglycaemia (classified as ≥ 5.6 mM by the 2006 IDF) are also symptoms of metabolic syndrome (Khunti and Davies, 2005; Reaven, 2005). Indeed, patients who suffer from metabolic syndrome and hypertension are more at risk of developing type II diabetes (Cooper-DeHoff et al., 2010). Both type I and type II diabetes have been associated with metabolic syndrome but a recent study found that
69.5% patients with type II diabetes had metabolic syndrome, whereas only 22% patients with type I diabetes suffered metabolic syndrome (AlSaraj et al., 2009). There is also considerable data to show that the increase in adipocytes in a high-fat diet model causes insulin resistance, which is a determining factor in the development of type II diabetes (Unger, 1991; Yki-Jarvinen, 2002; Despres and Lemieux, 2006). In fact only 5 days of high-fat feeding in healthy humans is necessary to raise plasma glucose by 0.5 mM, with an accompanying increase in insulin secretion due to hepatic insulin resistance (Brons et al., 2009).

Although the literature clearly indicates a very tight relationship between obesity and the onset of metabolic syndrome and type II diabetes (Figure 5.3) (Must et al., 1999; Mokdad et al., 2001; Knowler et al., 2009), there are few studies on renal glucose handling in obesity or metabolic syndrome as a model of prediabetes. To understand further renal glucose transport in diabetes, the factors leading to the disease were studied in feeding studies with both a junk-food diet, a model to reflect the etiology of dietary obesity in humans, and a high-fat diet, an established model of insulin-resistance and obesity.

### 8.1.2.1 Junk-food diet

The epidemic of type II diabetes has been attributed to an increase in obesity due to junk-food within the Western diet; therefore, to adequately simulate prediabetes, a feeding study using processed ‘human’ junk foods was established. Feeding a palatable junk-food diet to rats results in obesity and glucose intolerance, but the underlying mechanisms leading to these symptoms have not been adequately elucidated (Kiens and Richter, 1996; Petry et al., 1997).

In 1976, Sclafani and Springer set up a feeding study, the ‘cafeteria diet’, in control rats to study the effects of dietary junk-food-induced obesity, rather than overfeeding of chow, on feeding behavioural patterns (Sclafani and Springer, 1976). The cafeteria diet comprised of supermarket-purchased palatable foods, including chocolate chip cookies, salami, cheese, marshmallows and peanut butter. This diet has been used
more recently as a model of dietary obesity to study different facets of obesity such as the physiological and neurological causes and consequences of compulsive eating behaviour (Heyne et al., 2009), and the regulation of the leptin receptor (Milagro et al., 2006).

Interestingly, the junk-food diet has been shown to promote elongation of the small intestine, due to the reduced fibre content of the diet, increased food storage capacity and calorie intake occurring in response to the diet (Planas et al., 1992; Scoaris et al., 2010). These alterations in the handling of the diet are likely due to the atrophy in the small intestine that occurs from a lack of food in the diet, with reduced villus height and crypt depth in response to a low-fibre diet (Firmansyah et al., 1989; Scoaris et al., 2010). Furthermore, in rodents fed a high-fat diet, both alkaline phosphatase and α-glucosidase activities in the jejunum are increased compared with those maintained on a normal chow diet (Sefcikova et al., 2008), however, it has been shown that genetically obese mice exhibit significantly higher intestinal disaccharidase and alkaline phosphatase activity, which precedes the development of excess body weight (Flores et al., 1990).

The junk-food diet model used in the work presented here was set up in normal Wistar rats given *ad libitum* access to a diet high in saturated fats, sugar and salt, and low in protein and fibre, including food items such as flapjacks, crisps and chocolate bars over an 8 week period to reflect human feeding patterns resulting in obesity.

**8.1.2.2 High-fat diet**

The relationship between insulin resistance, metabolic syndrome, and obesity, and type II diabetes was also studied using a high-fat diet. Chen et al showed that a 50%-fat diet increases the plasma concentrations of total cholesterol and triglycerides, both of which are heavily associated with metabolic syndrome; indeed the 2006 IDF classified reduced high-density lipoprotein (HDL) cholesterol < 1.03 mM in males and < 1.29 mM in females and raised triglycerides > 1.7 mM as symptoms of metabolic syndrome (Chen et al., 2010). In the same study, chronically restricting the
high fat diet by 50% of baseline daily food intake caused a reduction in both HDL cholesterol and triglycerides.

The few renal studies using a high-fat model show conflicting data with regards to kidney function: a high-fat diet fed for 3 months to C57BL/6J mice caused an increase in urine flow rate and sodium excretion rate, although no hypertension or kidney dysfunction was apparent (Noonan and Banks, 2000). However, Song et al showed that rats fed a diet high in fats for 4 weeks had reduced fractional excretion of sodium and a blunted down-regulation of aldosterone-mediated effects, resulting in increased mean arterial pressure, which is associated with metabolic syndrome (Song et al., 2004).

The insulin-resistant nature of animals fed a high-fat diet, implies that a dysregulation of glucose handling occurs, therefore it was of interest to study renal glucose transport in the high-fat model. This insulin-resistant model was set up by allowing control Wistar rats ad libitum access to 60% fat-as-calories chow (Research Diets, New Brunswick, USA) largely comprised of soybean oil and lard for 5 weeks.

8.1.2.3 RYGB surgery

There are three main treatment methods for obesity: 1) lifestyle changes, including a controlled diet and exercise regime (Tuomilehto et al., 2001;Knowler et al., 2009), 2) pharmacotherapy such as orlistat and sibutramine (Wadden et al., 2005;Powell and Khera, 2010), and 3) gastric bypass surgery (Rand and Macgregor, 1991;Sagar, 1995). Gastric bypass surgery involves the formation of a small stapled proximal stomach which limits the capacity for food intake (Kral, 1987). Gastric bypass can be classed as either restrictive, including gastric banding and sleeve gastrectomy, or malabsorptive, such as jejunoileal bypass, a technique considered too extreme and therefore no longer used (Brolin et al., 2002) or hybrid, of which Roux-en-Y gastric bypass (RYGB) surgery is considered the ‘gold-standard’ treatment.
RYGB involves the transaction of the stomach into a small pouch, redirection of a 10 cm duodenal segment, and anastomosis to a 50 cm jejunal loop that joins a 25 cm segment of the ileum (Bueter et al., 2010). Thus, the majority of the stomach and the entire duodenum (biliopancreatic limb) are bypassed and food moves through the small gastric pouch immediately into the jejunum (alimentary limb), and then the ileum (common limb) before reaching the cecum (Figure 8.1).

RYGB can be performed laparoscopically and has been shown to abolish hypertension and hypertriglyceridemia, as well as resulting in weight loss (Wittgrove et al., 1996; Lima et al., 2010). This reduction in body weight, post-RYGB, has been attributed to an increase in postprandial PYY and GLP-1, thus providing enhanced satiety and an appropriate physiological environment for weight loss (Le Roux et al., 2006).

**Figure 8.1** – Schematic diagram of the physiological intestinal alterations in RYGB surgery – A: before RYGB operation ➔ indicates the movement of food, B: after RYGB operation ➔ indicates movement of food and grey area is the bypassed region of the gastrointestinal system.
Interestingly, RYGB surgery has also been shown to ablate both type I and type II diabetes (Pories et al., 1995; MacDonald, Jr. et al., 1997; Ukkola, 2009; Czupryniak et al., 2010), with the effect occurring rapidly, within 24 hours of surgery, and before any significant weight loss (Schauer et al., 2003; Andreelli et al., 2009; Hall et al., 2010). Follow-up studies between 6 and 168 months post-RYGB with human type II diabetic patients have shown on average 80% of patients exhibit abolished diabetes, and resolution was dependent on the length of diabetes and pancreatic reserve (Pories et al., 2001; Schauer et al., 2003; Sugerman et al., 2003; Torquati et al., 2005).

However there is limited data on the impact of gastric bypass surgery on glucose homeostasis, as mediated by the gut-renal axis, following gastric bypass surgery. Thus RYGB is an interesting model in which to study renal and intestinal glucose handling and transport because, as well as its ability to treat obesity, RYGB also results in the immediate normalisation of glycaemia in diabetes.

8.2 Aims of chapter

The aims of this chapter are to study renal glucose transport across the BBM in type II diabetes as a comparison with the type I diabetes study in the previous chapter. Furthermore, glucose transport at the proximal tubule BBM was also assessed in models of metabolic syndrome induced by feeding studies, as a precursor to type II diabetes. With the epidemic of metabolic syndrome and obesity escalating, renal glucose handling in models preceding type II diabetes is an increasing area of interest. The palliative RYGB surgery was studied in a rodent model to understand the role of renal and intestinal glucose transport in the improved glucose homeostasis following surgery.
8.3 Results

8.3.1 Type II diabetes

8.3.1.1 Animal parameters of the Goto-Kakizaki (GK) rat

The GK rats had significantly reduced body weight compared with the age-matched lean Wistar rats (316.6 ± 5.2 g compared with 397 ± 7.4 g) and, although there was no change in kidney weight, the ratio of kidney-to-body weight was 16% higher in the GK animals (Table 8.1 - 1). In keeping with the type II diabetic disease model, the rats exhibited significantly elevated plasma glucose concentrations of 20.3 ± 1.2 mM and an accompanying 7% increase in plasma osmolality (Table 8.1 - 2). There was a 23.9% increase in circulating insulin levels in GK rats compared with lean controls, although the elevation was not significant (Table 8.1 - 2). Interestingly, the urine glucose concentration in the initial five GK animals studied was 14.2 ± 0.5 mM, at a plasma glucose level of 17.8 ± 0.4 mM (Figure 8.2). However, in the final three type II diabetic rats assessed on consecutive days glycosuria was amplified to 59.3 ± 3.9 mM, at a plasma glucose level of 24.3 ± 0.9 mM (Figure 8.2).

Figure 8.2 – Scattergraph of the relationship between glycosuria and hyperglycaemia in 8-9 week lean (diamond points) and type II diabetic GK (square points) rats
Table 8.1 – 1) Body and kidney weight, and kidney-to-body weight ratio and 2) Plasma glucose, osmolality and basal circulating insulin measurements in 8-9 week lean and type II diabetic GK rats. Values are expressed as mean ± SEM, n=8, ***p<0.005, ****p<0.001 compared with lean.

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th>GK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>397 ± 7.4</td>
<td>316.6 ± 5.2   ****</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>2.51 ± 0.42</td>
<td>2.38 ± 1.05</td>
</tr>
<tr>
<td>Kidney:body weight ratio</td>
<td>6.33 ± 0.1</td>
<td>7.53 ± 0.35   ***</td>
</tr>
</tbody>
</table>

2

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th>GK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose (mM)</td>
<td>13.28 ± 0.41</td>
<td>20.26 ± 1.25  ****</td>
</tr>
<tr>
<td>Plasma osmolality (mOsM)</td>
<td>329 ± 2</td>
<td>353 ± 3      ****</td>
</tr>
<tr>
<td>Basal plasma insulin (ng.ml⁻¹)</td>
<td>4.83 ± 1.12</td>
<td>6.35 ± 1.38</td>
</tr>
</tbody>
</table>

8.3.1.2 Renal BBM vesicle glucose uptakes in GK type II diabetic rat

8.3.1.2.1 Validation of BBM vesicles

The BBM vesicles freshly-prepared from control lean Wistar animals and type II diabetic GK animals showed the same enrichment values (lean BBM vesicles were 6.26 ± 1.04-fold enriched compared with 6.26 ± 0.68-fold enrichment in type II animals) as assessed with alkaline phosphatase marker enzyme. There was also no significant change in intravesicular volume measured after 15 minute incubation using 100 μM glucose concentration, with vesicle trapped space in lean animals 2.84 ± 0.23 μl.mg protein⁻¹ compared with 3.6 ± 0.6 μl.mg protein⁻¹ in type II animals. To verify the ability of BBM vesicle to accumulate glucose, sodium-dependent glucose transport was determined at different time points using 100 μM glucose. At 40 seconds, an early peak in glucose uptake was noted (Figure 8.3), as has been previously shown (Marks et al., 2003), which is due to a rapid influx of Na⁺ ions into the BBM vesicle. There was complete inhibition of the overshoot in the presence of phloridzin, which was consistently shown throughout the glucose uptake experiments at this concentration of glucose (Figure 8.3).
8.3.1.2.2 Renal BBM glucose transport in GK type II diabetic rats

In renal BBM vesicles from GK animals there was a significant increase in SGLT-mediated glucose uptake between 30-960 μM glucose concentration (Figure 8.4 - 1), with a 40% increase in the $V_{\text{max}}$ of SGLT-mediated glucose transport (Figure 8.4- 2). This was accompanied by a significant increase in $K_m$ (lean $K_m$ $147.1 \pm 21.6$ μM compared with GK $K_m$ $264.3 \pm 46$ μM) of SGLT-mediated glucose transport (Figure 8.4 - 3). Although there was no effect of type II diabetes on total glucose transport at 20 mM, the phlorizin-insensitive uptake was augmented in the GK rats (Figure 8.5). GLUT-mediated transport was elevated from $1660.1 \pm 380.8$ pmoles.mg protein$^{-1}$ in lean animals to $3809.5 \pm 426.4$ pmoles.mg protein$^{-1}$ in type II diabetic animals.

Figure 8.3 – Representative graph of the time-dependent overshoot seen for renal BBM vesicle glucose uptake at 100μM glucose concentration, in the presence (dotted line) and absence of phloridzin (solid line)
Figure 8.4 – Effect of type II diabetes in GK rats (broken line) on SGLT-mediated glucose uptake (1) Glucose concentration curve (2) $V_{\text{max}}$ and (3) $K_m$ of glucose uptake derived from BBM vesicles incubated at glucose concentrations between 30-960 μM for 4 seconds. Values are expressed as mean ± SEM, n=6-8, *p<0.05, ***p<0.005 compared with lean control rats (solid line).

1

2

3
Figure 8.5 – Effect of type II diabetes on total glucose uptake (open bars) and GLUT-mediated glucose uptake, in the presence of phloridzin (closed bars) using renal BBM vesicles incubated in 20 mM glucose for 4 seconds. Mean ± SEM, n=6-8, ***p<0.005 compared with lean control rats.

8.3.1.3 Effect of type II diabetes on BBM expression of renal glucose transporters

The proximal tubule expression of GLUT2, PKC-βI, GLUT5 and SGLT1 was assessed in BBM prepared from the GK animals, to assess the cause of the increased uptake outlined in section 8.3.1.2.2. There was a significant elevation in both GLUT2 and GLUT5, with an increase of 1.4- and 1.5-fold, respectively (Figure 8.6), in response to type II diabetes. Expression of the GLUT2 activator PKC-βI exhibited a 2-fold increase in type II diabetic animals and BBM expression of the other glucose transporter studied, SGLT1, was also significantly augmented in GK animals (Figure 8.6).
**Figure 8.6** - Effects of type II diabetes in GK animals (D, closed bars) on expression of GLUT2 and GLUT5, PKC-βI and SGLT1 at the proximal tubule BBM. 1) Western blots 2) quantification of western blots (expressed as percentage BBM expression), standardised to β-actin. Values are expressed as mean ± SEM, n=6-8, *p<0.05, ****p<0.001 compared with lean control animals (C, open bars)
8.3.2 Junk-food model

The augmentation of glucose transport in both STZ-induced type I diabetes and the non-obese GK model of type II diabetes, raised the interesting question of whether a pre-diabetic metabolic model would also be associated with changes in expression of renal BBM glucose transporters. The junk-food model was set up to assess this using an energy-rich diet of palatable processed foods over an 8-week time period to mimic a Western diet.

8.3.2.1 Animal parameters following a junk-food diet

Experimental animals were allowed *ad libitum* access to both chow and a combination of sugary and fatty human foods, including marshmallows and cheese. Figure 8.7 – 1 shows that rats consumed a similar amount of junk-food (20.82 ± 0.61 g) as control animals maintained on normal chow (20.53 ± 0.19 g). 31.4% of the administered junk-food was consumed and Figure 8.7 – 2 shows the amounts of each food eaten per animal per day. The control animals had significantly reduced weight gain compared with the junk-food fed animals (Figure 8.8 - 1) and over the 8-week feeding period the experimental rats put on weight at a significantly accelerated rate compared with the control group (Figure 8.8 - 2). There was a 20.1% increase in plasma glucose concentration in the junk-food fed animals (Figure 8.9 - 1) and a 25% increase in urine glucose concentration. There was also a significant 327% elevation in circulating basal insulin levels in junk-food fed animals (Figure 8.9 - 2).
Figure 8.7 - 1) Daily weight of chow consumed by control and experimental animals (open bars) and junk-food consumed by experimental animals (closed bar) 2) Daily components of junk-food diet consumed by experimental rats. Values are expressed as mean ± SEM, n=6-12 animals monitored daily for 55 days.
Figure 8.8 - Body weight increase in response to 8 weeks junk-food diet 1) Total weight gain in control chow-fed animals and experimental junk-food-fed animals at time of cull 2) Progressive weight gain in chow-fed (diamond points) and junk-food-fed (square points) animals over 8 week feeding period. Values are expressed as mean ± SEM, n=6-12, *p<0.05, ***p<0.005 compared with chow-fed animals.
Figure 8.9 - 1) Effect of chow and junk-food diet on plasma (open bar) and urine (closed bar) glucose concentration and 2) plasma insulin levels. Values are expressed as mean ± SEM, n = 6-12, **p<0.01, ***p<0.005 compared with chow-fed controls.

8.3.2.2 Effect of junk-food diet on glucose transporter expression at the proximal tubule BBM

Kidneys from chow and junk-food fed animals exhibited no difference in weight, with chow-fed rat kidneys weighing 1.10 ± 0.02 g compared with 1.13 ± 0.03 g for junk-food rat kidneys. There was also no significant change in the enrichment values from the renal BBM vesicles prepared from both chow and junk-food fed animals (chow-fed rat BBM vesicles were 6.34 ± 0.41-fold enriched compared with 7.08 ± 0.5-fold enrichment in junk-food fed rats). There was a 5.8-fold increase in GLUT2 expression at the proximal tubule BBM in junk-food fed animals, which was accompanied by a 1.7-fold increase in PKC-βI expression (Figure 8.10). The junk-food diet also caused a significant elevation in SGLT1 transporter expression at the renal BBM (Figure 8.10), and a 3-fold increase in GLUT5 expression.
Figure 8.10 – Effects of 8-week junk-food diet (JF, solid bars) on expression of GLUT2 and GLUT5, PKC-βI and SGLT1 expression at the proximal tubule BBM. 1) Western blots 2) quantification of western blots (expressed as percentage BBM expression) standardised to β-actin. Values are expressed as mean ± SEM, n=6-8, *p<0.05, **p<0.01, ***p<0.005 compared with chow-fed control animals (C, open bars)
8.3.3 High-fat diet

The amplification of renal GLUT2, GLUT5, and SGLT1 expression in response to the energy-rich processed food fed during the junk-food study led to the question as to whether the transporters were responding to a fat-specific, obesity stimulus in the junk-food. The high-fat, insulin-resistant model was used (animals were prepared by Dr. Cleasby, Royal Veterinary College, London) to assess this; Wistar rats were given *ad libitum* access to a 60% fat-as-calories chow over a 5-week period.

8.3.3.1 Animal parameters following a high-fat diet

Animals fed a high-fat diet exhibited a significant increase in body weight (Figure 8.11), which was matched by a 9.3% increase in kidney weight (Figure 8.11). There was also a significant increase in epididymal (EPI) fat pad mass from 1.91 ± 0.07 g in chow-fed animals to 3.23 ± 0.34 g in high-fat fed animals. Although there was a significant 10.8% increase in fasted plasma glucose concentration of high-fat fed animals, values remained within the normal range (Table 8.2). Both the chow and high-fat fed animals had no detectable glucose in the urine. Basal insulin levels were increased by 51.3% in high-fat fed rats, with a 34.9% increase in circulating insulin after the insulin tolerance test (Table 8.2).

