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

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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

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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

$Ca_v1.3$ – 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

dH_2O – 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

$G\alpha_{gustducin}$ – α-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 peroxidise

IDDM – insulin-dependent diabetes mellitus

IDF – International Diabetes Foundation

IKKβ - IκB kinase β

IL-6 – interleukin-6

IP_3 – 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-PK_1 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-$_\kappa$B - 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

RLC_{20} – 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_{max}

TMB - tetramethylbenzidine

TNF-α – tumour necrosis factor-α

Tris HCl – tris hydrochloride

VEGF - vascular endothelial growth factor

VLDL – very low density lipoproteins

V_{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

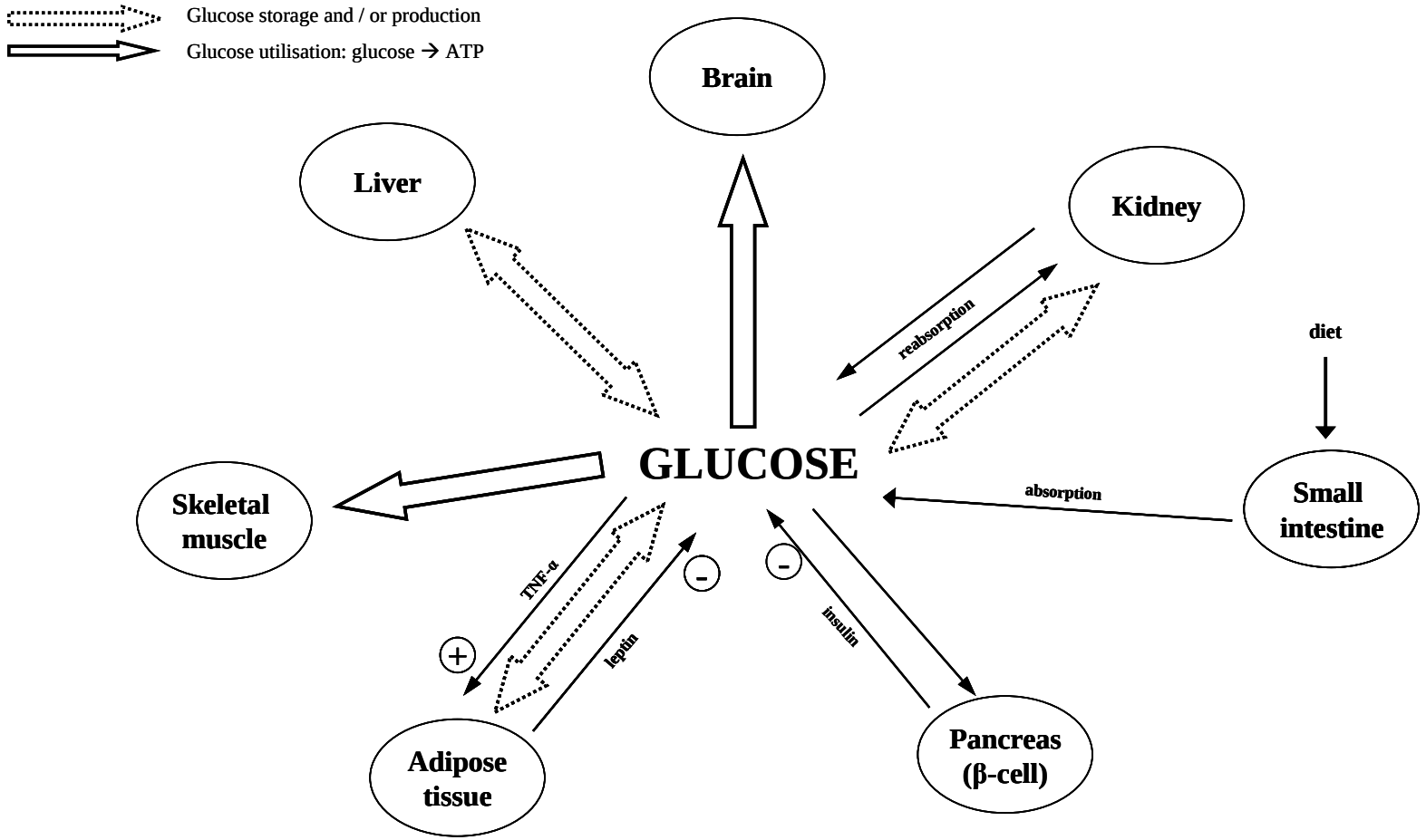
5 Introduction

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 adrenalin 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 afflicts 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.