**Figure 8.11** – Final body (bar chart) and kidney weights (broken line) in rats fed chow or high-fat diet. Values are expressed as mean ± SEM, n=6-8, *p<0.05 compared with chow body weight, #p<0.05 compared with chow kidney weight.
Table 8.2 – Fasted plasma concentrations of glucose, basal insulin and insulin concentration following the insulin tolerance test. Values are expressed as mean ± SEM, n = 6-8, *p<0.05, ***p<0.005 compared with chow-fed controls

<table>
<thead>
<tr>
<th></th>
<th>Chow</th>
<th>High-fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting plasma glucose (mM)</td>
<td>4.17 ± 0.14</td>
<td>4.67 ± 0.17 *</td>
</tr>
<tr>
<td>Basal plasma insulin (ng.ml⁻¹)</td>
<td>4.91 ± 0.87</td>
<td>10.07 ± 1.76 *</td>
</tr>
<tr>
<td>Plasma insulin post-tolerance test (ng.ml⁻¹)</td>
<td>15.5 ± 1.93</td>
<td>23.8 ± 1.94 ***</td>
</tr>
</tbody>
</table>

8.3.3.2 Effect of high-fat diet on glucose transporter expression at the proximal tubule BBM

There was no significant change in the alkaline phosphatase enrichment from renal BBM vesicles prepared from chow and high-fat fed animals, with 8.23 ± 0.75-fold enrichment and 7.25 ± 0.92-fold enrichment, respectively. A significant 3-fold increase in GLUT2 transporter expression was noted in the renal BBM vesicles prepared from animals fed the high-fat diet for 5 weeks compared with chow fed animals (Figure 8.12). There was also an increase in the GLUT2 activator, PKC-βI, although no change in SGLT1 or GLUT5 expression was seen (Figure 8.12).
Figure 8.12 – Effects of 5-week high-fat diet (HF, solid bars) on GLUT2 and GLUT5, PKC-βI and SGLT1 expression at the proximal tubule BBM. 1) Western blots 2) quantification of western blots (expressed as percentage BBM expression), standardised to β-actin. Values are expressed as mean ± SEM, n=6-8, *p<0.05, ***p<0.005 compared with chow-fed control animals (C, open bars)
8.3.4 Roux-en-Y Gastric Bypass (RYGB) surgery

The increase in GLUT2 transporter in the outlined models of diabetes and obesity was investigated further in a rodent model of RYGB surgery. As well as an effective treatment for weight loss in obesity, gastric bypass surgery also results in the rapid resolution of hyperglycaemia in type I and II diabetes. The response of glucose handling to duodenal bypass was studied in rats 60 days after RYGB surgery.

8.3.4.1 Animal parameters following RYGB surgery

Before surgery, animals in both the sham and RYGB group were non-obese and had matched body weight (355.6 ± 6.37 g and 346.2 ± 4.26 g, respectively). At 60 days post-surgery, the RYGB animals exhibited a 4% increase in mean body weight compared with a 30% increase in those subjected to sham surgery (Figure 8.13 - 1). There was no significant change in fasting plasma glucose concentrations between the sham (8.53 ± 0.59 mM) and RYGB (8.48 ± 0.83 mM) animals. Consequentially, there was no difference between plasma osmolality measurements (313.2 ± 1.4 mOsM and 313.4 ± 3.4 mOsM after sham and RYGB surgery, respectively); also both sham and RYGB-operated animals displayed no glycosuria but there was a 192.7% reduction in circulating insulin levels in animals 60 days after RYGB (Figure 8.13 - 2). Kidneys from RYGB animals exhibited a significant 20.6% decrease in weight compared with those from sham operated animals.
**Figure 8.13** – Animal parameters following RYGB surgery 1) Body weight and 2) fasted basal plasma insulin levels in animals 60 days after sham (solid line) or RYGB (broken line) surgery. Values are expressed as mean ± SEM, n = 8-20, ***p<0.005, ****p<0.001 compared with sham surgery control.
8.3.4.2 Effect of RYGB surgery on glucose transporter expression at the proximal tubule BBM

There was no change in renal BBM vesicle enrichment between the two surgical groups of animals, following sham surgery BBM vesicles were 5.66 ± 0.54-fold enriched compared with 6.34 ± 0.51-fold enrichment after RYGB surgery. Following RYGB surgery, there was a 2-fold decrease in GLUT2 expression at the proximal tubule BBM (Figure 8.14). This was accompanied by a significant reduction in PKC-βI expression and a 2-fold decrease in GLUT5 expression; however, no change in SGLT1 expression was seen (Figure 8.14).

Figure 8.14 – Effects of RYGB surgery (RY, solid bars) on GLUT2 and GLUT5, PKC-βI and SGLT1 expression at the proximal tubule BBM. 1) Western blots 2) quantification of western blots (expressed as percentage BBM expression), standardised to β-actin. Values are expressed as mean ± SEM, n=6, *p<0.05 compared with sham surgery controls (S, open bars)
8.3.4.3 Effect of RYGB surgery on gene expression of glucose transporters in the renal cortex

There was a trend for reduced mRNA expression of glucose transporters in the renal cortex from animals post-RYGB surgery. Although the decline in mRNA expression of SGLT1, SGLT2 and GLUT5 did not reach significance, the reduction was 52.1%, 74.7% and 59.4%, respectively (Figure 8.15). GLUT2 mRNA expression in renal cortex from animals post-RYGB surgery was significantly reduced, with a 79.8% decrease compared to that seen in animals after sham surgery (Figure 8.15).

**Figure 8.15** – Effects, after 60 day sham (open bars) or RYGB surgery (closed bars), on SGLT1, SGLT2, GLUT2 and GLUT5 mRNA expression in renal cortex. Values are expressed as mean ± SEM standardised to β-actin, n=6-8, ***p<0.005 compared with sham surgery control animals.
8.3.4.4 Effect of RYGB surgery on glucose transporter expression at the enterocyte BBM

The regional profile of BBM glucose transporter expression was studied in the small intestine after sham and RYGB surgery (Figure 8.16). There was a 2-fold increase in GLUT2 expression at the BBM in the alimentary limb of RYGB animals, although this did not reach statistical significance (Figure 8.16). Expression of GLUT2 in the bypassed biliopancreatic limb of RYGB animals was unchanged from that seen in the non-bypassed duodenum in sham operated animals (Figure 8.16). Expression of GLUT5 was significantly reduced in the biliopancreatic limb of animals post-RYGB compared with that seen in animals after sham surgery (Figure 8.16). However, no difference was noted between GLUT5 expression in the jejunum of sham animals and the alimentary limb of RYGB animals (Figure 8.16). There was a significant increase in the jejunal GLUT2 activator, PKC-βII, and SGLT1 expression in the alimentary limb of RYGB animals, but no change in expression of GLUT2 in the biliopancreatic limb (Figure 8.16).
Figure 8.16 - Effects of RYGB surgery (RY, closed bars) on GLUT2 and GLUT5, PKC-βII and SGLT1 BBM protein expression in the small intestine 1) Western blots 2) quantification of western blots (expressed as percentage BBM expression), standardised to β-actin. Values are expressed as mean ± SEM, n=4-6, *p<0.05, **p<0.01 compared with sham surgery controls (S, open bars)

<table>
<thead>
<tr>
<th></th>
<th>Jejunum</th>
<th>Duodenum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GLUT2</strong></td>
<td><img src="image" alt="Jejunum GLUT2" /></td>
<td><img src="image" alt="Duodenum GLUT2" /></td>
</tr>
<tr>
<td><strong>GLUT5</strong></td>
<td><img src="image" alt="Jejunum GLUT5" /></td>
<td><img src="image" alt="Duodenum GLUT5" /></td>
</tr>
<tr>
<td><strong>Actin</strong></td>
<td><img src="image" alt="Jejunum Actin" /></td>
<td><img src="image" alt="Duodenum Actin" /></td>
</tr>
<tr>
<td><strong>PKC-βII</strong></td>
<td><img src="image" alt="Jejunum PKC-βII" /></td>
<td><img src="image" alt="Duodenum PKC-βII" /></td>
</tr>
<tr>
<td><strong>SGLT1</strong></td>
<td><img src="image" alt="Jejunum SGLT1" /></td>
<td><img src="image" alt="Duodenum SGLT1" /></td>
</tr>
</tbody>
</table>

- GLUT2: 58 kDa
- GLUT5: 55 kDa
- Actin: 42 kDa
- PKC-βII: 79 kDa
- SGLT1: 75 kDa

Values are expressed as mean ± SEM, n=4-6, *p<0.05, **p<0.01 compared with sham surgery controls (S, open bars)
8.3.4.5 Effect of RYGB surgery on gene expression of glucose transporters in the small intestine

The mRNA expression profile of intestinal glucose transporters from sham and RYGB surgery animals was also studied (Figure 8.17). mRNA expression of GLUT2 was increased significantly by 266%, in the alimentary limb from RYGB rats (Figure 8.17 - 1). No change in GLUT2 mRNA was noted in the duodenum or ileum from animals following sham surgery compared with the biliopancreatic limb following RYGB surgery (Figure 8.17 - 1). There was a 250% reduction in GLUT5 expression in the biliopancreatic limb following RYGB surgery, whereas GLUT5 mRNA expression in the alimentary limb showed no significant change, as did GLUT5 mRNA expression in the common limb of RYGB animals (Figure 8.17 - 2).

Figure 8.17 – Regional mRNA expression profile, 60 days after sham (open bars) or RYGB surgery (closed bars), on 1) GLUT2 and 2) GLUT5 in the small intestine. Values are expressed as mean ± SEM, standardised to β-actin, n=5-6, *p<0.05, ***p<0.005 compared with sham control animals
8.4 Discussion

Type II diabetes in the western population is reaching epidemic levels and the relationship between obesity, metabolic syndrome and type II diabetes has been well established. In the studies presented here, renal glucose handling in these models has been assessed to understand potential mechanisms of glycaemia in type II diabetes, pre-diabetic diet-induced obesity, and following gastric bypass surgery. The animal parameters and expression of glucose transporters at the proximal tubule BBM in each metabolic model studied are summarised in Table 8.3.

Table 8.3 - Summary table of effects of type II diabetes, and metabolic models associated with the disease, on animal parameters and expression of glucose transporters at the proximal tubule BBM. # represents animals which were fasted before cull.

<table>
<thead>
<tr>
<th></th>
<th>Body weight</th>
<th>Kidney weight</th>
<th>Plasma glucose</th>
<th>Urine glucose</th>
<th>Plasma insulin</th>
<th>Proximal tubule BBM expression of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GLUT2</td>
</tr>
<tr>
<td>Type II diabetes</td>
<td>↓</td>
<td>↔</td>
<td>↑</td>
<td>↑</td>
<td>↔</td>
<td>↑</td>
</tr>
<tr>
<td>Junk food diet</td>
<td>↑</td>
<td>↔</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>High-fat diet #</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↔</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>RYGB #</td>
<td>↓</td>
<td>↓</td>
<td>↔</td>
<td>↔</td>
<td>↓</td>
<td>↑</td>
</tr>
</tbody>
</table>
Type II diabetes was studied using GK rats, since this model has been shown to have minimal effect on obesity and renal physiology, but still maintains the disease phenotype of glycaemia and insulin-era dysregulation. In accordance with human type II diabetic patients, body weight was reduced in GK rats, but the kidney-to-body-weight ratio was elevated. Interestingly, there was a progressive rise in glucose excretion (Figure 8.2) from the initial five GK rats studied, where modest glycosuria with characteristic glycaemia was presented, to the final three type II diabetic rats examined, which exhibited a small 4 mM rise in plasma glucose and a significant elevation in glycosuria to levels similar to those in STZ-induced type I diabetes. The development of glycosuria in the later animals did not coincide with changes in SGLT or GLUT-mediated glucose transport or transporter expression, suggesting that the progressive increase in plasma glucose, as part of the disease phenotype in GK rats, reaches a threshold concentration after which further rises in filtered glucose cannot be reabsorbed, and glycosuria is amplified. However, it is worth noting that glucose concentration measured in urine is a rough index of glucose excretion and glomerular filtration rate (GFR) experiments would be necessary to establish the net glucose excretion in these type II diabetic animals.

In type I diabetes, insufficient insulin is produced due to autoimmune destruction of pancreatic β-cells and patients present with reduced plasma insulin levels (Foulis et al., 1986). In contrast, type II diabetes is associated with normal or increased insulin levels due to peripheral insulin resistance (Saltiel and Kahn, 2001; Ahren et al., 2004; Reaven, 2005). At 4 weeks of age, GK rats have been found to exhibit deficient insulin secretion without the onset of peripheral insulin resistance (Picarel-Blanchot et al., 1996). However, data from GK rats obtained in the present study at 8-9 weeks of age exhibited no significant change in plasma insulin concentration, a finding also shown by Berthelier et al although this study showed a decrease in the efficacy of insulin on glucose utilisation in the whole body in GK rats (Berthelier et al., 1997).

To assess the efficacy of the purified BBM vesicles, sodium-dependent glucose transport across the proximal tubule BBM was demonstrated by measuring glucose uptake into the BBM vesicles (Figure 8.3). The time-dependent overshoot at 100µM glucose was inhibited by phlorizin, a competitive inhibitor of SGLT-mediated glucose uptake. The early phase of glucose uptake, in the absence of phlorizin, occurs where
sodium ions and glucose are co-transported into the vesicle by SGLT1 and SGLT2. When the sodium gradient of the intravesicular space matches that outside the vesicle, SGLT-mediated glucose uptake is abolished and accumulated glucose diffuses out of the intravesicular space via facilitative transporters. The time-dependent overshoot was not seen in the presence of phlorizin, demonstrating that SGLT1 and SGLT2, are responsible for the majority of sodium-dependent glucose uptake at low glucose concentrations (100µM) (Marks et al., 2003).

Uptake data using BBM vesicle prepared from type II diabetic rats show an elevation in SGLT-mediated glucose transport, more specifically, both V_max and K_m were augmented, and this was accompanied by an increase in SGLT1 expression at the proximal tubule BBM. This data suggests that type II diabetes in GK rats causes an increase in the maximum capacity for SGLT-mediated glucose transport across the proximal tubule BBM, as well as a rise in transporter insertion; thus an increase in SGLT1 transporter expression correlates with elevated SGLT-mediated glucose uptake. The lack of a working SGLT2 antibody prevents the assessment of total SGLT expression in the proximal tubule, although Hummel et al have recently shown that SGLT1 had 10-fold higher maximal transport rate for glucose transport than SGLT2 (Hummel et al., 2011). Furthermore, they showed that human SGLT1 and SGLT2 expressed in human embryonic kidney cells exhibited an affinity for glucose of 2 mM and 5 mM, respectively; therefore, it is likely that a high percentage of SGLT-mediated glucose transport seen in the type II diabetic GK rats is due to SGLT1 (Hummel et al., 2011). Despite this, the relationship between increased glucose uptake and SGLT2 expression in vivo has not been established. Albertoni Borghese et al showed that there was an increase in α-methyl glucose uptake at 14 days STZ-induced type I diabetes; although accompanied by a slight increase in SGLT2 expression at the BBM, the elevated transport could not be fully attributed to SGLT2. Indeed, there was no positive correlation between SGLT-mediated transport and SGLT2 expression (Albertoni Borghese et al., 2009). However, in STZ-induced type I diabetes it has been shown here (Chapter 7) and in previous studies (Dominguez et al., 1994a;Marks et al., 2003) that SGLT1 is not up-regulated in the insulin-dependent form of the disease. In 5/6 nephrectomised rats, the resulting decrease in V_max of SGLT-mediated glucose uptake was attributed to the marked reduction in SGLT2 mRNA, as no change was seen in expression of SGLT1 mRNA;
however, no data were available on the BBM expression of the transporter (Nakamura et al., 2004). In vitro perfused tubule studies have shown that SGLT2 has a higher capacity for glucose transport ($V_{\text{max}}$ of 83 pmol/min per mm) than SGLT1 ($V_{\text{max}}$ of 7.9 pmol/min per mm) (Barfuss et al., 1981). Therefore, an increase in SGLT-mediated glucose uptake when there is high glucose in the tubular fluid would be attributable to the high capacity SGLT2 transporter. Interestingly, SGLT2 inhibitors currently in clinical trials such as dapagliflozin result in increased urinary glucose excretion and lower glycaemia in diabetes; however, these inhibitors cause between 50-60% reduction in glucose uptake, strongly suggesting that SGLT1 and SGLT2 are responsible for approximately equal proportions of glucose reabsorption by the kidney in type II diabetes (Han et al., 2008; Komoroski et al., 2009). Human studies have shown that the isolation and subculture of exfoliated proximal tubular epithelial cells from the urine of type II diabetic patients exhibit an increase in SGLT-mediated glucose transport, as well as augmented SGLT2 mRNA and protein expression, although no studies on SGLT1 were performed in these cells (Rahmoune et al., 2005). Elevated SGLT1 protein expression or SGLT-mediated glucose uptake were not seen in either short-term (Chapter 7) or chronic (Marks et al., 2003) STZ-induced type I diabetes, despite glycaemia in the type I model far exceeding that seen in GK rats in the present work. Therefore, renal SGLT1 transporter expression does not respond solely to plasma (and therefore proximal tubular) glucose concentrations. This conclusion is reinforced by the absence of a correlation between plasma glucose and SGLT1 expression in the type II diabetic GK studies presented here.

In addition to the amplification in SGLT-mediated transport, a rise in GLUT-mediated transport at 20 mM glucose concentration was also noted in renal BBM vesicles from type II diabetic rats. However, despite the augmentation of both SGLT- and GLUT-mediated transport, total glucose uptake at 20 mM, in the absence of phlorizin, was not significantly altered. SGLT-mediated transport did not reach saturation for either the lean or type II diabetic rats thus it is possible that, despite a higher $V_{\text{max}}$ and $K_m$ for transport, glucose uptake into BBM vesicles by SGLT may be matched in both groups at saturation. Thus the increase in GLUT-mediated transport at this glucose concentration may not be sufficient to significantly elevate total glucose uptake at 20 mM. To study this, glucose transport, via both GLUT’s and SGLT’s, would need to
be measured between 960 µM and 20 mM. The use of the GLUT inhibitor, phloretin, and an SGLT2 inhibitor, such as dapagliflozin, would enable the identification of the specific transporters involved in glucose transport across the proximal tubule BBM in response to type II diabetes.

Elevated GLUT-mediated glucose transport was accompanied by increases in expression of GLUT2, its activator PKC-βI, and GLUT5 transporter, at the proximal tubule BBM. This supports the relationship, originally postulated by Goestemeyer et al., between PKC-βI recruitment to the proximal tubule BBM and its signalling to shuttle GLUT2 to the BBM (Goestemeyer et al., 2007). The higher expression of GLUT2 emphasises that increased GLUT-mediated glucose uptake occurs concomitantly with a rise in GLUT2 transporter expression. Previous studies have shown that chronic STZ-induced type I diabetes also promotes an elevation in GLUT-mediated transport, together with increases in both GLUT2 and GLUT5 proximal tubule BBM expression (Marks et al., 2003). Therefore, the expression and capacity for glucose transport of renal GLUT2 and GLUT5 transporters may be responding to hyperglycaemia in both diabetes models, suggesting that increased tubular fluid glucose concentration in type I and type II diabetes results in the recruitment of GLUT2 and GLUT5 to the BBM. However it is worth noting that GLUT-mediated transport in type II diabetes exceeds that seen in type I diabetes (Marks et al., 2003), despite higher plasma glucose levels in the latter model; therefore, GLUT-mediated glucose reabsorption does not correlate with hyperglycaemia in the GK rat.

In vitro studies on the small intestine from the same GK rats used for renal glucose transport studies presented here were performed in our laboratory by Dr. E Debnam. Interestingly, using the everted sleeve technique, an increase in both GLUT- and SGLT-mediated glucose absorption was seen in both the jejunum and ileum (data not shown). The up-regulation of glucose absorption in the small intestine in type II diabetes is also noted in the fatty type II diabetic OLETF rat, with hyperglycaemia in the disease believed to be the main cause, as insulin treatment reverses the augmented glucose absorption (Caspary, 1973;Csaky et al., 1981;Thomson, 1983;Fujita et al., 1998). Thus, glucose transport is up-regulated at the BBM via elevated abundance of GLUT’s and SGLT’s in both the small intestine and kidney during type II diabetes;
this represents how glucose homeostasis is likely regulated during the disease and poses a mechanism for the exacerbation of hyperglycaemia during diabetes.

Interestingly, in both diet-induced models of insulin-resistance, the junk-food and high-fat feeding studies, GLUT2 expression was up-regulated. The insulin receptor has been identified at sites all along the nephron, including the proximal tubule, with a higher proportion of expression at the basolateral, rather than brush-border, membrane (Hammerman, 1985; Feraille et al., 1995). Thus it is possible that high circulating insulin provides a basolateral stimulus to promote GLUT2 recruitment to the proximal tubule BBM. Circulating insulin levels in type II diabetes, another model of insulin resistance, are elevated (Alberti et al., 1992); however although there was a rise in plasma insulin levels in the GK rat, this did not reach significance. Furthermore, in type I STZ-induced diabetes, where plasma insulin levels are down-regulated, GLUT2 expression and GLUT-mediated glucose transport were elevated (Gepts, 1965; Marks et al., 2003). Therefore the data presented here suggests that GLUT2 may exhibit increased expression at the proximal tubule BBM via an insulin-dependent or – independent pathway, depending on glycaemia and disease state.

Diabetes and diet-induced insulin-resistance is associated with an up-regulation in the resistin-like molecule RELMβ, a gut-derived hormone responsible for energy homeostasis, for example the hormone affects GLUT2 and SGLT1 expression in the jejunum; therefore, it is interesting to consider whether the hormone is having a similar effect on renal GLUT2 and SGLT1 (Shojima et al., 2005; Krimi et al., 2009). This could lend support to the idea that gut-derived hormones, such as GLP-1, control glucose transporter recruitment to the proximal tubule BBM (Marks et al., 2003).

Although GLUT2 is able to transport fructose, GLUT5 is the main fructose transporter and is expressed in fructose-metabolising tissue such as the small intestine, adipocytes, skeletal muscle and kidney (Hajduch et al., 1998; Mate et al., 2001; Hajduch et al., 2003). Additionally, GLUT5 has a limited capacity for transporting glucose (Bell et al., 1990); therefore, the raised renal BBM expression of GLUT5 in type II diabetic rats, as well as in type I diabetes (Marks et al., 2003), may be associated with increased plasma and tubular fluid glucose concentration. This is
emphasised by the type II diabetic GK model exhibiting a positive correlation between GLUT5 expression at the proximal tubule BBM and plasma glucose.

Fructose is known to be involved in the pathogenesis of diabetic complications due to the non-enzymatic fructosylation of proteins, its action in the polyol pathway, and carbonyl stress (McPherson et al., 1988). Also, increased fructose consumption results in glucose intolerance and hyperlipidaemia (Thorburn et al., 1989; Lee et al., 1994) and it has been strongly correlated with the increased incidence of type II diabetes and obesity (Havel, 2005; Montonen et al., 2007). Furthermore, type II diabetic patients also exhibit elevated serum fructose levels and excretion rates (Kawasaki et al., 2002); GLUT5 may be elevated at the renal BBM to handle the high-fructose load in the proximal tubular fluid. The increased renal GLUT5 expression at the BBM in response to type II diabetes in GK rats has also been noted in skeletal muscle (Stuart et al., 2007). Additionally, GLUT5 was overexpressed in the duodenum from type II diabetic patients (Dyer et al., 2002). Whether this is due to diabetes-induced changes in circulating fructose levels or whether it can be attributed to the ability of GLUT5 to transport glucose is currently unknown. Although the possibility of both factors affecting the transporter cannot be overlooked, the elevated GLUT5 expression at the BBM in both type I and II diabetes, where hyperglycaemia is noted suggests the latter is more likely. However, GLUT5 transports low concentrations of glucose and has been shown to be unchanged by a glucose-rich diet (Gouron et al., 2003). Elevated GLUT5 in type II diabetes is more likely to be a symptom of the disease (Rand et al., 1993; Burant and Saxena, 1994). It is also seen in type I diabetes with elevated GLUT5 mRNA levels in the proximal straight tubule following chronic STZ-induced type I diabetes (Chin et al., 1997).

Although there are many cell studies on renal glucose handling in response to a high-glucose stimulus (Heilig et al., 1995; Phillips et al., 1999; Phillips et al., 1999; De Lima E Santos et al., 2009), there is limited data on the expression of renal GLUTs in type II diabetes. Interestingly, studies with mesangial cells cultured from type II diabetic patients show enhanced GLUT1 expression (Liu et al., 2001). Expression of GLUTs in GK rats was also altered in pancreatic β-cells, where a down-regulation of GLUT2 expression was noted, although the resulting reduction in GLUT-mediated transport is
not sufficient to explain the profound reduction in glucose-stimulated insulin secretion seen in the GK model (Ohneda et al., 1993). A reduction in GLUT4 recruitment to the plasma membrane in skeletal muscle from type II diabetic GK rats has also been demonstrated (Bitar et al., 2005), as well as a down-regulation of GLUT4 transporter in GK rat cardiac tissue (Desrois et al., 2004). Although SGLT expression in GK rats has not been characterised before, chronic administration of a potent SGLT inhibitor in this rodent model of type II diabetes resulted in improved glucose tolerance and insulin resistance (Ueta et al., 2005). Furthermore, SGLT-mediated glucose uptake in renal BBM vesicles was elevated in a genetic animal model of type II diabetes, C57BL/KsJ-db/db (Arakawa et al., 2001), suggesting that the up-regulation of SGLT-mediated glucose transport in GK rats is a whole-body response to type II diabetes.

The augmentation of glucose handling in type II diabetes in man has been previously shown and glucose uptake in the kidney is significantly elevated (Meyer et al., 2004). Furthermore, type II diabetic patients also exhibit a two-fold increase in post-prandial renal glucose release compared with those with normal glucose tolerance, which is due to renal gluconeogenesis (Meyer et al., 1998). The isolation and subculture of human exfoliated proximal tubular epithelial cells from the urine of type II diabetics has shown an increase in GLUT2 and SGLT2 expression (Rahmoune et al., 2005). Studies in proximal tubules from diabetic obese Zucker rats identified an increase in renal GLUT2 protein and mRNA; however, the membrane localisation of the transporter was not studied. Therefore, elevated expression of this transporter could have occurred at either the BBM or BLM (Kamran et al., 1997). Zucker diabetic rats also exhibited an increase in renal SGLT1 and SGLT2 mRNA expression (Tabatabai et al., 2009). However, it should be noted that the studies outlined above were carried out on models with obese phenotypes, whereas data presented in this chapter are from the GK rat model of type II diabetes that exhibit reduced body weight.

Previous studies with the cafeteria diet have shown it to be a valid and useful model of human obesity (Planas et al., 1992; Kretschmer et al., 2005; Burneiko et al., 2006), and the diet is characterised by its ability to induce lipid-storage in adipose tissue, and oxidative stress (Milagro et al., 2006). The diet of palatable junk-food does not necessarily stimulate a higher food intake; in fact, both Scoaris et al and studies presented here show that junk-food fed animals do not consume more food but, given
a choice of chow food, they consistently display a preference for junk-food (Scoaris et al., 2010). The consumption of energy-rich junk food over an 8-week period resulted in obesity, as was the case in animals fed a high-fat diet for 5 weeks. In this latter hyperlipidic diet, renal hypertrophy was also found, although no glycosuria was evident, whereas the junk-food diet was without effect on kidney weight, but caused glycosuria, albeit at supraphysiological levels. Both the junk-food and high-fat diet resulted in augmented plasma glucose levels, although the latter was measured in the blood from fasted animals and glycaemia was maintained within the normal blood glucose range of 6 – 10mM for unfasted Wistar rats (Chalkley et al., 2002; Maurer et al., 2010). The offspring from dams fed a junk-food diet throughout pregnancy and lactation exhibit increased insulin levels and adipocyte area, both of which are associated with the development of insulin resistance (Bayol et al., 2008). Rats in this study by Bayol et al fed a junk-food diet exhibited increased basal circulating insulin levels, and insulin-resistance is strongly associated with this model (Brandt et al., 2010). Furthermore, rodents fed a high-fat diet rapidly develop insulin resistance. Todd et al showed that high-fat feeding leads to the accumulation of lipids and lipid intermediates within skeletal muscle, which is associated with increased activation of specific serine kinases (IKKβ and JNK) causing a reduction in insulin action (Todd et al., 2007). This results in the impaired activation of the insulin signalling pathways from low-grade inflammation due to activation of the stress-activated NF-KB pathway; this inflammatory pathway interferes with proximal insulin signalling via the inhibition of IRS-1 function (Todd et al., 2007; Yaspelkis, III et al., 2007). In agreement with this, rats fed a high-fat diet exhibit elevated epididymal fat pad mass and insulin resistance, with basal circulating insulin levels increased compared with chow-fed controls (Chalkley et al., 2002). In contrast, others have noted that insulin-resistance in response to the junk-food diet is not associated with the impairment of insulin signalling (Brandt et al., 2010) and that the molecular mechanisms causing insulin resistance in response to the junk-food diet are different from high-fat diet-induced insulin resistance. The data presented here show that rats fed a high-fat diet for 5 weeks were not hyperglycaemic, presumably due to compensatory hyperinsulinaemia, which is in agreement with data from the 5 weeks of age genetic Zucker rat model of obesity (Litherland et al., 2004).
In response to high-fat induced obesity, PKC-βI expression was higher at the proximal tubule BBM and is likely to have resulted in the observed increase in GLUT2 at the BBM. This elevation of GLUT2 and its activator PKC-βI was also seen in the junk-food fed animals, but to a much higher degree than in the high-fat model, suggesting an amplified response of GLUT2 in response to other components of the junk-food diet besides its high-fat content. These data also signify that GLUT2 expression at the proximal tubule BBM occurs in the pre-diabetic model (diet-induced obesity) and is likely to be an early-diabetic renal response to metabolic syndrome.

Although fasted plasma glucose concentrations were raised in the high-fat model, they remained within the normal range; this implies that the increase in facilitative glucose transporter was independent of tubular glucose concentrations. Furthermore, although elevated plasma glucose levels in the junk-food model were noted, the levels remained within a ‘normal’ range; this suggests it is unlikely that GLUT2 was recruited to the BBM in response to high glucose levels, as was the case in both type I and type II diabetes. It is possible that insulin-resistance-induced sustained glycaemia at levels higher than control rats for a 5-week period for the high-fat diet and an 8-week period for the junk-food study, which may result in recruitment of GLUT2 at the BBM via PKC-βI activation. However, the physiological ramifications of this are uncertain, as the high-capacity transporter GLUT2, at the proximal tubule BBM, would not receive an elevated tubular load of glucose for reabsorption, as is the case in type I and II diabetes.

Interestingly, elevated GLUT2 expression in response to a high-fat diet has been shown previously in neonatal brains from dams fed 30% and 40% fat-as-calories during pregnancy (Cerf et al., 2010). The authors suggested that GLUT2 expression is up-regulated to alter neuronal glucose sensing in neonatal rats and results in dysregulation of the feeding response to programme the offspring to consume more food, and become more likely to develop obesity. There is currently no data on renal glucose transporters in response to junk-food feeding in adult rats, although it has been shown that adipocytes from the offspring of dams fed a junk-food diet exhibit increased GLUT1 and GLUT3 expression (female offspring), and increased GLUT4 mRNA (male offspring) (Bayol et al., 2008).
In contrast to the type II diabetic GK rat, BBM expression of SGLT1 was unaffected by the high-fat diet, a response also seen in type I diabetes. The presence of euglycaemia in this model may explain the lack of response of SGLT1 to high-fat diet-induced obesity; however, in the junk-food model there was elevation in expression of SGLT1, despite modest glycaemia. Although both obesity models exhibit insulin resistance and increased glycaemia, high-fat feeding may be considered a radical dietary intervention, whereas the junk-food diet has a more balanced caloric composition that better resembles a Western diet. Renal hypertrophy is noted in response to high-fat diet, whereas the junk-food diet had no effect on kidney size. Therefore, it is likely that SGLT1 expression at the proximal tubule BBM is unaffected by renal damage associated with hypertrophy and is not dependent on circulating basal insulin levels. Furthermore, the high-fat diet had no effect on glycosuria and no change in SGLT1 expression, whereas the junk-food diet resulted in glycosuria, as well as an increase in SGLT1 expression. This implies that the junk-food diet creates a renal environment in which proximal tubule fluid contains glucose at a higher concentration than the increased levels of GLUT2 and SGLT1 are able to transport. Thus, the junk-food diet presents a more realistic pre-diabetic model in which renal glucose transporter expression mirrors that seen in type II diabetes, whereas the high-fat diet also represents a clinically relevant model of pre-diabetes, because of the renal hypertrophy exhibited which reflects that seen in both type I and type II diabetes.

Unlike both the STZ-induced type I diabetic model and the type II diabetic GK rat, the high-fat model had no effect on recruitment of GLUT5 to the proximal tubule BBM. GLUT5 is primarily a fructose transporter, and although fructose feeding leads to obesity, there is little evidence that hyperlipidaemia affects circulating fructose levels; furthermore, animals were euglycaemic in response to the high-fat diet and there was no physiological stimulus for renal GLUT5 expression. In addition to elevated dietary fructose resulting in obesity, it also increases urinary fructose excretion (Kizhner and Werman, 2002). Nakayama et al showed proximal tubular hyperplasia in response to 60% fructose feeding. The elevated fructose uptake in the junk-food model and type II diabetes via high GLUT5 expression at the proximal tubule BBM may result in tubulointerstitial injury (Nakayama et al., 2010). It is worth noting that this is not the case for other obesity models, as demonstrated in
obese Zucker rats in which GLUT5 expression in adipocytes is up-regulated in response to insulin resistance (Litherland et al., 2004), suggesting that the absence of GLUT5 regulation in response to hyperlipidaemia may be specific to the kidney.

Interestingly, GLUT5 expression at the proximal tubule BBM was elevated in response to the junk-food diet. This is likely to be due to the high inverted sugar, also known as high-fructose corn syrup (HFCS) content within the components of the junk-food administered; this would increase fructose concentration in the plasma and thus proximal tubular fluid and may recruit GLUT5 to transport fructose across the BBM. Renal GLUT5 mRNA and protein expression are low in prenatals, but rapidly increase during the weaning period on exposure to fructose in the diet, which implies that renal GLUT5 expression responds to fructose in the junk-food diet (Rand et al., 1993).

The RYGB model was used to try to understand the regulation of glucose transporters and to hypothesise mechanisms for the improved glycaemic status observed in obese patients with type II diabetes post-surgery. However it is worth noting that this RYGB surgical model was studied in non-obese, non-diabetic control animals. As expected, animals exhibited body-weight loss after RYGB surgery due to reduced intake of food; furthermore, a reduction in kidney weight was also observed (Bueter et al., 2010). It has been suggested that improved glycaemia in patients post-RYGB, may be due to the early and more exaggerated peak in insulin levels, which is not seen in patients with gastric bands (Le Roux et al., 2006). However, a time-course study of insulin resistance in patients after RYGB revealed that the abolition of insulin resistance occurs 6 days after surgery, before weight loss, and so it does not explain the lower blood glucose levels exhibited within 24 hours of RYGB (Wickremesekera et al., 2005). In the studies presented here, although there was no alteration in plasma glucose or osmolality, basal circulating insulin levels were considerably reduced, which correlates with the elevated insulin sensitivity seen in type II diabetic and obese patients. However, the lack of insulin resistance in the rats in these studies before surgery (they were neither obese nor diabetic) implies that lowered plasma insulin is a consequence of the removal of the duodenum, rather than in response to resolved pre-surgery hyperinsulinaemia. This adds weight to the ‘foregut’ theory for improvement of glycaemia, whereby bypassing of duodenum and proximal jejunum results in the
release of incretins, from the lower small intestine, which improve glycaemic control following RYGB surgery (Hickey et al., 1998; Pories et al., 2001).

RYGB surgery resulted in lowered GLUT2 mRNA and protein expression, and PKC-βI levels at the proximal tubule BBM and the reduction in GLUT2 expression at the BBM would result in lower glucose reabsorption and the normalisation of glycaemia. Although my data have shown higher GLUT2 and its activator PKC-βI expression at the BBM after 7 days hyperglycaemia, it is known that GLUT2 moves away from the BBM relatively quickly in response to overnight fasting (Marks et al., 2003). This abolition of GLUT2 from the proximal tubule BBM occurred within 16 hours of food deprivation, a time-point which is similar to the elimination of hyperglycaemia in type II diabetic patients (Pories et al., 1995; MacDonald, Jr. et al., 1997). Furthermore, proximal tubule BBM expression of the sugar transporters GLUT2 and GLUT5 is down-regulated after RYGB surgery, implying a renal halting of fructose and glucose transport. Although there was a trend for reduced gene expression of GLUT5 this did not reach significance, whereas GLUT2 gene expression was significantly down-regulated following RYGB surgery. This suggests that the removal of GLUT5 from the proximal tubule BBM is a trafficking response to the surgery, as opposed to the GLUT2 response where RYGB surgery also reduced cellular levels of the transporter.

In contrast, RYGB did not alter gene or protein expression of SGLT1 in the proximal tubule, implying that the renal involvement in the improvement of glycaemia immediately following RYGB surgery is solely modulated by GLUT2 and GLUT5. This is one of the few studies on renal handling of glucose in response to gastric bypass surgery and highlights the potential involvement of gut signals regulating renal glucose homeostasis. However, the contribution of down-regulated GLUT2 expression, at the proximal tubule BBM, to the abolished hyperglycaemia noted in patients following RYGB surgery is not known. This could be further characterised by performing RYGB surgery on rodent models of metabolic syndrome, such as the high-fat or junk-food feeding-induced models of obesity, or type II diabetes, such as the ZDF or GK rat.

Following RYGB surgery, gene expression of GLUT5 in the duodenum was reduced, as was expression of the transporter at the enterocyte BBM. In rodents,
distribution of GLUT5 is higher in the duodenum and proximal jejunum than more distal regions of the small intestine. Therefore, since the main site of GLUT5 expression is bypassed in RYGB surgery, nutrient exclusion at the duodenal BBM likely regulates GLUT5 expression. Although this expression pattern was not seen for other sugar transporters in the duodenum in response to RYGB, it is known that GLUT5 in the small intestine is remarkably responsive to its substrate fructose, with increased expression in response to either high-fructose diets or in vivo perfusion of the small intestine with fructose (Jiang and Ferraris, 2001).

GLUT2 levels at the jejunal BBM were unaffected by RYGB surgery, although the cellular pool of the transporter was elevated; furthermore, in these studies, the jejunal GLUT2 activator PKC-βII is up-regulated at the jejunal BBM, suggesting that signalling for GLUT2 expression may also be amplified in RYGB jejunum in response to a luminal glucose stimulus. Additionally, GLUT2 at the jejunal BBM is known to rapidly increase in response to elevated levels of luminal sugars, a process that is rapidly reversed after the digestive period (Kellett and Helliwell, 2000); however, in the present studies, no feeding study was undertaken. Therefore, the absence of changes in jejunal GLUT2 expression may not be an accurate reflection of alterations resulting from RYGB per se, but more a result of animals not having access to food immediately before being culled. It has been shown previously that there is no phloridzin-insensitive, GLUT-mediated glucose uptake in the jejunum from sham or RYGB operated rats (Stearns et al., 2009). However Stearns et al did not record details of food intake prior to killing animals and it is therefore possible that rapid shuttling of GLUT2 away from the enterocyte BBM, in response to reduced jejunal glucose content, could also explain their data (Stearns et al., 2009). Therefore the elevated gene expression of GLUT2 in the jejunum seen in the data in the present study may serve to increase glucose absorption when the proximal intestine is removed.

Intriguingly, expression of SGLT1 at the jejunal BBM was higher following RYGB surgery, as seen previously with expression of SGLT1 mRNA at 10am and 10pm (Stearns et al., 2009). The 10am sampling time matched the time of cull for the present RYGB experiments. However, Stearns et al also noted that jejunal glucose absorption was lower after RYGB surgery; they attributed this finding to a shift in cell
lineage, from absorptive to secretory cells and post-translational modifications of SGLT1 at the BBM. This leads to the confounding situation in the jejunum where cellular levels and BBM expression of SGLT1 are increased, but the rate of SGLT-mediated glucose transport is reduced.