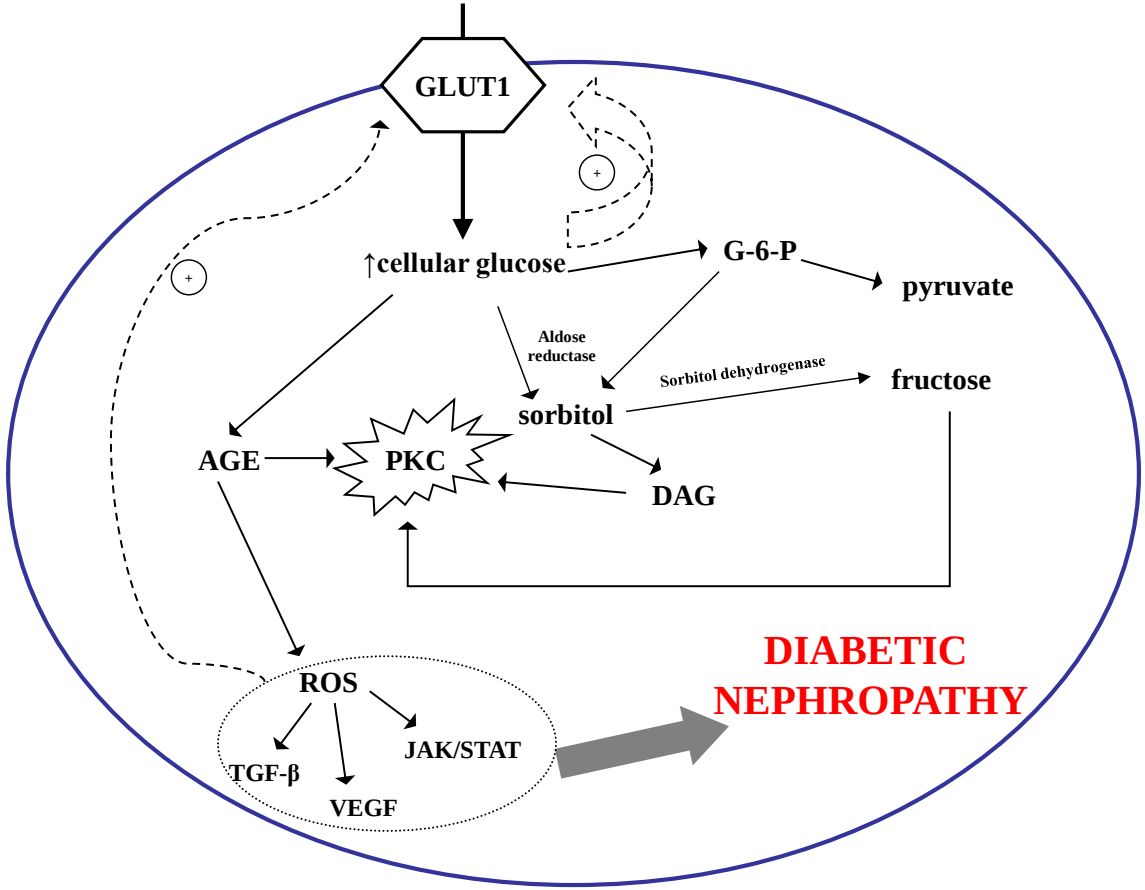
5.2.4 Diabetic nephropathy

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 int 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 diacylglyerol (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-$_K$B; 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 sulfoylureas 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 sulfoylureas.