Morphologically, an increase in jejunum length and villus height occurs in response to RYGB surgery, which is likely to be due to the increased nutrient flow directly from the gastric pouch into the jejunum (Stearns et al., 2009). Expanding jejunal mucosa has also been shown to be a consequence of increased incretin GLP-2 release, which, in contrast to the study by Stearns et al, causes an increase in SGLT1 jejunal BBM transport and expression (Cheeseman, 1997; Ramsanahie et al., 2004; Kaji et al., 2009). Therefore, the increase in SGLT1 at the jejunal BBM may be a result of the GLP-2 hormone up-regulation in response to RYGB surgery. Furthermore, Moriya et al showed that SGLT-mediated glucose transport across the jejunal BBM triggers GLP-1 secretion, in addition to its’ role in recruiting GLUT2 to the BBM. Glucose absorption in the jejunum is closely linked with GLP-1 release (Moriya et al., 2009). Intriguingly, it has been shown by Au et al that the rapid shuttling of GLUT2 to the jejunal BBM occurs via elevated GLP-2 secretion; however, at present, no data are available on circulating GLP-2 following RYGB surgery (Au et al., 2002). The regulation of glucose transporters by incretins is an interesting phenomenon and there are many current studies based on using a peptidyl mimetic of GLP-1, such as exenatide (Young et al., 1999; Edwards et al., 2001), as a treatment for type II diabetes. Exenatide has been shown to cause a blunted GLP-1 effect which stimulates insulin biosynthesis and secretion, as well as causing modest weight loss (Knop et al., 2003). Furthermore, alterations in GLP-1 levels are related to GLUT2 in different tissues: in the small intestine, the gut-derived incretin hormone GLP-1 is secreted in a GLUT2-dependent manner and in type I diabetes, GLP-1 treatment reduces liver GLUT2 mRNA (Villanueva-Penacarrillo et al., 2001; Cani et al., 2007). Furthermore, the relationship between GLP-1 and GLUT2 has been shown in aging rats with down-regulated glucose tolerance. In response to GLP-1, reversal of the age-related decline in glucose tolerance occurred via modifications in pancreatic GLUT2 occurs (Wang et al., 1997). It is possible that the post-prandial up-regulation of GLP-1 in rodents following RYGB surgery is associated with a reduction in renal GLUT2 expression, whereas the impaired GLP-1 signalling in type II diabetes may be associated with the
increase in GLUT2 expression at the proximal tubule BBM shown in the data presented here (Lugari et al., 2002; Le Roux et al., 2006). Interestingly, duodenal-jejunal bypass surgery causes an increase in jejunal GLP-1, but no impact was noted on glucose tolerance; however, the same study with GK rats showed a GLP-1 receptor-mediated impact of bypass surgery on glucose tolerance in the type II diabetic model (Kindel et al., 2009; Kindel et al., 2010). Furthermore, studies with GLP-1 receptor KO mice have shown blunted development of obesity and related insulin-resistance in response to high-fat feeding (Scrocchi et al., 1998; Ayala et al., 2010).

GLP-1 has been shown to cause an increase in renal blood flow and GFR (Unwin et al., 1990) and the GLP-1 receptor expression has been localised to the proximal convoluted tubules (Marks et al., 2003). Furthermore, type I diabetes reduces the expression of renal GLP-1 receptor, potentially due to the activation of PKC-βI, which is known to desensitise GLP-1 receptor expression (Fehmann et al., 1996; Widmann et al., 1996). Therefore, the up-regulation of renal GLUT2, in response to both junk-food and high-fat diet may be a GLP-1 receptor-mediated response. Interestingly, in normoalbuminuric type II diabetic patients, an increase in urinary excretion of GLP-1 has been shown, with a further increase in response to the development of diabetic nephropathy (Lugari et al., 2001). Thus, the regulation of renal glucose transporters by locally-produced incretins presents an interesting perspective on physiological glucose homeostasis and RYGB surgery may prove a useful model to study these effects.
8.5 Conclusions

The metabolic models studied in this chapter have provided a better understanding of renal glucose handling. Increased GLUT2 recruitment to the proximal tubule BBM, via PKC-βI activation, occurs in both experimental type I and type II diabetes, accompanied by an increase in GLUT-mediated glucose transport. However, PKC-βI-mediated GLUT2 expression at this membrane does not exclusively respond to hyperglycaemia, since proximal tubule BBM levels of GLUT2 are promoted in euglycaemic models of obesity and the well-established junk-food, pre-diabetic model. Therefore PKC-βI-stimulated GLUT2 presence at the proximal tubule BBM may be influenced by augmented incretin levels in varying metabolic models. Furthermore, GLUT2 recruitment to the proximal tubule BBM is down-regulated in response to duodenal bypass, and operates via an insulin-independent pathway.

GLUT2 and GLUT5 recruitment to the renal BBM are regulated by the same metabolic models used in the present study, with the exception of any GLUT5 response to the euglycaemic, insulin-resistant high-fat model. In contrast, SGLT1 expression at the proximal tubule BBM is up-regulated in response to the pre-diabetic, insulin-resistant, junk-food diet model, and glucose transport is also elevated in type II diabetes, but not type I diabetes. Therefore, SGLT1 is not solely regulated by tubular fluid glucose concentrations, but it is unaffected by nutrient exclusion and incretin release from the small intestine in response to RYGB surgery. Furthermore, the regulation of intestinal glucose intake may be altered by nutrient exclusion following RYGB surgery.

The data presented in this chapter show that increased GLUT-mediated glucose transport, noted in type I diabetes, is also seen in type II diabetes, as well as in a pre-diabetic junk-food model and hyperlipidaemia-induced obesity, emphasising the strong clinical link between lifestyle and the onset of type II diabetes. Furthermore, in response to RYGB, which alleviates obesity and hyperglycaemia, GLUT2 expression at the renal BBM is abolished, suggesting that proximal tubule glucose transport is greatly influenced by disorders in carbohydrate metabolism.
9 Modulation of renal glucose transport by sweet taste sensing at the proximal tubule brush-border
9.1 Introduction

Chapter 8 was concerned with the alterations in glucose handling at the proximal tubule BBM in response to metabolic changes during diabetes and the related metabolic syndrome and RYGB surgery. However, the mechanisms controlling the expression of GLUT2, and other renal glucose transporters, are still largely unknown and therefore it was of interest to further study the regulation of glucose handling at the proximal tubule BBM.

Taste sensing is vital for the recognition of nutrients in response to energy requirements and is mediated by a number of highly-promiscuous seven transmembrane receptors which have been cloned and characterised in the last few years (Conigrave and Brown, 2006; Egan et al., 2008; Engelstoft et al., 2008). These chemosensors are located throughout the body, depending on their role, such as the pancreas (Taniguchi, 2004; Nakagawa et al., 2009), liver (Taniguchi, 2004; Kuang et al., 2005), and adipose tissue (Kuang et al., 2005; Wellendorph and Brauner-Osborne, 2009), as well as in the classical location in lingual taste tissue (Matsumura et al., 2009). Taste receptors include the free fatty acid (FFA) sensing receptors, FFA1-3, and the calcium-sensing receptor (CaSR), with the latter able to sense both calcium (Ca$^{2+}$) and L-amino acids (Chattopadhyay et al., 1997; Conigrave et al., 2000; Conigrave et al., 2006). Amino acids are also sensed by the T1R1/3 heterodimer, which comprises of the taste receptors T1R1 and T1R3; these receptors belong to the same G-protein-coupled receptor family C as the CaSR (Nelson et al., 2002). This family of taste receptors, T1R and T2R, was originally identified in taste buds of the oral cavity and has recently been shown to be distributed throughout the small intestine (Nelson et al., 2001; Dyer et al., 2005; Bezencon et al., 2007; Mace et al., 2007; Egan et al., 2008) and in airway smooth muscle and chemosensory cells (Deshpande et al., 2010; Tizzano et al., 2011).

The T1R1/3 heterodimer is stereo-specific and can perceive most of the 20 standard L-amino acids, although not aromatic amino acids; T1R1/3 functions as an umami taste sensor, corresponding to its ability in lingual taste buds, where it is highly expressed, to respond to savoury taste of glutamate and aspartate (Li et al.,
2002; Nelson et al., 2002; Damak et al., 2003; Zhao et al., 2003). Interestingly, this taste sensor, T1R1/3, has also been found in brush cells in the small intestine, along with its mediator, the G-protein transducin, where its role in nutrient sensing is associated with glucose transport across the BBM (Hofer et al., 1996; Dyer et al., 2005; Sbarbati and Osculati, 2005; Bezençon et al., 2007; Mace et al., 2009). The activation of the T1R1/3 heterodimer by L-glutamate stimulates glucose absorption in the rat jejunum via an up-regulation in BBM expression of glucose transporter GLUT2 (Mace et al., 2009). In addition, amino acid stimulation of T1R1/3 promotes BBM recruitment of the high-affinity glutamate transporter EAAC1 and internalises the oligopeptide transporter PepT1 in the enterocyte; thus, amino acid taste sensing, via T1R1/3, at the jejunal BBM mediates absorption of specific nutrients (Mace et al., 2009).

In addition to forming the umami taste sensing heterodimer, the T1R class of the G-protein-coupled receptor family C also contains T1R2, which dimerises with T1R3 to form the sweet taste sensor T1R2/3. Originally identified in taste buds in oral taste tissue, this sweet taste heterodimer demonstrates an intriguingly broad specificity for sweet substrates, including the sugars glucose, sucrose and fructose, as well as sweet proteins such as brazzein, sweet taste D-amino acids, and artificial sweeteners such as aspartame, cyclamate, saccharin and sucralose (Raybould, 1998; Hofer and Drenckhahn, 1999; Nelson et al., 2001; Li et al., 2002; Jiang et al., 2004; Assadi-Porter et al., 2010; Servant et al., 2010). However, the sweet taste heterodimer is stereoselective for certain molecules, for example, unlike T1R1/3, T1R2/3 can respond to D-tryptophan, but not L-tryptophan (Li et al., 2002). Furthermore, Xu et al showed that the artificial sweeteners aspartame and neotame bind the N-terminal domain of T1R2, whereas T1R3 is stimulated by cyclamate and down-regulated by the taste inhibitor lactisole (Xu et al., 2004). The sweet taste heterodimer has, like T1R1/3, been identified at the brush-border of enterocytes, the same localisation as α-gustducin, with the proximal and mid-intestine linked to peak levels of T1R2/3 (Hofer et al., 1996; Dyer et al., 2005; Stearns et al., 2010).

As with the umami taste heterodimer, the sweet taste sensor, T1R2/3, was identified as a regulator of glucose absorption at the enterocyte BBM; the addition of membrane-impermeable glucose analogues to the lumen of the intestine caused the
stimulation of SGLT1, which implicated a glucose sensor in the modulation of this high-affinity glucose transporter (Dyer et al., 2003). This finding was mirrored in weaning piglets, which exhibited an enhanced capacity to absorb dietary sugars via SGLT1 when fed high-saccharin in their diet (Moran et al., 2010). Furthermore, Margolskee et al showed that the stimulation of SGLT1 in the small intestine in response to a 70% sucrose diet, or sucralose-sweetened water feeding, was blocked in T1R3 KO mice (Margolskee et al., 2007). T1R-mediated alterations in SGLT1 synthesis in response to artificial sweeteners have been hypothesised to be a result of signalling via cAMP (Margolskee, 2002; Mace et al., 2007).

Interestingly, Mace et al have shown that intestinal sweet taste sensing also has an effect on enterocyte glucose absorption via the facilitative transporter GLUT2 (Mace et al., 2007). A glucose concentration of 75 mM in the jejunal lumen is normally required for the rapid insertion of GLUT2 to the enterocyte BBM and this GLUT2 response is mediated by SGLT1-dependent accumulation of glucose in the enterocyte. Elevated intracellular glucose levels cause a series of events involving the depolarisation of the BBM and an influx of Ca\(^{2+}\) ions into the cell, which result in the contraction of the terminal web and GLUT2 shuttling to the BBM (Figure 5.7) (Morgan et al., 2007; Mace et al., 2007). At lower glucose concentrations (20 mM), irrespective of Ca\(^{2+}\) stimuli, GLUT2 does not shuttle to the BBM; however, the addition of 1 mM sucralose, together with 20 mM glucose, causes the same GLUT2 response as that seen using 75 mM glucose (Mace et al., 2007). The response occurs within 15 minutes of luminal exposure to the stimulus and thus this short-term effect of sweet taste sensing via T1R2/3 was hypothesised to be a mode of secondary signalling to the glucose transporter GLUT2. On binding to its substrates, the sweet taste heterodimer undergoes a conformational change that causes the G-protein subunit α-gustducin to dissociate from the enterocyte BBM into the cytosol, while the βγ-gustducin subunits remain anchored to the membrane, where they bind to and activate PLCβ2. This activation promotes further inactive cytosolic PLCβ2 to be translocated to the BBM, which generates DAG, the lipid secondary messenger able to activate PKC-βII present at the enterocyte BBM (Rhee, 2001; Mace et al., 2009). Indeed, sucralose resulted in the up-regulation of PKC-βII, which when activated is responsible for GLUT2 recruitment to the jejunal BBM (Mace et al., 2007). Additionally, Mace et al showed that short-term T1R2/3 stimulation was without
effect on SGLT1 in the jejunum and hypothesised that sweet taste receptors may regulate short-term regulation of GLUT2 via PLCβ2 and long-term effects on SGLT1 synthesis via cAMP (Mace et al., 2007).

Sweet taste sensing, via the stimulation of T1R2/3 at the jejunal BBM results in the up-regulation of the glutamate transporter EAAC1 and internalisation of the oligopeptide transporter PepT1 (Mace et al., 2009). Thus, as with umami taste sensing via T1R1/3, the sweet taste heterodimer can also mediate nutrient absorption across the jejunal BBM in response to luminal substrate load.

T1R2/3, has been shown to be sensitive to sugars, such as sucrose, used at high levels (more than 100 mM), whereas the intense sweetness of artificial sweeteners, such as saccharin and aspartame, means that they are able to elicit a response from the sweet taste sensor at concentrations between 1-5 mM (Nelson et al., 2001;Li et al., 2002). Artificial sweeteners are widely used as a weight-loss aid and sugar-substitute for diabetics, although their efficacy in both is currently under debate in the light of their influence on glucose absorption as outlined above (Stellman et al., 1986;Ma et al., 2010;Swithers et al., 2010). Saccharin is a commonly used artificial sweetener commercially available as ‘Sweet n Low’ and rapidly absorbed by the small intestine (Renwick, 1985;Renwick, 1990), with peak concentrations in the blood 30-60 minutes post-feeding; however, a large proportion of this absorbed saccharin, between 66-99%, is not metabolised (Kennedy et al., 1972;Byard and Goldberg, 1973;Lethco and Wallace, 1975;Colburn et al., 1981). Renal clearance of saccharin is via filtration and tubular secretion and, therefore, the artificial sweetener is present at high concentrations at the proximal tubule BBM in response to oral or IV-infused saccharin administration; furthermore, saccharin is approximately 300 times sweeter than sucrose and has no effect on blood glucose concentration (Goldstein et al., 1978;Bekersky et al., 1980).

Although there are similarities between the process of glucose transport across the enterocyte and proximal tubule BBM, important differences have been identified. For example, in the early S1 region of the proximal tubule SGLT2 the high capacity glucose transporter, is present at the BBM (Kanai et al., 1994;Wallner et al., 2001;Wright, 2001). Additionally, the studies presented in this thesis (Chapter 7)
reveal that GLUT2 recruitment to the proximal tubule BBM in response to glucose infusion is slower than that reported for GLUT2 shuttling to the jejunal BBM. The movement of GLUT2 to the proximal tubule BBM is thought to occur via signalling from its activator PKC-βI and is observed in experimental type I diabetes, type II diabetes, and metabolic syndrome induced by either a high-fat or junk-food diet (Chapter 8). Therefore, the recruitment of GLUT2 to the renal BBM is not mediated by hyperglycaemia alone, as previously believed (Chin et al., 1997; Marks et al., 2003; Freitas et al., 2007). To further understand this phenomenon, it was of interest to investigate the involvement of the known regulators of jejunal glucose absorption, such as taste receptors, in the kidney. There are no previous studies on renal expression of T1R1, T1R2 or T1R3; although human embryonic kidney cells (HEK) have been widely used to express these taste receptors (Kuang et al., 2005; Assadi-Porter et al., 2010).

9.2 Aims of chapter

The presence of taste receptors in the small intestine, where they modulate glucose transport by GLUT2 and SGLT1, together with the similarities between glucose transport across the BBM in the small intestine and proximal tubule, led to the aim of this chapter, which was to establish whether sweet taste receptors are expressed in the kidney. The response of renal glucose transport, upon activation of these sweet taste receptors, was investigated. The activity of these sensors, and their involvement on proximal tubule glucose transport was analysed using the same metabolic models used in Chapter 8, which are known to influence glucose transport across the proximal tubule BBM. Furthermore intestinal sweet taste receptor expression was studied following RYGB surgery to analyse the effect of gastric bypass on sweet taste sensing by the small intestine.
9.3 Results

9.3.1 Taste receptor mRNA expression in the kidney

The presence of taste receptors in the kidney was established initially using RT-PCR to analyse mRNA in the renal cortex of control rats. Figure 9.1-1 shows the mRNA expression of the components of the sweet taste heterodimer T1R2 and T1R3 in the kidney, where T1R3 has 2-fold higher expression that T1R2. The positive control tissue, rat jejunum, also expresses mRNA for the sweet taste receptors, with T1R3 exhibiting 3-fold elevated expression compared with T1R2 (Figure 9.1-1). Both T1R2 and T1R3 are expressed at an increased level in the kidney compared with the jejunum, with a 652% and 394% increase respectively. Figure 9.1-2 shows that the components of the umami taste heterodimer T1R1 and T1R3 are also expressed in the kidney cortex of control rats. As with the sweet taste components, expression in the kidney is higher than in the jejunum, with T1R1 exhibiting a 350% elevation. As previously shown (Bezencon et al., 2007; Mace et al., 2009), the umami taste heterodimer T1R1/3 is expressed in the jejunum of control rats, with mRNA expression of T1R3 exhibiting a 2.4-fold increase compared with T1R1 (Figure 9.1-2).
Figure 9.1 – Detection of taste receptor mRNA in the jejunum and kidney cortex: 1) mRNA expression of sweet taste heterodimer subunits, T1R2 (pink) and T1R3 (black), in control jejunum (solid bars) and kidney (slatted bars). 2) mRNA expression of umami taste heterodimer subunits, T1R1 (blue) and T1R3 (black), in control jejunum (solid bars) and kidney (slatted bars). Values are expressed as mean ± SEM, n=7-12, **p<0.01, ***p<0.005, ****p<0.001 compared with jejunum.
9.3.2 Detection of sweet taste dimer component proteins in the kidney

To study further sweet taste sensing receptors in the kidney, commercially available antibodies (Santa Cruz Biotechnology) raised against the extracellular domain of T1R2 and T1R3 were used in renal membrane preparations from different species.

9.3.2.1 Detection of T1R2 and T1R3 protein in whole membrane from kidney

To assess whether the mRNA detected in section 9.3.1 translated into detectable protein levels, whole membrane preparations from control rat kidney cortex and jejunal mucosa were analysed using the ‘dot blot’ method. Figure 9.2 shows the expression of T1R2 in the whole membrane from kidney and jejunum; as with the mRNA data, expression of T1R2 protein appeared to be higher in the kidney than the jejunum. T1R3 was also expressed in the whole membrane preparation from kidney although its expression was similar in the kidney and jejunum (Figure 9.2).

**Figure 9.2** – Dot blot detection of sweet taste heterodimer components T1R2 and T1R3 in whole membrane prepared from control rat kidney cortex and jejunal mucosa.
9.3.2.2 Detection of renal sweet taste receptors in other renal cell types

To establish the specificity of the sweet taste receptor antibodies and the expression of these receptors in other renal cell types, whole membrane preparations from mouse kidneys and the porcine proximal tubule cell line (LLC-PK₁) were studied using western blotting. Bands were detected between the 128 kDa and 93 kDa molecular weight markers on the gel, corresponding to the reported molecular weight of T1R2 (95.8 kDa) and T1R3 (93.4 kDa) respectively. Figure 9.3 shows the expression of the sweet taste receptor T1R2 in mouse kidney whole membrane preparation; however, there was no expression in cultured porcine proximal tubule cells. This result was matched with T1R3 probing (Figure 9.3), with the antibody detecting the receptor in mouse kidney whole membrane preparation, but not in LLC-PK₁ cells.

Figure 9.3 – Detection of sweet taste receptor heterodimer components T1R2 and T1R3 by western blotting of whole membrane prepared from control mouse kidney cortex and porcine proximal tubule cells (LLC-PK₁)
9.3.2.3 Detection of renal sweet taste receptors at the proximal tubule BBM

The expression of sweet taste receptors T1R2 and T1R3 was studied in BBM preparations from the kidney cortex of control rats to assess whether, as in the jejunum, T1R2/3 could be associated with transport systems at the BBM in the proximal tubule. Renal BBM vesicles were probed for the sweet taste heterodimer components with western blotting. Figure 9.4-1 shows the detection of both T1R2 and T1R3, at 95.8 kDa and 93.4 kDa respectively, in BBM vesicles prepared from control rat kidney cortex. Both proteins were also detected in jejunal BBM prepared from the positive control tissue, where T1R2 and T1R3 are known to be strongly expressed in the jejunal mucosa (Dyer et al., 2005; Mace et al., 2007). As seen with the mRNA and whole membrane protein results, T1R2 and T1R3 levels at the proximal tubule BBM were significantly higher than at the jejunal enterocyte BBM. There was a 2.9- and 3-fold elevation in T1R2 and T1R3 expression at the renal compared with the jejunal BBM (Figure 9.4-2).
**Figure 9.4** – Detection of sweet taste heterodimer components, T1R2 (pink) and T1R3 (black) in BBM prepared from the kidney (slatted bars) and jejunum (solid bars) from control rats: 1) western blotting 2) quantification of western blots (expressed as percentage BBM expression) standardised to β-actin. Values are expressed as mean ± SEM, n=6, **p<0.01, ***p<0.005 compared with jejunum.
9.3.3 Effects of acute high plasma glucose and osmolality on expression of sweet taste receptors at the proximal tubule BBM

The response of renal sweet taste receptors at the proximal tubule BBM to either acute hyperglycaemia induced by a 2 hour IV infusion of 1.1 M glucose or high plasma osmolality induced by a 2 hour IV infusion of 1.09 M mannitol was studied. As shown in Chapter 7, acute hyperglycaemia caused an immediate and significant elevation in plasma glucose levels and therefore proximal tubular lumen levels. Within 15 minutes, plasma glucose concentration was elevated to 23 ± 2.6 mM, levels which were maintained for the 2 hour period. Figure 9.5 - 1 shows the western blotting of renal BBM probed for T1R2 and T1R3, with IV infusion of 154 mM saline or 0.08 M mannitol in saline, used as controls for the effects of glycaemia and high osmolality effect, respectively. Quantification of T1R2 and T1R3 at the proximal tubule BBM showed no alteration to their expression in response to acute hyperglycaemia (Figure 9.5 - 2). It was also shown that mannitol infusions at either low (0.08 M) or hyperosmotic concentrations (1.09 M) had no impact on sweet taste receptor expression at the proximal tubule BBM (Figure 9.5 - 2).
Figure 9.5 – Effect of acute hyperglycaemia or high osmolality, induced by IV infusion of 1.1 M glucose (with 154 mM saline as control) or 1.09 M mannitol (with 0.08 M mannitol as control) respectively on sweet taste heterodimer components, T1R2 (pink) and T1R3 (black) at the proximal tubule BBM: 1) Western blots 2) quantification of western blots (expressed as percentage BBM expression of saline control) and standardised to β-actin. Values are expressed as mean ± SEM, n=6, p>0.5 compared with 154 mM saline

1

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Glucose</th>
<th>mannitol</th>
<th>mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1R2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

95.8 kDa

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Glucose</th>
<th>mannitol</th>
<th>mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1R3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

93.4 kDa

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

42 kDa

2

% BBM expression

saline glucose 0.08 M mannitol 1.09 M mannitol
9.3.4 Effect of chronic hyperglycaemia on sweet taste receptors at the proximal tubule BBM

To assess further the response of the renal sweet taste sensing heterodimer to simple sugars, chronic hyperglycaemia was used to assess the effects of long-term, high-glucose tubular fluid luminal concentration. As was the case in Chapter 7, chronic hyperglycaemia was studied using the STZ-induced model of type I diabetes.

9.3.4.1 Effect of type I diabetes on mRNA expression of sweet taste receptors in the kidney and jejunum

The effect of chronic hyperglycaemia, established with STZ-induced type I diabetes, on gene expression of sweet taste receptors in the kidney and jejunum was studied using RT-PCR. Since acute hyperglycaemia following a high-glucose infusion (section 9.3.3) had no effect on sweet taste receptor expression at the proximal tubule BBM, longer-term gene expression of T1R2 and T1R3 was studied 10 and 30 days after induction of type I diabetes. Figure 9.6 - 1 shows that mRNA expression of T1R3 in the kidney cortex was significantly elevated by 58-fold at 10 and 30 days after induction of diabetes when compared with T1R2 mRNA expression at the same time point after induction of diabetes. There was also a significant decrease in renal T1R2 mRNA expression after 10- and 30-day hyperglycaemia, with 97% and 96% reduction, respectively; T1R3 mRNA expression in the kidney was unaffected by both 10- and 30-day type I diabetes (Figure 9.6 - 1). In the jejunum, chronic hyperglycaemia induced by 10-day type I diabetes had no significant effect on T1R2 or T1R3 expression; however, after 30 days there was a 643% increase in T1R2 and a 443% elevation in T1R3 expression (Figure 9.6 - 2).
Figure 9.6 – Effect of chronic hyperglycaemia studied in 10- and 30-day STZ-induced type I diabetic rats on mRNA expression of sweet taste receptors T1R2 (pink) and T1R3 (black) in the kidney cortex (1) and jejunum (2), standardised to β-actin. Values are expressed as mean ± SEM, n=7-12, *p<0.05, **p<0.01, ***p<0.005 compared with control animals.
9.3.4.2 Effect of type I diabetes on the expression of sweet taste receptor proteins, T1R2 and T1R3, at the proximal tubule BBM

Protein levels of the components of the sweet taste heterodimer T1R2 and T1R3 were studied in response to chronic hyperglycaemia established by 7-day STZ-induced diabetes. Figure 9.7 – 1 shows the western blot of renal BBM vesicles probed with T1R2 and T1R3 antibodies. Despite changes in T1R2 and T1R3 renal mRNA expression (Figure 9.6), renal hypertrophy (Table 7.3), and chronic hyperglycaemia for 7 days, there was no significant change in T1R2 or T1R3 protein levels at the proximal tubule BBM following 7-day type I diabetes (Figure 9.7 - 2).

Figure 9.7 – Effect of chronic hyperglycaemia studied with 7-day STZ-induced diabetes (slatted bars) on sweet taste receptors T1R2 and T1R3 at the proximal tubule BBM: 1) Western blots 2) quantification of western blots (expressed as percentage BBM expression) standardised to β-actin. Values are expressed as mean ± SEM, n=4-6, p>0.5 compared with vehicle injected controls (open bars)
9.3.5 Effects of different metabolic models on the expression of sweet taste receptors at the proximal tubule BBM

Studies presented in this thesis have established the influence of different metabolic conditions on glucose transport at the proximal tubule BBM (Chapter 8). Therefore to further understand the renal sweet taste heterodimer, it was of interest to study whether the expression of T1R2 and T1R3 at the proximal tubule BBM was also affected by metabolic dysregulation.

9.3.5.1 Type II diabetic Goto-Kakizaki (GK) rat model

Protein levels of both components of the sweet taste receptor heterodimer were studied at the proximal tubule BBM of 8-9 weeks old GK rats. Figure 9.8 – 1 shows that the protein levels of T1R2 and T1R3 in renal BBM vesicles prepared from GK rats were significantly elevated compared with lean rats. Quantification of western blots for both T1R2 and T1R3 sweet taste receptors, showed a significantly higher expression, with a 3.5-fold increase for both at the proximal tubule BBM of GK rats compared with lean animals (Figure 9.8 - 2).
Figure 9.8 – Effect of type II diabetes (slatted bars) on sweet taste receptors T1R2 and T1R3 at the proximal tubule BBM in lean control rats (C) and GK type II diabetic rats (D): 1) Western blots 2) quantification of western blots (expressed as percentage BBM expression) standardised to β-actin. Values are expressed as mean ± SEM, n=8, **p<0.01, ***p<0.005 compared with lean controls (open bars)
9.3.5.2 High-fat diet-induced metabolic syndrome model

Expression of sweet taste receptors at the proximal tubule BBM were assessed in response to metabolic syndrome, induced by feeding a high-fat diet (60% fat-as-calories chow) for 5 weeks (Figure 9.9 - 1). There was no significant change in T1R2 and T1R3 expression at the renal BBM in response to the high-fat diet (Figure 9.9 - 2).

**Figure 9.9** – Effect of high-fat diet (slatted bars) on sweet taste receptor expression at the proximal tubule BBM: 1) Western blots 2) quantification of western blots (expressed as percentage BBM expression) standardised to β-actin. Values are expressed as mean ± SEM, n=6, p>0.5 compared with chow-fed controls (open bars)
9.3.5.3 Junk-food diet-induced metabolic syndrome model

Expression of T1R2 and T1R3 in response to metabolic syndrome induced by feeding rats a junk-food diet for 8 weeks is shown in Figure 9.10 - 1. This well-established model of the western diet resulted in significantly elevated levels of T1R2 and T1R3 protein at the proximal tubule BBM compared with the chow-fed control rats, with a 2.2- and 2-fold increase in BBM expression, respectively (Figure 9.10 - 2).

Figure 9.10 – Effects of junk-food diet (slatted bars) on sweet taste receptor expression at the proximal tubule BBM: 1) Western blots 2) quantification of western blots (expressed as percentage BBM expression) standardised to β-actin. Values are expressed as mean ± SEM, n=6-12, ****p<0.001 compared with chow-fed controls (open bars)
9.3.5.4 Roux-en-Y gastric bypass (RYGB) model

9.3.5.4.1 Effect of RYGB surgery on renal sweet taste receptor mRNA expression

RYGB surgery was performed on control rats and studies were performed 60 days following the gastric bypass surgery. T1R2 mRNA expression in the renal cortex was significantly down-regulated by 73%, compared with the sham-operated controls (Figure 9.11). However, there was no statistically significant difference between T1R3 mRNA expression in the kidney cortex following sham and RYGB surgery (Figure 9.11).

**Figure 9.11** – Effects of RYGB surgery (slatted bars) on sweet taste receptor mRNA components T1R2 (pink) and T1R3 (black) in the renal cortex. Values are expressed as mean ± SEM, n=6-10, ****p<0.001, compared with sham-operated control animals (open bars).
9.3.5.4.2 Effect of RYGB surgery on expression of renal sweet taste receptor proteins at the proximal tubule BBM

The effect of RYGB surgery on renal sweet taste receptors T1R2 and T1R3 at the proximal tubule BBM was assessed using western blotting (Figure 9.12). Protein levels of both components of the sweet taste heterodimer were unaffected 60 days after RYGB surgery (Figure 9.12 - 1), with no statistical difference between proximal tubule BBM expression of T1R2 and T1R3 compared with the sham-operated controls (Figure 9.12- 2).

**Figure 9.12** – Effects of RYGB surgery (slatted bars) on sweet taste receptor components T1R2 (pink) and T1R3 (black) at the proximal tubule BBM: 1) Western blots 2) quantification of western blots (expressed as percentage BBM expression) standardised to β-actin. Values are expressed as mean ± SEM, n=6, p>0.5 compared with sham surgery controls (open bars).

1

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>RYGB</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1R2</td>
<td><img src="image1.png" alt="Western blot" /></td>
<td><img src="image2.png" alt="Western blot" /></td>
</tr>
<tr>
<td>T1R3</td>
<td><img src="image3.png" alt="Western blot" /></td>
<td><img src="image4.png" alt="Western blot" /></td>
</tr>
<tr>
<td>Actin</td>
<td><img src="image5.png" alt="Western blot" /></td>
<td><img src="image6.png" alt="Western blot" /></td>
</tr>
</tbody>
</table>

2

<table>
<thead>
<tr>
<th></th>
<th>T1R2</th>
<th>T1R3</th>
</tr>
</thead>
<tbody>
<tr>
<td>% BBM expression</td>
<td><img src="image7.png" alt="Graph" /></td>
<td><img src="image8.png" alt="Graph" /></td>
</tr>
</tbody>
</table>
9.3.5.4.3 Effect of RYGB on expression of sweet taste receptor mRNA expression in specific regions of the intestine

The gene expression profile of sweet taste receptors T1R2 and T1R3 in mucosal scrapes from three specific regions of the small intestine was studied 60 days after RYGB surgery (Figure 9.13).

A significant 60% decrease in T1R2 mRNA expression in the by-passed biliopancreatic limb, corresponding to the duodenum of sham-operated control animals, was seen (Figure 9.13 - 1). However, no difference between T1R2 mRNA expression in the alimentary limb corresponding to the jejunum of the sham-operated control was noted (Figure 9.13 - 1). Following RYGB surgery, there was also no difference between T1R2 mRNA expression in the common limb corresponding to the ileum of sham-operated controls (Figure 9.13 - 1).

The expression profile of T1R3 in all three regions of the small intestine was unaffected by RYGB surgery. Although a 25% increase in T1R3 expression was seen in the alimentary limb compared with the jejunum from sham-operated control animals, this did not reach statistical significance (Figure 9.13 - 2). Furthermore, there was no significant difference between T1R3 expression in the duodenum or ileum compared with the biliopancreatic and common limbs, respectively (Figure 9.13 - 2).
Figure 9.13 – Effects of RYGB surgery (closed bars) on sweet taste receptor mRNA components (1) T1R2 (pink) and (2) T1R3 (black) in specific regions of the small intestine. Following RYGB, the duodenum becomes the biliopancreatic limb, the jejunum becomes the alimentary limb, and the ileum becomes the common limb. Values are expressed as mean ± SEM, n=5-8, ****p<0.001, compared with sham-operated control animals (open bars).

1 T1R2

![Graph 1 T1R2](image)

2 T1R3

![Graph 2 T1R3](image)
9.3.5.4.4 Effect of RYGB surgery on expression of T1R2 and T1R3 protein at the intestinal BBM

The expression of T1R2 and T1R3 proteins following RYGB surgery was studied with western blotting using BBM prepared from mucosal scrapes from each region of the small intestine (Figure 9.14 - 1 / Figure 9.15 – 1). Following RYGB surgery a significant 2-fold decrease in T1R2 in the by-passed biliopancreatic limb was noted, together with a 3.5-fold reduction in T1R2 in the alimentary limb compared with duodenum and jejunum, respectively, of sham-operated rats (Figure 9.14- 2). Despite an increase in T1R2 at the BBM of the common limb after RYGB surgery, this did not reach statistical significance (Figure 9.14 - 2). Levels of T1R3 protein at the BBM of the by-passed biliopancreatic limb were reduced, however, this was not statistically significant when compared with the duodenum from sham-operated control animals (Figure 9.15 - 2). T1R3 protein levels at the BBM of the alimentary limb were significantly decreased following RYGB surgery, although there was no significant difference in levels of T1R3 protein at the BBM from the ileum from control rats in comparison with the common limb from rats 60 days post-RYGB surgery (Figure 9.15 - 2).
**Figure 9.14** – Effects of RYGB surgery (closed bars) on expression of sweet taste receptor T1R2 protein in the specific regions of the small intestine. Following RYGB, the duodenum becomes the biliopancreatic limb, the jejunum becomes the alimentary limb and the ileum becomes the common limb: 1) Western blots 2) quantification of western blots (expressed as percentage BBM expression) standardised to β-actin. Values are expressed as mean ± SEM, n=6, ***p<0.005, ****p<0.001 compared with sham-operated control animals (open bars).
**Figure 9.15** – Effects of RYGB surgery (closed bars) on expression of sweet taste receptor T1R3 protein in specific regions of the small intestine. Following RYGB, the duodenum becomes the biliopancreatic limb, the jejunum becomes the alimentary limb and the ileum becomes the common limb: 1) Western blots 2) quantification of western blots (expressed as percentage BBM expression) standardised to β-actin. Mean ± SEM, n=6, ***p<0.005, ****p<0.001 compared with sham-operated control animals.

1

**Duodenum**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>RYGB</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1R3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

20 40 60 80 100 120 140 160 180 200

**Jejunum**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>RYGB</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1R3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Ileum**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>RYGB</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1R3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2

![Graph showing T1R3 expression in duodenum, jejunum, and ileum](image)
9.3.6 Effect of artificial sweetener infusion on glucose transport and sweet taste receptor expression in the proximal tubule

9.3.6.1 Animal parameters following saccharin infusion

The function of sweet taste receptors localised at the proximal tubule BBM was assessed using a 2 hour IV infusion of 154 mM saline alone or containing saccharin at 1.37 mM concentration. The infusate osmolality was not statistically different from the osmolality of the saline infusate used as a control (Table 9.1 - 1). Also, there was no difference in plasma osmolality in response to the artificial sweetener infusion compared with saline infusion (Table 9.1 - 1). Although there was a 1.2-fold increase in plasma glucose concentration following the saccharin infusion, this was not statistically significant (Table 9.1 - 1). The infusion of 1.37 mM saccharin had no impact on the body weight of animals or renal hypertrophy (Table 9.1 - 2).

Table 9.1 – Animal parameters following saccharin IV infusion 1) Infusate osmolality and its effect on plasma osmolality 2) Body and kidney weights and renal BBM vesicle enrichment of alkaline phosphatase marker enzyme in rats following IV infusion of either 154 mM saline or 1.37 mM saccharin in 154 mM saline for 2 hours. Mean ± SEM, n=6, no significant change between infusions, p>0.5.

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Saccharin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma glucose (mM)</strong></td>
<td>5.39 ± 0.2</td>
<td>6.27 ± 0.8</td>
</tr>
<tr>
<td><strong>Infusate osmolality (mOsM)</strong></td>
<td>327 ± 4</td>
<td>329 ± 8</td>
</tr>
<tr>
<td><strong>Plasma osmolality (mOsM)</strong></td>
<td>309 ± 0.9</td>
<td>310 ± 0.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Saccharin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>398 ± 11.8</td>
<td>409.7 ± 15.3</td>
</tr>
<tr>
<td><strong>Kidney weight (g)</strong></td>
<td>1.04 ± 0.08</td>
<td>1.01 ± 0.02</td>
</tr>
<tr>
<td><strong>BBM vesicle enrichment</strong></td>
<td>6.81 ± 0.61</td>
<td>6.64 ± 0.57</td>
</tr>
</tbody>
</table>
9.3.6.2 Effect of saccharin infusion on the expression of renal sweet taste dimer components

Renal BBM prepared from saccharin-infused animals displayed no difference in BBM vesicle purity compared to the response of saline infusion alone (Table 9.1 - 2). Following a 2 hour saccharin infusion, renal BBM vesicles were prepared and probed for T1R2 and T1R3 using antibodies for western blotting (Figure 9.16 - 1). A significant 2- and 2.8-fold increase was seen in levels of T1R2 and T1R3 protein respectively, at the proximal tubule BBM after 2 hour saccharin infusion compared with saline infusion alone (Figure 9.16 - 2).

Figure 9.16 – Effect of artificial sweetener on expression of sweet taste receptors, T1R2 (pink) and T1R3 (black), at the proximal tubule BBM, studied by IV infusion of 1.37 mM saccharin in 154 mM saline (slatted bars) for 2 hours 1) western blotting 2) quantification of western blots (expressed as percentage BBM expression) standardised to β-actin. Values are expressed as mean ± SEM, n=6, *p<0.05, ***p<0.005 compared with 154 mM saline infusion (open bars)
9.3.6.3 Renal BBM glucose transport in response to saccharin infusion

In renal BBM vesicles prepared from rats following a 2 hour IV infusion of saccharin, there was a significant increase in $V_{\text{max}}$ of SGLT-mediated glucose transport, as measured by glucose uptake between 30-960 μM concentrations, from $959.4 \pm 90.5$ pmol.mg prot$^{-1}$ in saline-infused control animals to $2333.2 \pm 447.1$ pmol.mg prot$^{-1}$ in saccharin-infused animals (Figure 9.17 - 1). Despite a 26% increase in $K_{m}$ of SGLT-mediated glucose transport, this did not reach statistical significance (Figure 9.17 - 2). Furthermore, renal GLUT-mediated glucose transport, as measured by phloridzin-sensitive uptake at 20 mM glucose concentration, was unaffected by saccharin IV infusion (Figure 9.18).