The α-glucosidase inhibitors, acarbose and miglitol, target the small intestinal brush-border membrane (BBM) enzyme, α-glycosidase; this enzyme breaks down dissacharides 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

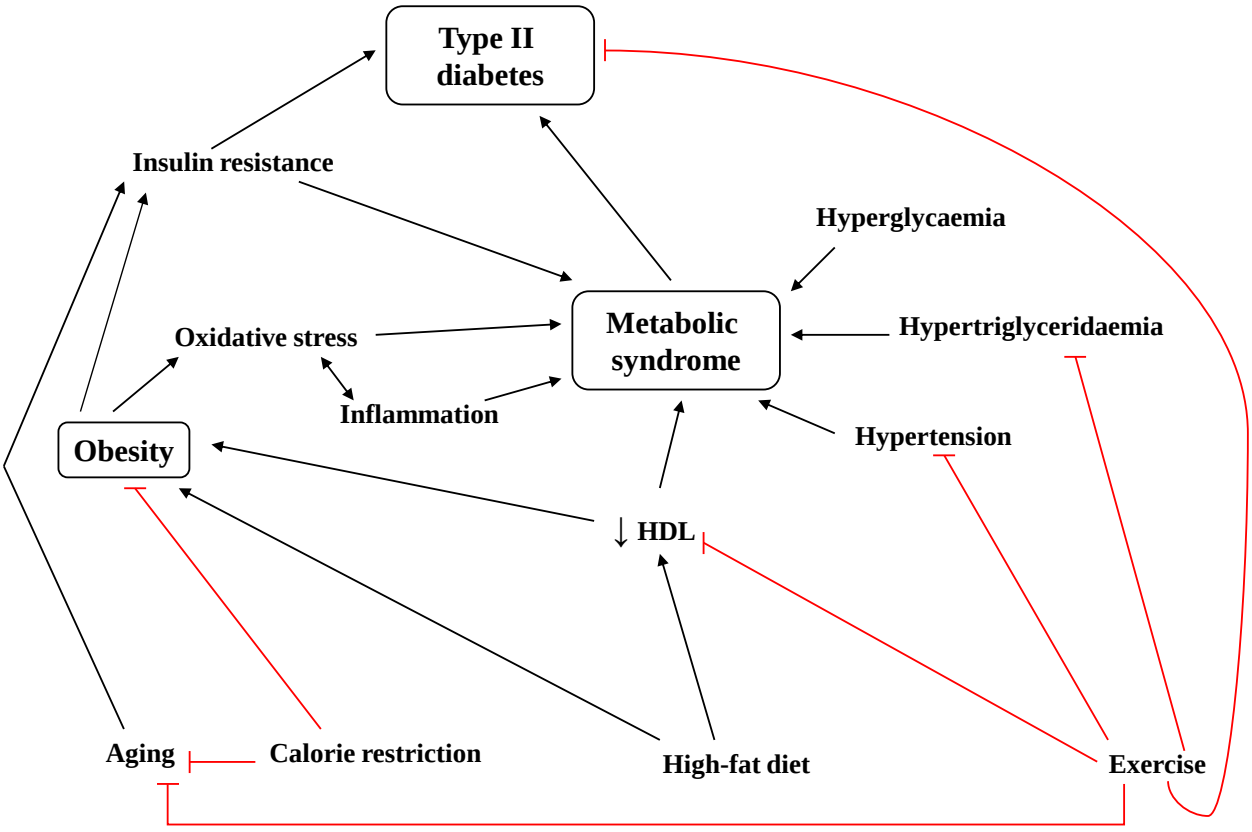
The progressive, polymorphic nature of type II diabetes means that combination oral anti-diabetic therapy is most often prescribed for patients (Inzucchi, 2002); the UK Prospective Diabetes Study (UKPDS) found that 3 years after treatment with only one drug, only 50% patients were adequately controlled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

1992;Pender *et al.*, 2004). 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;Laferrere *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-phsophates 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; Swithers *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

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

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

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

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 tweleve 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 fouteen 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.