Figure 9.17 – Effect of artificial sweetener on SGLT-mediated glucose uptake, studied by IV infusion of 1.37 mM saccharin in 154 mM (closed bars) for 2 hours (1) $V_{\text{max}}$ and (2) $K_{m}$ of glucose uptake derived from BBM vesicles incubated at glucose concentrations between 30-960 μM for 4 seconds. Values are expressed as mean ± SEM, n=6-8, *p<0.05 compared with 154 mM saline infusion (open bars)
**Figure 9.18** – Effect of artificial sweetener on total glucose uptake (open bars) and GLUT-mediated glucose uptake (closed bars) studied by IV infusion of 1.37 mM saccharin in 154 mM for 2 hours, using renal BBM vesicles incubated in 20 mM glucose for 4 seconds. Mean ± SEM, n=6-8, p>0.5 compared with 154 mM saline infusion.

9.3.6.4 Effect of saccharin infusion on the expression of renal glucose transporters

The 2 hour IV infusion of the sweetener saccharin was without significant effect on expression of the facilitative fructose transporter GLUT5 at the proximal tubule BBM (Figure 9.19). Although GLUT2 exhibited a decrease in response to saccharin infusion, this was not statistically significant (Figure 9.19). There was no significant change in the levels of the GLUT2 activator PKC-βI at the proximal tubule BBM, however, this also did not reach statistical significance (Figure 9.19). The sodium-dependent glucose transporter SGLT1 was significantly up-regulated in response to saccharin infusion, with a 2.3-fold increase in protein levels at the proximal tubule BBM (Figure 9.19).
**Figure 9.19** – Effect of artificial sweetener on expression of GLUT2 and GLUT5, PKC-βI and SGLT1 at the proximal tubule BBM studied by IV infusion of 1.37 mM saccharin in 154 mM saline (closed bars) for 2 hours: 1) western blotting 2) quantification of western blots (expressed as percentage BBM expression) standardised to β-actin. Values are expressed as mean ± SEM, n=6, **p<0.005 compared with 154 mM saline infusion (open bars)

1

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Saccharin</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT2</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>GLUT5</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>Actin</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>PKC-βI</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td>SGLT1</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
</tr>
<tr>
<td>Actin</td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>

2

![Graph](image13.png)

* * *

**58 kDa**

**55 kDa**

**42 kDa**

**79 kDa**

**75 kDa**

**42 kDa**
9.3.7 Relationship between T1R2 and T1R3, and SGLT1 expression at the proximal tubule BBM

The expression of components of the sweet taste heterodimer T1R2/3 at the proximal tubule BBM in different metabolic models was found to match the expression of SGLT1 in these models (data from Chapter 8). Figure 9.20 displays the expression of T1R2, T1R3 and SGLT1 as compared with the controls for each metabolic model. In the junk-food model and type II diabetic model, T1R2, T1R3 and SGLT1 levels were significantly elevated at the proximal tubule BBM compared with the respective control groups (Figure 9.20). However after 7-day STZ-induced type I diabetes or a high-fat diet or ost-RYGB surgery, there was no significant alteration in renal T1R2, T1R3 or SGLT1 observed (Figure 9.20).

Figure 9.20 – Effect of different metabolic models on the expression of T1R2 (pink), T1R3 (black) and SGLT1 (grey) at the proximal tubule BBM. Values expressed as a percentage BBM expression, standardised to β-actin and compared with control groups for each study.
9.3.8 Relationship between expression of T1R2 and T1R3 at the proximal tubule BBM

As indicated by mRNA analysis in section 9.3.1, in control conditions T1R3 mRNA in the kidney was expressed at higher levels than T1R2 mRNA (Figure 9.1). Therefore, the expression of the sweet taste heterodimer T1R2/3 assessed at the proximal tubule was also expressed as a percentage of expression of T1R2 compared with that of T1R3 in each metabolic model used (Table 9.2). T1R3 was consistently present at the proximal tubule BBM at higher levels than T1R2, as shown by the percentage of T1R2:T1R3 not exceeding 100% (Table 9.2); these increased levels of T1R3 relative to T1R2 reached statistical significance in every metabolic model studied, irrespective of whether the sweet taste receptors in the experimental group were elevated compared with the control for the study (Table 9.2).

Table 9.2 – Relationship between the expression of renal sweet taste receptors T1R2 and T1R3 in response to various metabolic models quantified as percentage expression of T1R2 to T1R3. Values expressed as mean % ± SEM, n=5-12, *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 compared with T1R3 expression.

<table>
<thead>
<tr>
<th>Model</th>
<th>T1R2:T1R3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-day type I</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>48 ± 2.4*</td>
</tr>
<tr>
<td>Diabetic</td>
<td>35.7 ± 2.3*</td>
</tr>
<tr>
<td>Type II</td>
<td></td>
</tr>
<tr>
<td>Lean</td>
<td>54 ± 5.5*</td>
</tr>
<tr>
<td>Diabetic</td>
<td>51.7 ± 5.7*</td>
</tr>
<tr>
<td>Junk-food diet (metabolic syndrome)</td>
<td></td>
</tr>
<tr>
<td>Chow</td>
<td>57.1 ± 0.3****</td>
</tr>
<tr>
<td>Junk-food</td>
<td>61.4 ± 2.7*</td>
</tr>
<tr>
<td>High-fat diet (metabolic syndrome)</td>
<td></td>
</tr>
<tr>
<td>Chow</td>
<td>56.1 ± 9.6**</td>
</tr>
<tr>
<td>High-fat</td>
<td>58.2 ± 0.8*</td>
</tr>
<tr>
<td>RYGB</td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>45.8 ± 4.8***</td>
</tr>
<tr>
<td>RYGB</td>
<td>50.8 ± 3.3****</td>
</tr>
</tbody>
</table>
9.4 Discussion

Glucose reabsorption in the kidney is an area of considerable interest because of its contribution to glucose homeostasis in both normal and metabolic disease models such as diabetes. The importance of renal glucose uptake has prompted the development of SGLT2 inhibitors as a method to reduce glucose reabsorption in diabetes, and thus as a means of lowering blood glucose concentrations. Furthermore, glucose accumulation within the proximal tubule cell, arising from increased glucose uptake, is a potential cause of diabetic nephropathy, a major cause of ESRD. Therefore, the identification of novel agents to influence glucose reabsorption, such as the SGLT2 inhibitor dapagliflozin, is of potential therapeutic value in the treatment of diabetes by lowering blood glucose levels and preventing diabetic nephropathy. This chapter is concerned with the presence of sweet taste sensors in a newly-identified location at the proximal tubule BBM. Furthermore, this chapter focuses on the function of sweet taste receptors in glucose transport across the proximal tubule BBM and the response of these sweet sensors in different models of metabolic dysregulation. Table 9.3 summarises the results of this chapter and conclusions have been made on the involvement of T1R2/3 in renal glucose reabsorption which raises the possibility that sweet taste receptors may be vital therapeutic targets in the treatment of hyperglycaemia and diabetic nephropathy in diabetes.

Table 9.3 - Summary table of effects of type II diabetes, and metabolic models associated with the disease, on expression of sweet taste receptors and SGLT1 at the proximal tubule BBM. SGLT1 data taken from chapter 8. * represents animals which were fasted before cull.

<table>
<thead>
<tr>
<th></th>
<th>T1R2</th>
<th>T1R3</th>
<th>SGLT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I diabetes (7 day)</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>Type II diabetes</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Junk-food diet</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>High-fat diet*</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>RYGB*</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
</tbody>
</table>
The heterodimer of T1R2 and T1R3 is a broadly-acting sweet taste receptor that acts through α-gustducin, to activate adenyly cyclase leading to an increase in cAMP, or which acts through the βγ-subunit of gustducin to activate a PLCβ2-dependent pathway (Cui et al., 2006; Bachmanov and Beauchamp, 2007). The sweet taste heterodimer has been shown to be located throughout the intestinal tract, where it is associated with glucose absorption, as well as in lingual taste tissues (Hoon et al., 1999; Dyer et al., 2005; Bezencon et al., 2007; Stone et al., 2007; Bigiani and Cuoghi, 2007; Mace et al., 2009; Moon et al., 2010). Although a previous study identified TAS1R3 gene expression in the swine kidney, as well as in other tissues, including the heart, lung and pancreas, the finding in the kidney was not investigated further and has not been noted in other species (Kiuchi et al., 2006). Interestingly, Pluznick et al. have identified olfactory receptors, traditionally involved in nasal detection of odorants, in distal tubules in the kidney (Pluznick et al., 2009). In the kidney, these receptors signal via adenylate cyclase and the olfactory G protein Go1f to modulate GFR and rennin secretion.

In the present study, gene expression of both components of the sweet taste sensing heterodimer T1R2 and T1R3 in the renal cortex has been identified under normal conditions. Furthermore, these taste receptors have been shown to be present in both whole membrane and BBM preparations, suggesting a similarity to the enterocyte taste sensing mechanism, where T1R2/3 is localised at the jejunal BBM (Mace et al., 2007). The present study has also shown the presence of the mRNA for both components of the umami taste heterodimer T1R1 and T1R3 in the kidney cortex; this novel expression poses interesting questions about possible amino-acid sensing in the proximal tubule. However, T1R1/3 expression in the kidney cortex was considerably higher than in the jejunum, suggesting a role for these receptors under normal conditions in the kidney, which may not be applicable in the small intestine.

9.4.1 The umami taste sensor

A potential role for the renal T1R1/3 heterodimer could be in the sensing of glutamate in the proximal tubule during ammoniagenesis. This process of generating ammonia
in the proximal tubule cell for secretion across the BBM and into the lumen is vital in regulating acid excretion by the kidney (Good and Knepper, 1985; Nagami, 2004; Van de Poll et al., 2004). In states of insulin-resistance such as obesity, metabolic syndrome and type II diabetes, there is reduced ammonium excretion, and this increases the tendency for acidic urine, leading to uric acid and mixed urate and oxalate stones (Powell et al., 2000; Daudon and Jungers, 2007; Palladino and Stanley, 2010). Indeed approximately 20% of diabetics suffer from renal stones compared with only 8% in non-diabetics; interestingly, metabolic syndrome patients also have hyperuricaemia, a more acid urine and increased risk of renal stones (Maalouf et al., 2004; Liebman et al., 2007). Although the reasons for this reduced ammonium excretion are not yet fully understood, possible explanations are that either there is impaired proximal tubular ammoniagenesis or that there are disturbances in the complex pathway of ammonium excretion by the kidney. Decreased ammoniagenesis could result from a defect in glutamine uptake into proximal tubular cells, for example, reduced SNAT3 protein for glutamine and EAAC1 for glutamate (Mu and Welbourne, 1996; Moret et al., 2007; Busque and Wagner, 2009). A reduction in ammoniagenesis could also be a result of a defect in the production of ammonia, for example, through altered mitochondrial metabolism. Furthermore, it could be explained by disturbed ammonium secretion along the proximal tubule via NHE3 disruption (Biemesderfer et al., 1993; Ambuhl et al., 1996; Gagnon et al., 1999).

In the jejunum, the umami taste receptors T1R1 and T1R3 are responsible for sensing glutamate and aspartate in the lumen and the heterodimer T1R1/3 signals via transducin to activate PKC-βI through a similar pathway to T1R2/3 sweet taste sensing. Activated PKC-βII promotes BBM expression of EAAC1, the glutamate transporter; EAAC1 and T1R1/3 have been shown to have the same specificity for glutamate. Furthermore, glutamate transport via EAAC1 provides the substrate for ammoniagenesis (Li et al., 2002; Mace et al., 2009). In contrast to the small intestine, PKC-βI activation in the S2/S3 segment of the proximal tubule reduces the BBM expression of EAAC1 and NHE3. These PKC-mediated effects could influence ammoniagenesis: first, by limiting the uptake of glutamate into the proximal tubule cell via EAAC1; second, by reducing NHE3-mediated ammonium secretion into the tubular fluid (Karim et al., 1999; Pedrosa et al., 2004; Fournier et al., 2004; Padovano
et al., 2009). Renal taste sensing, in a similar manner to that in the jejunum, might be involved in the regulation of renal EAAC1, and potentially NHE3 (Mace et al., 2009).

Another role for umami sensing in the proximal tubule could be in oligopeptide transport via the proton-dependent PepT1 and PepT2; both these transporters have been found in the proximal tubule BBM, with the Pep-T1 high-capacity transporter localised in the early S1 segment and the PepT2 high-affinity transporter present in the late S2/S3 segment (Shen et al., 1999). Interestingly, in the jejunum, stimulation of the umami taste receptor with glutamate results in the internalisation of PepT1 at the BBM in a PKC-βII-dependent manner (Mace et al., 2009). Due to the similarities in nutrient transport between the jejunum and proximal tubule, it is interesting to speculate whether the umami taste receptor in the kidney is involved in the regulation of PepT1 and PepT2 oligopeptide transporters. Further studies on the effect of stimulating the renal umami taste heterodimer, T1R1/3, on PepT1 and PepT2 activity is specifically intriguing as these oligopeptide transporters are also responsible for the uptake of a variety of pharmacological agents into the proximal tubule cell (Meredith et al., 2000; Daniel and Kottra, 2004).

### 9.4.2 The sweet taste sensor

The sweet taste heterodimer T1R2/3 was considered more applicable to my work, because it is known to be involved in the control of intestinal glucose absorption. Thus, T1R2/3 was studied in relation to glucose transport across the proximal tubule BBM. In addition to the mRNA expression of T1R2 and T1R3 observed in the kidney cortex, expression of these sweet taste receptors was also identified in whole membranes prepared from the kidney cortex of rats and mice. This is in contrast to a study by Kitagawa et al., which showed T1R3 mRNA to be absent in the mouse kidney, with expression only noted in the circumvallate papillae of the tongue, and in the testis (Kitagawa et al., 2001). However, the porcine proximal tubule cell line did not express either T1R2 or T1R3, although this is more likely to be a result of antibody specificity than expression per se in LLC-PK₁ cells. Interestingly, the cell line originates from pigs which exhibit close homology to humans (Valentin et al.,
therefore it would be interesting to study the expression of T1R2 and T1R3 in human kidney tissue.

Sweet taste receptors were also found at the proximal tubule BBM, and, as with the mRNA expression for the umami taste sensor, levels were considerably elevated when compared with those seen in the jejunum. This may indicate that sweet taste sensing in the kidney is more significant than in the jejunum. However, expression of renal sweet taste receptors is unaffected by acute or chronic hyperglycaemia, despite chronic hyperglycaemia being the stimulus for the increased levels of GLUT2 at the proximal tubule BBM via PKC-βI. This suggests that unlike in the jejunum, renal sweet taste receptors are not related to GLUT2 expression at the BBM. Interestingly, STZ-induced type I diabetes affected expression of T1R2 mRNA in the kidney cortex, implying that although BBM levels of the sweet taste receptor are not affected by diabetes at an early stage of the disease, gene-related alterations in their expression do occur. Although renal T1R3 mRNA expression was unaffected in type I diabetes, its heterodimer partner T1R2 was decreased to the same degree at early (10 days) and late (30 days) time points following the induction of type I diabetes. Therefore, the only alterations in the sweet taste heterodimer occur in T1R2 early in type I diabetes and are maintained through the disease.

Human studies have shown that T1R2 and T1R3 transcripts are both down-regulated in the duodenal mucosa biopsies from type II diabetics, and the sweet taste receptors exhibit an inverse correlation with plasma glucose; thus, the duodenal sweet taste receptors are responsive to type II diabetes (Young et al., 2009). The relationship between type II diabetes and sweet taste receptors was studied in the kidneys from normotensive GK rats. These type II diabetic rats were shown in Chapter 8 to exhibit up-regulated glucose transport via PKC-βI activated GLUT2, GLUT5, and SGLT1. In this type II diabetic model, the sweet taste receptors T1R2 and T1R3 were also elevated at the proximal tubule BBM. Therefore, unlike GLUT5, GLUT2, and its activator PKC-βI, the sweet taste receptor expression profile in type II diabetes is different from that seen in type I diabetes.

The elevated renal sweet taste expression seen in kidneys from GK rats was also evident in kidneys from animals fed a junk-food diet for an 8-week period. Renal
T1R2 and T1R3 expression was increased in response to this Western diet, although to a lesser degree than during type II diabetes. This could be explained by the insulin-resistance obesity in the junk-food diet model. However, in response to a high-fat diet, another insulin-resistant model, there were no alterations in sweet taste receptor expression at the proximal tubule BBM. This raises the question of whether there is a component of the junk-food diet that can specifically affect the sweet taste receptors in this dietary model. Intriguingly, there has been a massive rise in HFCS use in soft drinks and junk foods, such as pastries, desserts and various processed foods within the last thirty years. HFCS is a widely-used sweetener, commonly consisting of a mixture of 55% fructose and 45% glucose, which varies from the 50:50 ratio in sucrose, and this causes its intense sweetness, making it a cost-effective sweetener to add to foods (Fulgoni, III, 2008; Moeller et al., 2009). In the present study, long-term feeding of junk-foods high in inverted sugar syrup, another name for HFCS, such as digestive biscuits, flapjacks and marshmallows, would be expected to raise levels of fructose and glucose in the plasma. As a result, the elevated levels of these sugars in tubular filtered fluid may result in enhanced stimulation of sweet taste receptors at the renal BBM; an effect which would not occur in rats fed a high-fat diet alone. Recent studies highlight that HFCS may be detrimental to health and a major reason for the global obesity epidemic, with insulin resistance established as a side-effect of a high-fructose intake (Forshee et al., 2007; Johnson et al., 2009). Thus elevated fructose consumption correlates with the development of type II diabetes. Furthermore, fructose consumption is also associated with renal tubular injury and the very early stages of diabetic nephropathy, potentially as a side-effect of elevated uric acid in response to raised levels of circulating fructose (Boot-Handford et al., 1981; Sanchez-Lozada et al., 2007; Bomback et al., 2010). The enhanced renal sweet taste sensing in junk-food fed animals may even be involved in fructose-induced renal tubular injury.

9.4.3 Sweet taste sensing and hormonal regulation

Interestingly, dysregulation of sweet taste sensing in diabetes is not confined to the kidney. Sweet hypoguesia, the reduced ability to taste sweet substances, was
identified in 73% of type I diabetic patients, whereas the incidence was only 16% in non-diabetics (Le Floch et al., 1989). Also, type II diabetics display a blunted taste response to sweet foods (Perros et al., 1996; Gondivkar et al., 2009). Therefore impaired sweet taste sensing in diabetes may result in the increased consumption of sugary foods to achieve sweet satiety, and may exacerbate the hyperglycaemia associated with these disorders.

Chronic alterations in plasma leptin levels have been hypothesised to underlie the changes in sweet taste threshold and sensitivity, and increased serum leptin has been shown to affect gustatory mechanisms in diabetic and obese conditions (Ninomiya et al., 2002; Shimizu et al., 2003; Nakamura et al., 2008). Leptin, the product of the obese (ob) gene, is a hormone predominantly released from adipose tissue and regulates food intake and body weight by binding to the leptin receptor (Ob-Rb) in the hypothalamus, however, the Ob-Rb has also been identified in peripheral organs such as adipose tissue, lung, liver and kidney (Cao et al., 1997; Wang et al., 1997a; Pralong and Gaillard, 2001). Leptin binding to Ob-Rb on taste-sensitive cells causes the specific inhibition of peripheral neural and behavioural responses to sweet taste sensing in lean mice; inhibition occurs due to Ob-Rb activation causing increased outward currents and reduced cell excitability, followed by a reduction in sweet taste sensing conveyed to the brain (Kawai et al., 2000). In Ob-Rb deficient db/db diabetic mice there is no suppression of sweet taste sensing because leptin cannot bind and activate its receptor.

Energy excess or deprivation may affect the sensitivity of some nuclei in the central nervous system (CNS) that are involved in taste perception, which in turn act on the gustatory receptors (Plata-Salaman et al., 1993; Sudakov, 1993). This was shown in diabetic and high-fat diet-induced obese rats where a heightened response to sweeteners was seen in the chorda tympani nerve, a branch of the facial nerve that serves taste buds in the front of the tongue (Kawai et al., 2000; Shimizu et al., 2003). Patients have shown a reduced acuity for sweet tastes following RYGB surgery, whereas the bitter taste threshold was unaffected (Bray et al., 1976; Burge et al., 1995). This has also been shown in rodent studies with sham-operated animals exhibiting a higher preference for sucrose than RYGB-operated (Tichansky et al., 2010). In the studies presented here, the sweet taste heterodimer in the proximal
tubule was unaffected by RYGB surgery, however there was reduced expression in the proximal small intestine, therefore reduced sugar preference following RYGB may be related to intestinal T1R2/3 expression. Interestingly, following RYGB surgery, patients exhibit reduced preference for meat and lower protein intake, which can result in malabsorption, and this may be caused by changes in T1R1/3 umami taste sensing (Burge et al., 1995).