5.6.1.1.1 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 *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 *et al.*, 1997;Santer *et al.*, 2002;Taha *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 *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 *et al.*, 1997;Guillam *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 *et al.*, 1999). However no mutational hotspots have been identified within the *GLUT2* gene; there are 33 different mutations detected in 49 sufferers of Fanconi-Bickel syndrome, making gene localisation difficult (Santer *et al.*, 2002).

The genetic variation of the *GLUT2* promoter has also been associated with the onset of type II diabetes, for example, the *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 *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 *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 duplicon 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-transports 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). This

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-transports 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).

5.6.2.2.2 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).

5.6.2.2.2.1 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 is it 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

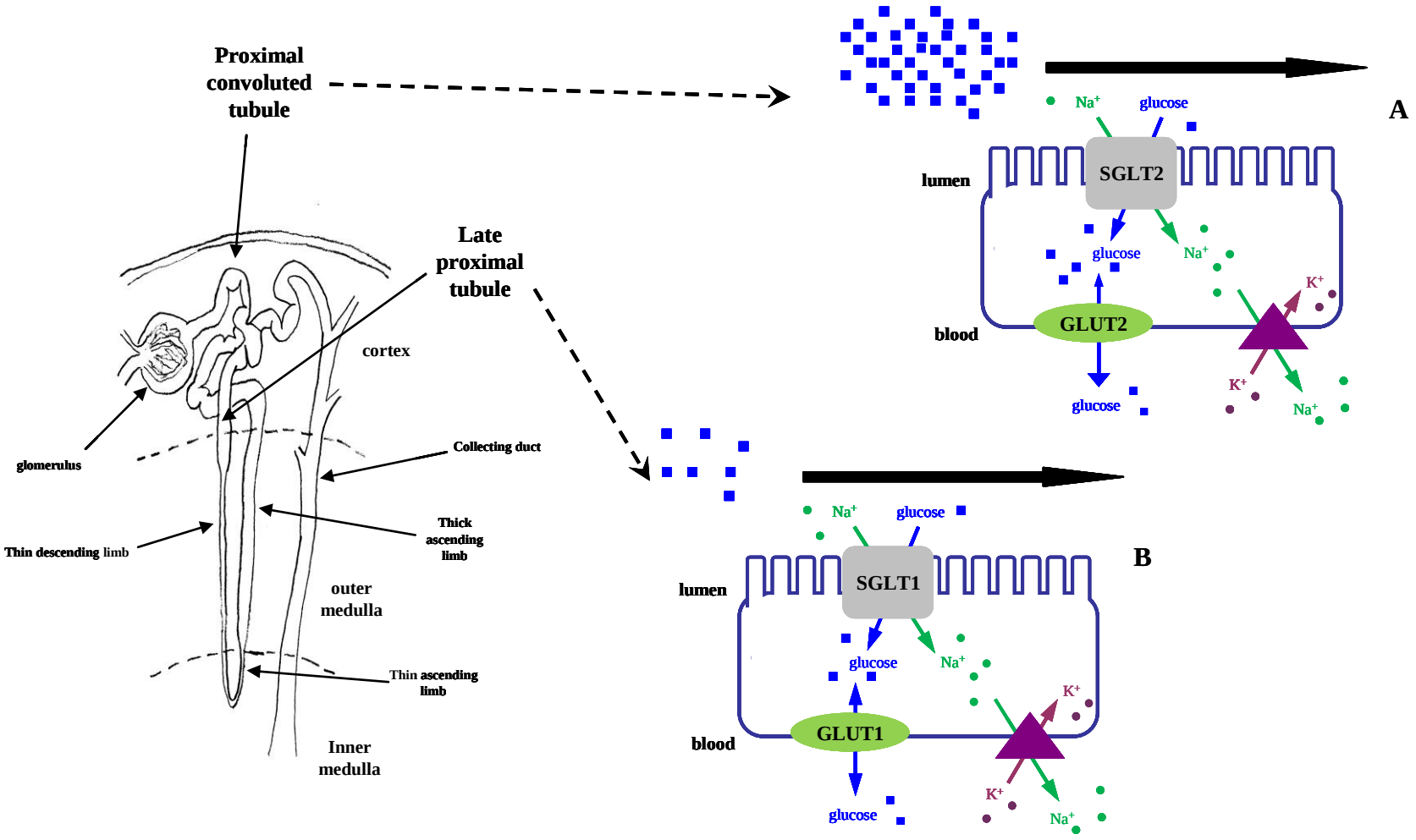
Under normal conditions, when glycaemia is approximately 5.5 mM, since 180 L of plasma is filtered by the kidneys per day, 180 g of glucose is filtered by the kidney over this time period. Circulating glucose moves from the glomerular capillaries into the Bowman's capsule, together with low molecular 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 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.*,

1991). 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 prolonger 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;Bamri-

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.

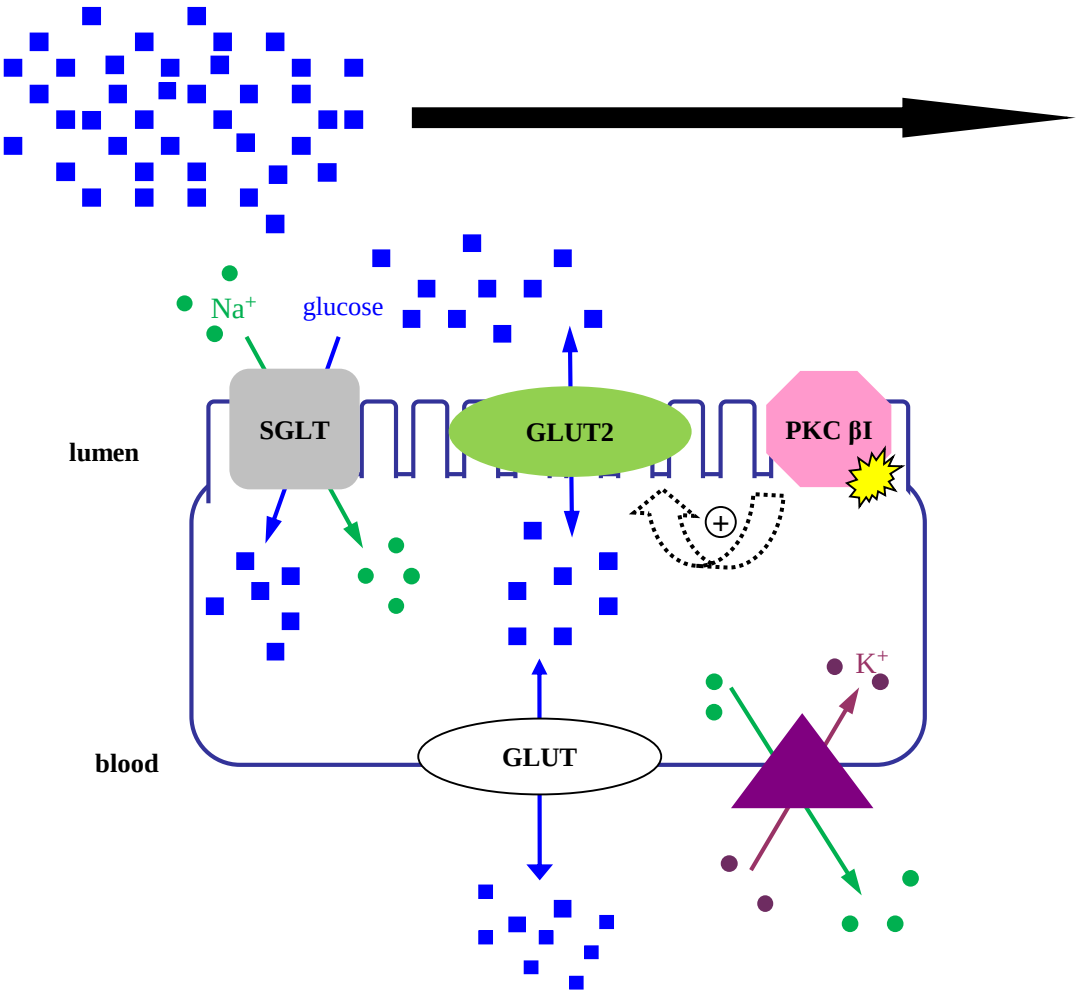
5.7.2.1.4.2 Involvement of PKC in glucose transporter expression