In addition to a reduced preference for high concentrations of saccharin, rats fed a high-fat diet exhibited a significant decrease in mRNA expression of T1R3 in taste buds, as well as a trend towards reduction of the molecules involved in the signalling pathway for taste transduction (Chen et al., 2010). In contrast, chronically restricting the control chow diet by 50% caused an increase in preference for saccharin when the sweetener was used at a concentration of 0.04 M (Chen et al., 2010). Although there was no change in T1R2 or T1R3 mRNA expression in taste buds, there was a significant increase in expression of leptin receptor (Chen et al., 2010). A junk-food diet results in raised leptin levels, primarily due to elevated adiposity, since when leptin concentration was corrected for body fat mass, there was no difference between junk-food fed and controls (De et al., 1998). Furthermore, a high-fat diet causes an elevation in circulating leptin levels in rodent models and man (Maffei et al., 1995; Considine et al., 1996). Therefore, this hormone has a depressive effect on sweetener intake and restricting the diet causes increased Ob-Rb and increased preference for saccharin via elevated taste cell excitability. In response to RYGB surgery, plasma leptin is significantly decreased, a finding in both patient and rodent RYGB models due to a reduction in adiposity, similar to that seen in chronically-fasted conditions (Rubino et al., 2004; Borg et al., 2006; Cariani et al., 2008; Korner et al., 2009). This emphasises that changes in metabolic state are able to modulate the sensitivity to gustatory stimuli, as well as the sensory pleasure of sweetness and the hedonic reactions of rats to sweeteners.

Although these studies were based on oral taste signalling pathways, it is interesting to note that the leptin receptor is present in the kidney. Leptin is cleared principally by the kidney and serum leptin concentrations are increased in patients with chronic renal failure; as well as the binding of the hormone to the leptin receptor (Ob-Rb) in the kidney, leptin can also bind megalin and cubulin at the proximal tubule BBM.
Therefore, leptin-mediated effects on sweet taste sensing may not only have a bearing on taste in the mouth and intestinal tract, but leptin may have a whole-body effect on sweet taste sensing, including in the proximal tubule.

GK type II diabetic rats are hyperleptinaemic whereas in type I STZ-induced diabetes, circulating leptin levels are decreased (Havel et al., 1998; Maekawa et al., 2006). A high-fat diet and RYGB surgery were without effect on renal sweet taste receptors, whereas junk-food feeding caused an elevated expression of sweet taste receptors at the proximal tubule BBM, suggesting a dissociation between oral taste signalling and renal taste sensing. Plasma leptin may be responsible for the changes in sweet taste sensing at the proximal tubule BBM as, with the exception of increased leptin in response to the high-fat diet, changes in circulating leptin levels are mirrored by T1R2/3 expression. However, in response to long-term saccharin feeding, diabetic ob/ob mice exhibit delayed onset of hyperglycaemia, thus the stimulation of sweet taste sensors can occur in this leptin-deficient rodent model (Bailey et al., 1997). It would therefore be of interest to study the influence of circulating leptin on the renal sweet taste sensor.

Unlike sweet taste sensing in the oral taste cells, which stimulates peripheral gustatory nerves and brain gustatory pathways, sensing in small intestinal enteroendocrine cells results in the release of incretins. These gut-derived hormones comprise GIP and GLP-1 and are released from the BLM of enteroendocrine cells; incretins can either act locally on enterocytes or activate afferent neurones in the gut villi or enter the bloodstream to act as systemic hormones to augment the release of pancreatic insulin (Jang et al., 2007; Shin et al., 2008). Therefore, sweet taste sensing in the small intestine is not only involved in paracrine control of glucose absorption, but also affects whole-body glucose homeostasis, such as the increased appetite and weight gain associated with increased circulating insulin; a theory known as the Egan & Margolskee hypothesis (Margolskee et al., 2007). However, knowledge of plasma insulin levels in these metabolic models, type I and II diabetes and metabolic syndrome offers no further understanding of the mechanisms involved in taste receptor activation. The present study showed that type I diabetes was associated
with low levels of circulating insulin, whereas there was no change in insulin levels in GK rats, and high levels were seen in both the metabolic syndrome models; therefore, sweet taste receptors are probably activated via an insulin-independent pathway. Furthermore, the plasma glucose levels in these metabolic models were extremely varied, although all exhibit significantly augmented levels compared with controls. Blood glucose levels of the STZ-induced type I diabetic model was nearly two-fold higher than in the GK type II diabetic model, and both high-fat feeding and junk-food diet increased plasma glucose levels, but maintained them within the normal range. Under normal conditions, the late S3 region of the proximal tubule would not be exposed to high luminal glucose levels as the majority is reabsorbed in the early S1/S2 region. However, in the STZ-induced type I and GK type II model of diabetes T1R2/3 expression is unchanged and up-regulated, respectively; hyperglycaemia in these models would result in elevated glucose in the tubular fluid and thus, it is likely that the renal sweet taste heterodimer exerts a response that is independent of a glucose stimulus. This is an unusual finding considering the specificity for the sweet taste heterodimer, T1R2/3, for simple sugars, as well as other sweet substrates including artificial sweeteners. Li et al have previously shown a dose-dependent activation of T1R2/3 to sucrose in the very high millimolar range and, although acute IV infusion of glucose was administered at an infusate concentration of 1.1 M, the equivalent concentration in the plasma, and thus early proximal tubular fluid, never exceeded 50 mM (Li et al., 2002). Despite the tendency for these supraphysiological levels of glucose to cause osmotic diuresis and dehydration, they are unlikely to elicit a sweet taste response (Small and MacCuish, 1987; Tanaka et al., 2000; Chiasson et al., 2003). This is in agreement with data presented here, where IV infusion of glucose and mannitol, which incurred hyperglycaemia and osmotic shock respectively, had no effect on T1R2/3 expression at the proximal tubule BBM.

Interesting studies by Shin et al found that GLP-1 is specifically co-localised with T1R3 and the sweet taste signalling molecules α-gustducin and PLCβ2 in a subset of lingual taste cells (Shin et al., 2008). The presence of gut hormones in the taste bud highlights an important parallel between gustatory and intestinal epithelia; furthermore, it has been postulated that GLP-1 and glucagon signalling modulate oral sweet taste sensitivity (Shin et al., 2008). Au et al have shown that the structurally
related GLP-2 can rapidly promote expression of GLUT2 at the intestinal BBM. This suggests that the glucagon family of peptide hormones is potentially important in the control of epithelial glucose transport (Au et al., 2002). Intriguingly, Marks et al. have identified the expression of glucagon receptor mRNA in the proximal tubule and showed that glucagon stimulates GLUT-mediated glucose transport (Marks et al., 2003•). Therefore, it is possible that glucagon signalling may influence sweet taste sensing in the kidney. Glucagon, the 29-amino acid hormone synthesised by pancreatic α-cells, binds to the GLP-1 receptor and, through GTP-binding heterotrimeric Gs-proteins, activate adenylate cyclase activity, leading to increased intracellular cAMP levels (Mayo et al., 2003). Furthermore, glucagon binding to the GLP-1 receptor also results in increased signalling through the PLC/IP3/Ca2+ pathway, resulting in PKC activation and elevated gluconeogenesis (Li et al., 2006).

Clinical studies have shown that hyperglucagonaemia is often associated with the onset and progression of type II diabetes; also raised levels of blood glucagon in both obese and non-obese type II diabetes have been well-established (Unger, 1978). Indeed, the long-term infusion of glucagon into mice produced metabolic and renal phenotypes of type II diabetes and diabetic nephropathy, including increased fasting blood glucose, impaired glucose tolerance, development of microalbuminuria, mesangial expansion and increased extracellular matrix (ECM) deposition in the glomeruli (Li et al., 2008).

Studies carried out in type I diabetic rodents and humans have shown that proximal tubule levels of GLP-1 receptor are down-regulated, possibly due to PKC-mediated effects; the infusion of GLP-1 into type I diabetics results in restoration of glycaemia by reducing the high circulating glucagon levels (Creutzfeldt et al., 1996;Behme et al., 2003;Marks et al., 2003;Marks et al., 2003). Patients suffering from metabolic syndrome exhibit high plasma levels of GLP-1; however, in response to duodenal-jejunal exclusion gastric bypass surgery, improved glucose tolerance occurs in GK diabetic rats. The GLP-1 antagonist exendin-[9-39] abolished this improvement (Kindel et al., 2009;Yamaoka-Tojo et al., 2010). Thus glucose tolerance was hypothesised to be a result of elevated GLP-1 levels in plasma.
9.4.4 Sweet taste sensing and renal glucose transport

As previously stated in this section, an increase in adiposity and obesity does not appear to be the cause of the elevated expression of sweet taste receptors, otherwise the high-fat diet would have an impact on T1R2 and T1R3. However, it is possible that the adiposity and obesity in the junk-food diet is a more physiological pre-diabetic state than the high-fat diet. These data from the different diet-induced metabolic syndrome model echo the conclusion from data obtained in the present study on type I diabetes. The augmented PKC-βI-mediated expression of GLUT2 at the proximal tubule BBM occurs independently of activation of the sweet taste receptors T1R2 and T1R3. This is emphasised by the unaffected T1R2/3 levels in response to hyperosmotic mannitol, which, as described in Chapter 7, results in the recruitment of GLUT2 and PKC-βI to the proximal tubule BBM. Thus, it is likely that GLUT2 recruitment to the proximal tubule BBM can occur via a sweet taste receptor-dependent pathway, as is the case in type I diabetes and high-fat diet-induced metabolic syndrome, as well as in osmotic shock conditions. Alternatively, GLUT2 is shuttled to the proximal tubule BBM via a sweet taste receptor-dependent pathway, as seen in type II diabetes and junk-food-induced metabolic syndrome. It cannot be overlooked that there is increased mRNA expression of T1R2 in response to short and long-term type I diabetes; however, 7 days hyperglycaemia, though sufficient to result in the recruitment of GLUT2 to the proximal tubule BBM, does not stimulate an increase in T1R2 protein. It is possible that T1R2 responds at a slower rate than GLUT2, as shown by type II diabetes, diet-induced metabolic syndrome and RYGB models, which were studied over longer time periods (5-weeks to 8-9 weeks). Interestingly, altered GLUT5 transporter expression at the proximal tubule BBM parallels changes in T1R2/3 expression, with STZ-induced type I diabetes being the exception to this relationship, where GLUT5, but not T1R2/3, is up-regulated. Therefore, it is possible that fructose transport in the proximal tubule may be stimulated by sweet taste sensing at the tubular epithelium.
Artificial sweeteners are increasingly used to aid weight-loss and as a sugar-substitute for diabetics, since they have no calorific value and are not believed to affect blood glucose levels. However, these aspects of artificial sweeteners are currently under re-examination in light of their effect on intestinal glucose absorption. Sucralose, commercially available as ‘SucraPlus’ or ‘Splenda’, is often used to stimulate T1R2/3 due to its stability and intense sweetness (it is approximately 600-fold sweeter than sucrose and twice as sweet as saccharin). However, clinical studies suggest that artificial sweeteners blunt sugar sensing due to their intense sweetness; indeed, the consumption of sucralose results in reduced food intake and decreased body weight (Mann et al., 2000). Sucralose would be interesting to study, as it is able to enhance the recruitment of GLUT2 to the enterocyte BBM to a higher degree than saccharin (Mace et al., 2007). Sucralose induces a dose-dependent increase in kidney weight in long-term, 52 week, sucralose feeding studies; furthermore, high sucralose feeding has also been associated with an increased incidence of chronic nephropathy (Mann et al., 2000; Sims et al., 2000). Only 5-10% of an oral sucralose dose is absorbed by the small intestine and plasma sucralose has a half-life between 2 and 5 hours with absorbed sweetener found to accumulate in the liver, kidney and gastrointestinal tract (Sims et al., 2000). The more intense sweetness of sucralose, coupled with its established effects on intestinal glucose transport, make this sweetener an interesting artificial sweetener to study in relation to the involvement of renal sweet taste receptors and plasma glucose uptake in long-term, high glucose-induced tubular damage.

Since artificial sweeteners are able to elicit a stronger response from T1R2/3 than simple sugars at a lower concentration, the question was posed whether sweet taste receptors in the proximal tubule are responsive to artificial sweeteners in the circulation (Li et al., 2002). The present study shows that the stimulation of sweet taste receptors at the proximal tubule BBM, via IV infusion of saccharin, induced a rapid up-regulation in T1R2 and T1R3 expression. Interestingly this was accompanied by elevated SGLT-mediated glucose transport and SGLT1 expression but the artificial sweetener had no influence on GLUT-mediated glucose transport or
renal expression of PKC-βI, GLUT2 or GLUT5. Thus it is clear that unlike in the jejunum, acute activation of the renal sweet taste heterodimer does not elicit GLUT2 recruitment to the BBM. Longer-term studies using STZ-induced type I diabetes or the GK rat model of type II diabetes, where GLUT2 expression is elevated at the proximal tubule BBM would result in the proximal cell being primed with glucose; examination of the effect of saccharin on glucose-primed proximal tubule cells may be useful, since data in Chapter 7 show that renal GLUT2 cannot be recruited to the BBM in the short-term, unless in response to high osmolality. The necessity for a glucose stimulus, in addition to an artificial sweetener, to stimulate GLUT2 recruitment to the BBM has been shown in the jejunum where the administration of 20 mM glucose with sucralose was necessary to elicit the translocation of GLUT2 (Mace et al., 2007; Mace et al., 2009).

Intriguingly the elevated SGLT-mediated glucose transport at the proximal tubule BBM occurred despite the absence of changes in blood glucose levels. Although plasma saccharin levels were not analysed in these experiments, it is likely that levels of plasma saccharin were raised. Lethco et al observed 56-87% saccharin, which was fed in the diet over 7 days, was not metabolised and excreted via renal tubular secretion as well as filtration (Lethco et al., 1975). Therefore, in response to IV infusion of the sweetener in the studies presented here, the levels in the tubular fluid are likely to be high; however the artificial sweetener may exert an effect at either the BLM or BBM. Furthermore, SGLT1 expression in the proximal tubule was elevated in junk-food fed and type II diabetic rats, where levels of the sweet taste receptors T1R2 and T1R3 were also augmented. Thus, renal sweet taste receptor activation occurs in parallel with SGLT1 up-regulation at the proximal tubule BBM suggesting a strong relationship between this high-affinity glucose transporter and the sweet taste heterodimer.

Previous studies have identified the involvement of the SLC5 family of glucose transporters in sugar sensing; human SGLT3, expressed in Xenopus oocytes, depolarises plasma membranes in response to extracellular glucose and thus can sense, but not transport, sugars (Diez-Sampedro et al., 2003). Interestingly, a recent study identified a single amino acid mutation in the SGLT3 protein that can convert the sugar sensor into a sugar transporter (Bianchi et al., 2010). Data in the present
study shows that SGLT1 expression is related to the sweet taste sensors at the proximal tubule BBM, while the high-capacity transporter GLUT2 is unaffected by saccharin-mediated sweet taste receptor stimulation. Thus, the renal T1R2/3 heterodimer appears to mediate a finely-tuned mechanism for the control of renal glucose reabsorption, whereby glucose uptake occurs via the up-regulation of SGLT1. This echoes data from the small intestine, where sweeteners have been shown to increase SGLT1 and SGLT-mediated glucose transport, in addition to their role in the recruitment of GLUT2 to the enterocyte BBM (Mace et al., 2007; Stearns et al., 2010). It is important to study SGLT2 in this context, since it is a higher capacity transporter at the proximal tubule BBM; indeed studying the expression of SGLT2 in response to sweeteners would aid the further understanding of the relationship of T1R2/3 to SGLT-mediated glucose transport.

9.4.6 Sweet taste sensing and glucose absorption in response to gastric bypass surgery

In response to duodenal bypass as a result of RYGB surgery, the expression of GLUT2 was down-regulated at the proximal tubule BBM, along with PKC-βI, but SGLT1 and sweet taste receptor expression was unaffected. Although these data emphasise the relationship between the expression of SGLT1 and T1R2/3, they also provide further evidence that T1R2/3 is not involved in the expression of GLUT2. However, it is worth noting that mRNA expression of the sweet taste receptor T1R2 was reduced in the renal cortex from animals after RYGB surgery. Therefore, the intracellular pool of only one component of the renal sweet taste heterodimer was depleted in response to RYGB surgery. RYGB surgery has been shown to affect hormone signalling, in particular GLP-1 signalling; however, as described above, these factors do not appear to be solely responsible for sweet taste sensing.

Interestingly, the reduction in renal expression of T1R2 following RYGB surgery was also seen in T1R2 protein and mRNA expression in the bypassed duodenal biliopancreatic limb. RYGB surgery involves the re-sectioning of the duodenum to the alimentary limb, therefore dietary load moves directly into the jejunum and renders the biliopancreatic limb ineffectual (Figure 8.1). However, the T1R2 sweet
taste receptor is also down-regulated at the alimentary limb BBM, which is the first part of the small intestine to be exposed to high dietary glucose; therefore, expression of T1R2 is unlikely to be responsive to a direct sweet stimuli present in the intestinal fluid. Protein levels of the T1R3 sweet taste receptor expressed in the alimentary limb BBM were reduced, despite T1R3 mRNA expression being unaffected by RYGB surgery, along the entire small intestine. This is the first analysis of T1R2/3 expression in the small intestine following this surgical model and may provide an explanation for the reduced sweet taste preference observed following RYGB surgery in both human and rodent models (Burge et al., 1995; Tichansky et al., 2010; Hajnal et al., 2010).

Studies of intestinal glucose transport following RYGB surgery discussed in Chapter 8 showed reduced expression of GLUT5 in the duodenum; therefore, its expression may be a consequence of sweet taste sensing of the carbohydrate load in the lumen, as hypothesised above for proximal tubule GLUT5–sweet taste heterodimer expression. However T1R2/3 expression in the RYGB model provides evidence that sweet taste receptors and SGLT1 expression at the enterocyte BBM are unrelated, since studies in Chapter 8 show that expression of SGLT1 is up-regulated in the alimentary limb following RYGB surgery, whereas expression of both T1R2 and T1R3 are reduced.

Although hormones, such as insulin, GIP and GLP-2 are known to modulate enterocyte glucose transport, glycaemic status is also known to influence glucose absorption (Csaky et al., 1981; Maenz and Cheeseman, 1986; Pennington et al., 1994; Cheeseman et al., 1996; Cheeseman, 1997). Thus, STZ-induced type I diabetes promotes glucose uptake across the jejunal BBM via SGLT1 and GLUT2 (Debnam et al., 1988; Burant et al., 1994; Debnam et al., 1995; Kellett and Brot-Laroche, 2005). Interestingly, in the present study using the jejunum from 30-day STZ-induced diabetic rats, there was an elevation in mRNA expression of T1R2 and T1R3. Therefore, unlike in RYGB surgery, where glucose transport is not related to the expression of sweet taste receptors, in type I diabetes the previously established positive relationship between sweet taste sensing and enterocyte glucose absorption is confirmed (Dyer et al., 2005; Margolskee et al., 2007; Mace et al., 2007; Mace et al., 2009).
9.4.7 Characteristics of renal sweet taste sensing

It is worth noting that previous studies have suggested that sweet taste receptors are internalised on activation (Tan et al., 2004). Thus, the elevated brush-border expression of T1R2/3 seen in the proximal tubule in type II diabetic and junk-food fed rats may be a consequence of, under basal conditions, reduced activation of the renal sweet taste heterodimer. However, this does not appear to be the case in renal sweet taste sensing, since in response to saccharin expression of both T1R2 and T1R3 were elevated. Therefore, this represents a major difference in sweet taste receptor activation in the kidney in comparison with that in the small intestine and in lingual taste buds.

In the present work in all metabolic models studied, the renal expression of T1R3 mRNA and protein was significantly higher than T1R2 (Table 9.2). Interestingly, mRNA expression of the umami taste receptor component T1R3 was also found to be higher compared with T1R1 expression. Previous studies have shown that T1R3 is able to function as a homodimer, as well as part of the sweet and umami taste heterodimer: for example, for sensing of the disaccharide trehalose (Nelson et al., 2001; Ariyasu et al., 2003). T1R3 has been found to be located in the liver, bile duct carcinoma cells, stomach, lung, and pancreatic α and β-cells (Taniguchi, 2004; Toyono et al., 2007; Nakagawa et al., 2009). Li et al identified a fraction of T1R3-expressing cells that did not express T1R1 or T1R2; furthermore, allelic variations of only the Tas1r3 gene influence taste responses to a wide range of artificial sweetener concentrations and sugars (Li et al., 2002; Inoue et al., 2007). Studies using the T1R3 knockout mouse have emphasised the importance of this taste receptor, since in this model animals display no preference for artificial sweeteners with diminished behavioural and nerve responses to sweet and umami compounds (Damak et al., 2003). Hass et al suggested that T1R3 dimerises with different, and hitherto unknown, components in brush-border cells of the small intestine. Interestingly, T1R3 has also been shown to function as a gustatory calcium-magnesium receptor, as well as its involvement in sweet and umami taste sensing (Tordoff et al., 2008; Hass et al., 2010).
Therefore in the kidney, T1R3 may be more highly expressed, than T1R1 or T1R2, because of its role in sweet and calcium-magnesium tasting, as well as being a component of the sweet (T1R2/3) or umami (T1R1/3) taste heterodimer.

9.4.8 Novel locations and roles for taste sensors

Recent studies have linked chemosensation of luminal constitutes not only to the regulation of central nervous system activity, but also to local control of absorptive and secretory processes (Sclafani et al., 1976; Mace et al., 2007; Gulbransen et al., 2008). Bitter and sweet taste receptors recently identified in airway smooth muscle have been shown to trigger protective reflexes such as bronchoconstriction (Deshpande et al., 2010; Tizzano et al., 2011). Bitter taste receptors have also been found in nasal chemosensory cells where stimulation of these receptors invokes changes in respiration; whereas T1R3 in pancreatic β-cells has been implicated in stimulating insulin secretion (Nakagawa et al., 2009; Tizzano et al., 2010).

The presence of taste receptors in the kidney raises interesting questions concerning their potential role in nutrient sensing and reabsorption. As hypothesised above, renal T1R1/3 umami taste sensing is potentially involved in amino-acid transport and oligopeptide transport, with implications for ammoniagenesis or drug reabsorption. Expression of T1R2/3 sweet taste heterodimer in the kidney is altered by metabolic status, and data in the present study suggest that T1R2/3 sensing may have implications for glucose reabsorption during the feeding of a junk-food diet and in type II diabetes. There are many possibilities for taste sensing in the proximal tubule that may have a wider involvement in the regulation of nutrient reabsorption by the kidney, for example, the CaSR, located at the proximal tubule BBM is likely to have a sensory function (Riccardi et al., 1998). Recent evidence implicates the stimulation of the widely-distributed CaSR as mediating the japanese *kokumi* taste, as well as the established role of CaSR in amino-acid sensing (Conigrave et al., 2000; Conigrave et al., 2006; Ohsu et al., 2010). Thus taste sensing in the kidney may affect nutrient transport via mechanisms other than those studied in this chapter.
9.5 Conclusion

Sweet taste sensing is important for nutrient recognition in response to energy requirements. Data presented in this chapter have established that components of the sweet taste heterodimer T1R2 and T1R3 are expressed in the kidney, specifically at the proximal tubule BBM. Furthermore, the stimulation of T1R2 and T1R3 by artificial sweeteners, and their up-regulation in response to junk-food feeding and type II diabetes is associated with the increased $V_{\text{max}}$ of SGLT-mediated glucose transport and expression of SGLT1 at the renal BBM. However, a high glucose stimulus, acutely or chronically induced, did not alter levels of T1R2 and T1R3 at the proximal tubule BBM, suggesting a specific response of the heterodimer to sweetener intensity. GLUT2 at the proximal tubule BBM was up-regulated by a sweet taste receptor-SGLT1-dependent and -independent pathway, depending on the nature of the metabolic disease stimulus. This may have implications for the widespread use of artificial sweeteners and the potential relationship with the increasing incidence and prevalence of obesity-related diabetes. Glucose reabsorption across the proximal tubule BBM may be one cause of diabetic nephropathy; thus, the identification of sweet taste receptors and their influence on glucose reabsorption implicates these sensors in the novel treatment of junk-food and sweetener-induced metabolic syndrome and type II diabetes. Targeting renal sweet taste receptors would reduce blood glucose levels in these conditions, which could, in turn, slow the progression of nephropathy; however, the question remains ‘How do renal taste receptors affect the renal pathophysiology associated with metabolic syndrome and diabetes?’. By answering this question it may be possible to elucidate the role of taste receptors in a renal regulatory system that is responsive to changes in tubular fluid composition.
10 Discussion
10.1 Aims of thesis

Diabetic nephropathy occurs as a consequence of hyperglycaemia-induced renal cell damage with tubular injury in the disease postulated to be the result of elevated GLUT-mediated glucose entry into proximal tubule cells, in a manner similar to that seen in mesangial cells (Brosius and Heilig, 2005). Previous studies have established that in type I diabetes PKC-βI-dependent GLUT2 recruitment to the proximal tubule BBM occurs as a direct result of hyperglycaemia, resulting in up-regulated glucose transport across this membrane (Marks et al., 2003;Goestemeyer et al., 2007). However type II diabetes is the prevalent form of the disease in which sufferers develop insulin resistance; therefore type II diabetes presents an interesting model to characterise the factors modulating glucose reabsorption across the proximal tubule cell in the presence of a basolateral insulin stimulus. The development of type II diabetes is strongly linked to the current epidemic of obesity and metabolic syndrome; consequently, it was important to characterise glucose transport across the proximal tubule BBM in rodent models of metabolic dysregulation. In addition, the novel modulation of renal glucose transport across the BBM, by sweet taste receptors, was studied using recently characterised mediators of jejunal glucose transport as a model.
10.2 Discussion

The work presented here shows that, in euglycaemic conditions, renal GLUT2 is expressed in low abundance at the proximal tubule BBM. This finding contrasts with that of previous studies (Marks et al., 2003; Marks, 2004; Goestemeyer et al., 2007). The expression of GLUT2 at the renal BBM during euglycaemia parallels findings seen in the jejunum, where GLUT2 is expressed at low levels at the enterocyte BBM before a meal (Gouyon et al., 2003). In addition, elevated levels of GLUT2 occur at the BBM of proximal tubule cells and enterocytes in response to type I diabetes (Corpe et al., 1996; Boyer et al., 1996; Sharp et al., 1997; Marks et al., 2003; Marks, 2004). However, a major difference between BBM glucose transport in the small intestine and kidney has been shown in the studies presented here: in the proximal tubule increased GLUT2 expression at the BBM requires a glucose stimulus of at least 7 days, whereas Helliwell et al found higher levels of GLUT2 at the jejunal BBM within minutes following raised luminal levels of glucose (Helliwell et al., 2003). This highlights the importance of a rapid-response high-capacity glucose transporter in the small intestine to absorb the high-luminal carbohydrate load seen postprandially. In contrast, glucose concentration in proximal tubular fluid does not normally reach the levels seen in the small intestine; thus, renal glucose reabsorption at the BBM utilises SGLT1 and SGLT2, with elevations in GLUT2 occurring during periods of metabolic dysregulation. Interestingly, studies presented in this thesis have also shown that extreme osmotic stress increases the recruitment of GLUT2 to the proximal tubule BBM via a separate, rapid-response, PKC-β1-dependent signalling pathway; however the physiological relevance of this model is limited.

In type I and II diabetes, glucose is stored in the proximal tubule cell as glycogen; however, during obesity, the glycogen status of the cell is not known (Rasch, 1984; Williams et al., 2002; Bamri-Ezzine et al., 2003; Ohta et al., 2007). In models of both forms of diabetes, GLUT2-mediated glucose uptake into the proximal tubule cell is elevated; however, GLUT2 is also expressed at the BLM. Thus, under hyperglycaemic conditions, GLUT2 is able to transport glucose into the proximal tubule cell from the circulation, as well as from the tubular lumen. The facilitative transport of glucose is dependent on the glucose levels in the proximal tubule cell,
which will be altered if glucose is rapidly converted into glycogen. Further studies are therefore necessary to elaborate on the directional flow of glucose across the proximal tubule cell, as well as cellular glucose utilisation during periods of elevated GLUT2 expression. Such studies could use polarised proximal tubule cells such as the porcine LLC-PK1 cell line plated on transwell plates to study glucose fluxes, following exposure of the basolateral and brush-border surfaces to medium containing high glucose levels. This in vitro experimental set-up would also enable the addition of specific GLUT and SGLT inhibitors to the medium to allow analysis of specific transporters contributing to glucose flux across the proximal tubule cell.

Previous studies have shown that type I diabetes stimulates glucose transport across the proximal tubule BBM via GLUT2; a strong positive correlation between the expression of GLUT2 and PKC-βI has also been noted in the disease (Marks et al., 2003; Goestemeyer et al., 2007). The present study has validated this finding in 7-day STZ-induced diabetic rats, as well as observing similar effects of type II diabetes on elevated GLUT-mediated glucose transport across the BBM, and augmented GLUT2 and PKC-βI levels. Additionally, in all the metabolic models used, expression of GLUT2 at the BBM is paralleled by PKC-βI abundance. Indeed, following gastric bypass surgery the reduced GLUT2 levels were matched by a decline in PKC-βI expression. Therefore, it is likely that PKC-βI at the proximal tubule BBM provides the signal for trafficking of GLUT2 from intracellular or basolateral locations to the brush-border. The activation of PKC-βI at the proximal tubule BBM is an important area of interest and could be studied using the specific PKC-βI inhibitor Ruboxistaurin (LY-333531). Ruboxistaurin is currently under development by Eli-Lilly for the treatment of diabetic complications such as retinopathy and nephropathy (Tuttle et al., 2005). To study further the relationship between PKC-βI and GLUT2, Ruboxistaurin could be administered to rodents suffering metabolic dysregulation such as the feeding-induced obesity models used in this thesis. The data presented here suggests the need for a specific PKC-βI inhibitor in the treatment of diabetes to abolish the recruitment of GLUT2 to the proximal tubule BBM, so reducing glucose entry into the cell in this disease with its pathological consequences.

In the studies presented here, PKC-βI-mediated GLUT2 expression at the proximal tubule BBM can occur in conditions of metabolic dysregulation accompanied by
unaltered blood glucose concentration, such as the response to high-fat feeding or gastric bypass surgery. In both models, neither PKC-βI nor GLUT2 exhibit any correlation with plasma glucose levels; therefore, GLUT2 appears to respond to factors that are not directly associated with glycaemia. The stimulus responsible for increased GLUT2 expression at the BBM is unknown, but it may be endocrine, involving GLP-1 and GIP. The studies presented here indicate that expression of GLUT2 at the proximal tubule BBM operates via an insulin-independent pathway. Data from the junk-food-induced pre-diabetic and hyperlipidaemia-induced obesity models demonstrate a close association between dietary intake and the onset of altered renal glucose handling, which was also observed in diabetes. Interestingly, GLUT2 may be responding to a basolateral insulin stimulus, similar to the relationship seen in the small intestine (Pennington et al., 1994; Tobin et al., 2008). However, in STZ-induced type I diabetes, where circulating insulin levels are low, augmented GLUT-mediated glucose uptake and GLUT2 expression have been noted (Alberti et al., 1992; Marks et al., 2003). This implies that insulin alone is not responsible for GLUT2 expression, although further studies to ascertain the response of the insulin receptor in the proximal tubule to different models of metabolic dysregulation are necessary to confirm this. Furthermore, to fully understand the causative agent of renal GLUT2 up-regulation in diabetes and metabolic syndrome, analysis of a wide array of cytokines, such as IL-6, and hormones would be important.

Studies presented here provide further evidence for a relationship between obesity and the development of type II diabetes. GLUT2 at the proximal tubule BBM appears to be a renal response to metabolic stress, as well as the observed response to mannitol-induced osmotic shock. The implications of increased facilitative glucose uptake across the proximal tubule BBM during physiological stress for the cell are unknown.

The recruitment of GLUT2 to the jejunal BBM has been shown to be dependent on the activation of PKC-βII: the movement of PKC-βII to the BBM and its activation in this location occurs via a Ca^{2+}-dependent and sweet taste receptor pathway (Mace et al., 2007; Morgan et al., 2007; Mace et al., 2009). The study by Goestemeyer et al implies that the stimulation of GLUT-mediated glucose transport into proximal tubule cells required elevated cellular calcium levels (Goestemeyer et al., 2007). However, no studies have been performed to ascertain whether a sweet taste sensing pathway...
occurs in the kidney. In the studies presented in this thesis, components of the sweet taste heterodimer sweet taste receptors T1R2 and T1R3 were identified in the kidney. Although the localisation of these sweet taste receptors has not been studied, the presence of mRNA in the kidney cortex and protein at the proximal tubule BBM strongly implies that one renal location is the proximal tubule. In addition to identifying other areas of the kidney where T1R2 and T1R3 resides immunofluorescence could also provide information as to whether the expression of the sweet taste receptors corresponds to the heterodimerisation of the T1R2/3 complex. Alternatively, the use of a pull-down assay with BBM vesicles would allow the further understanding of the formation of the T1R2/3 complex.

The up-regulation of T1R2 and T1R3 at the proximal tubule BBM occurred in response to the artificial sweetener saccharin. The T1R2/3 heterodimer has been previously shown to rapidly stimulate GLUT2-mediated absorption across the jejunal BBM (Mace et al., 2009). However, studies in Chapter 9 of this thesis have shown that augmented renal expression of T1R2 and T1R3 occurs in the same metabolic models that cause the up-regulation of SGLT1. The relationship between T1R2/3 and SGLT1 has been demonstrated further by assessing the short-term response to saccharin. The rise in $V_{\text{max}}$ of SGLT-mediated glucose transport across the proximal tubule BBM following saccharin IV infusion was accompanied by elevated levels of SGLT1 at the proximal tubule BBM. However, the membrane expression of SGLT2 could not be elucidated in response to artificial sweeteners, or indeed any of the metabolic models used in this thesis, due to lack of a reliable antibody for SGLT2. As a major contributor to glucose reabsorption, it is important to evaluate SGLT2 expression in these models, especially in the model of type II diabetes, where an elevation in both $V_{\text{max}}$ and $K_T$ for SGLT-mediated glucose transport was observed, which was accompanied by augmented SGLT1 expression. Although recent studies by Hummel et al strongly indicate that SGLT1 plays a larger role in glucose reabsorption than originally believed (Hummel et al., 2011), it is important to study the SGLT2 response to metabolic dysregulation. In particular, the influence of saccharin-mediated sweet taste receptor activation on SGLT-mediated glucose transport may not solely be a result of elevated SGLT1 expression, but also an effect on SGLT2.
The relationship between glucose transporters and T1R2/3 in the proximal tubule is intriguing. The data presented in this thesis suggest that PKC-βI-dependent GLUT2 recruitment to the BBM can occur via a T1R2/3-dependent and -independent pathway (Figure 10.1 - 1). In response to type I diabetes, osmotic shock, and a high-fat diet, PKC-βI mediated GLUT2 recruitment to the BBM is elevated, whereas T1R2/3 levels remain unchanged (Figure 10.1 - 1). The same is true in response to RYGB surgery, where PKC-βI mediated GLUT2 movement away from the BBM is not associated with changes in T1R2/3 levels. Furthermore, in these models, SGLT1 expression is also unaffected, suggesting that PKC-βI-induced GLUT2 expression at the proximal tubule BBM involves an unknown factor.

In type II diabetes and pre-diabetes induced by junk-food feeding, PKC-βI-mediated GLUT2 recruitment to the BBM is elevated, together with augmented levels of T1R2/3 and SGLT1. Therefore, in these models, T1R2/3 may signal via PKC-βI to recruit GLUT2 to the proximal tubule BBM by a similar pathway to that seen at the jejunal BBM, that is SGLT-mediated glucose entry can stimulate GLUT2 expression at the proximal tubule BBM. In addition, elevated SGLT-mediated transport at the renal BBM observed in type II diabetes and following artificial sweetener infusion, but not in type I diabetes, is likely to involve up-regulation of T1R2/3 (Figure 10.1 - 2). The relationship between sweet taste sensing and glucose uptake across the proximal tubule BBM can be further studied by long-term stimulation of T1R2/3 by artificial sweeteners. In addition, the use of T1R2/3 inhibitors in models where renal SGLT-mediated glucose uptake is significantly up-regulated, for example type II diabetes and saccharin infusion, would further study the role of sweet taste sensing in glucose transport described in this thesis. Furthermore, KO mice for both T1R3 and gustducin, the major signalling molecule involved in sweet taste sensing, may provide insights into alternative roles, other than for glucose reabsorption, of renal sweet taste sensing. The hypothesised role of T1R2/3 in the stimulation of glucose reabsorption implicates the heterodimer as a potential therapeutic target for patients suffering both pre-diabetes or type II diabetes.
Figure 10.1 – The two proposed signalling pathways responsible for the regulation of glucose transport across the proximal tubule BBM in response to metabolic dysregulation and stress models; A: Recruitment of GLUT2 to the proximal tubule BBM, mediated by PKC-βI, occurs independently of SGLT1 and T1R2/3, in response to high-fat feeding, osmotic shock and type I diabetes; B: Recruitment of GLUT2 to the proximal tubule BBM, mediated by PKC-βI, occurs concomitantly with up-regulated SGLT1 and T1R2/3, in response to a junk-food diet and type II diabetes.
11 Conclusions

In conclusion, the studies described in this thesis lead to a hypothesis implicating two distinct pathways in the regulation of glucose uptake across the proximal tubule BBM. During periods of metabolic dysregulation associated with diabetes, sodium-dependent and –independent glucose transport is altered, which can lead to hyperglycaemia, the main diabetic phenotype. Exploitation of the mediators of these transporters, such as T1R2/3, may pave the way for the development of potential anti-hyperglycaemic treatments for diabetes and pre-diabetes.
12 References

Reports


International Diabetes Federation. 2010. pg 32-36. Annual report

Diabetes UK. Key statistics on diabetes 2010. pg 4-6. Report


Journals


and function in rat jejunal and ileal villus-attached enterocytes. *Pflugers Arch*, 430, 151-159.


Fulgoni V, III (2008) High-fructose corn syrup: everything you wanted to know, but were afraid to ask. *Am J Clin Nutr*, 88, 1715S.


Goestemeyer AK, Marks J, Srai SK, Debnam ES, and Unwin RJ (2007) GLUT2 protein at the rat proximal tubule brush border membrane correlates with protein
kinase C (PKC)-betal and plasma glucose concentration. *Diabetologia*, 50, 2209-2217.


Graff JC, Hanson DJ, and Hatanaka M (1973) Differences in cytochalasin B inhibition of 3-O-methylglucose uptake between BALB-3T3 cells and a murine sarcoma virus transformed clone. *Int J Cancer*, 12, 602-612.


Hong Y, Pedersen NL, Egberg N, and de FU (1997) Moderate genetic influences on plasma levels of plasminogen activator inhibitor-1 and evidence of genetic and environmental influences shared by plasminogen activator inhibitor-1, triglycerides, and body mass index. *Arterioscler Thromb Vasc Biol*, 17, 2776-2782.


Li X (2009) T1R receptors mediate mammalian sweet and umami taste. *Am J Clin Nutr*, 90, 733S-737S.


Melstrom LG, Salabat MR, Ding XZ, Milam BM, Strouch M, Pelling JC, and Bentrem DJ (2008) Apigenin inhibits the GLUT-1 glucose transporter and the


Milagro FI, Campion J, and Martinez JA (2006) Weight gain induced by high-fat feeding involves increased liver oxidative stress. *Obesity (Silver Spring)*, 14, 1118-1123.


Ridout JH, Ham AW, and Wrenshall GA (1944) The correlation of the insulin content and the histological picture of the pancreas at intervals after the administration of alloxan. *Science,* 100, 57-58.


Tofovic SP, Dubey RK, and Jackson EK (2001) 2-Hydroxyestradiol attenuates the development of obesity, the metabolic syndrome, and vascular and renal dysfunction in obese ZSF1 rats. J Pharmacol Exp Ther, 299, 973-977.