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 (Ca^{2+}) in proximal tubule cells due to the increased expression of L-type Ca^{2+} 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 Ca^{2+} 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).

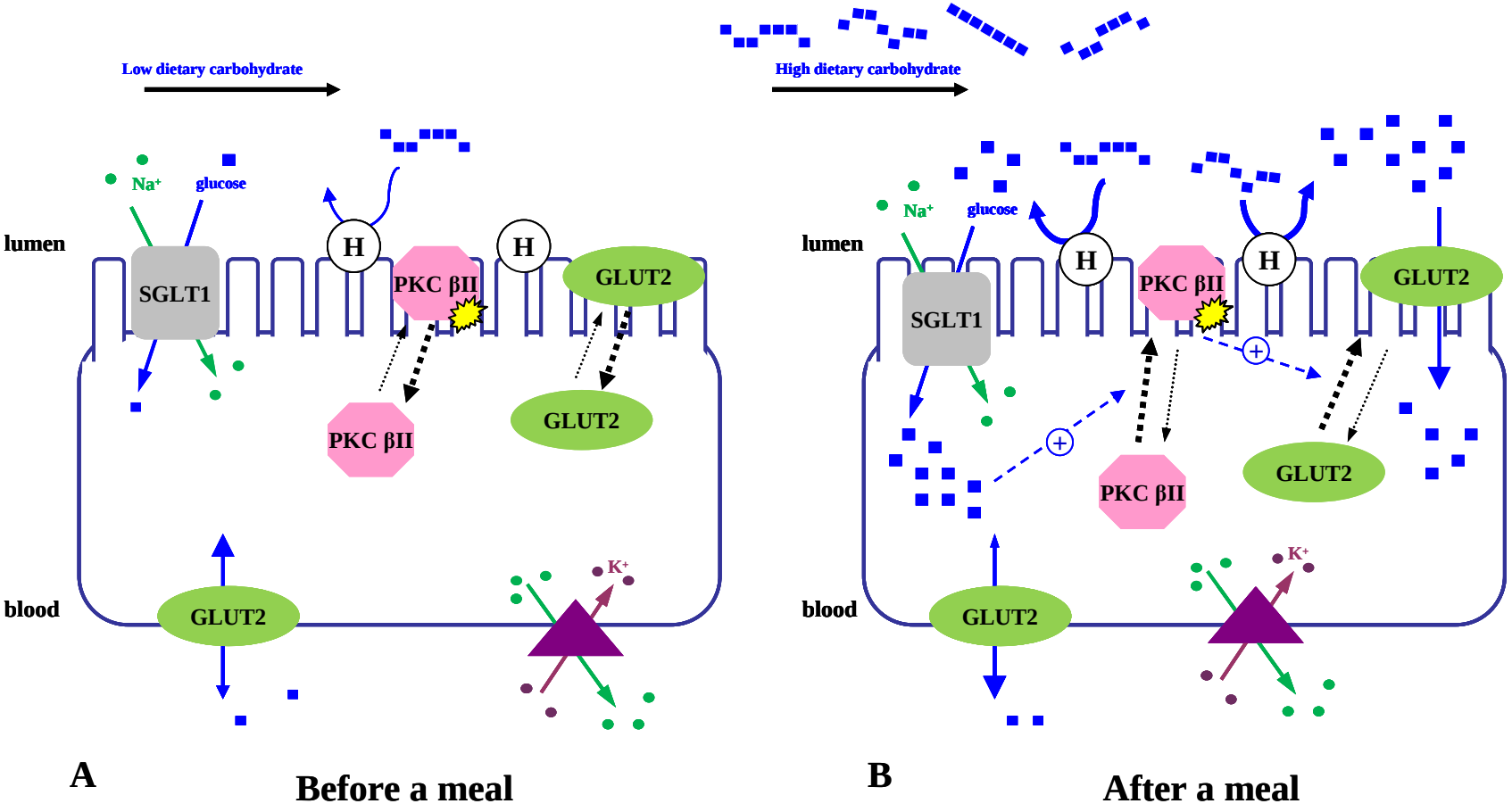
5.8.1 Before a meal

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 jejnual 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 (H) 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.

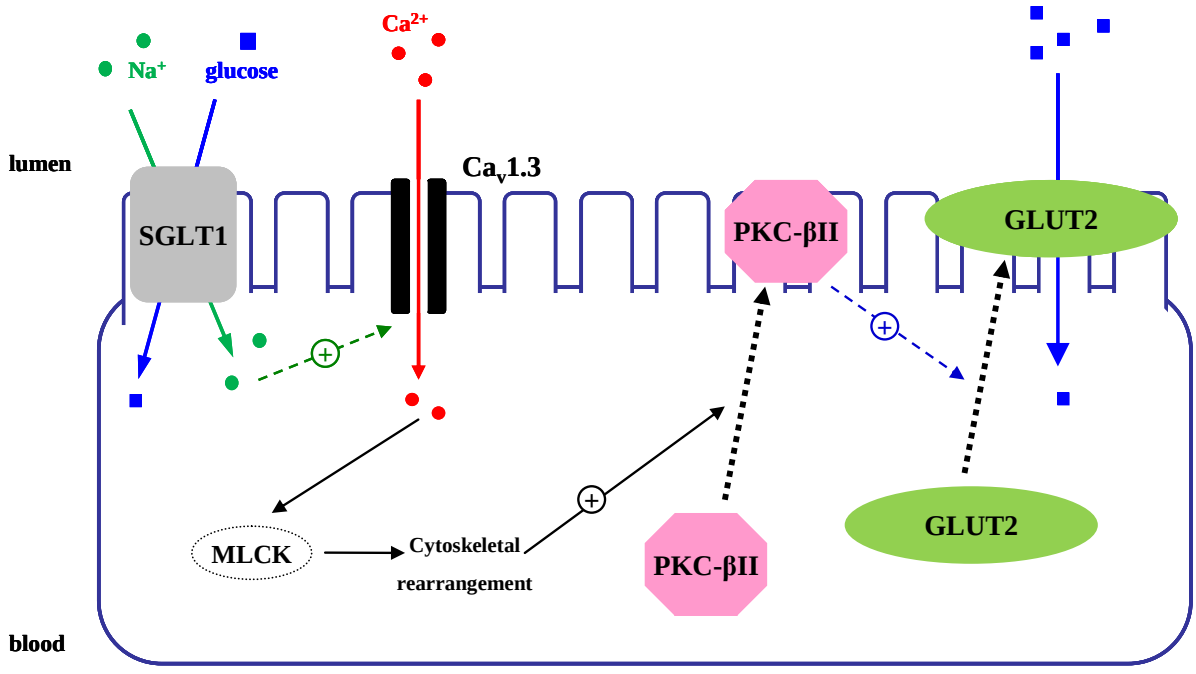
5.8.2.4 PKC-βII and calcium signalling

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 $Ca_v1.3$, the L-type calcium channel at the jejunal BBM (Morgan *et al.*,

2003;Morgan *et al.*, 2007). 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 $Ca_v1.3$, 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 $Ca_v1.3$ 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 Ca^{2+} 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 Ca^{2+} 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-metabolisable artificial sweeteners promoted SGLT1 expression and caused a cAMP-dependent rise in glucose absorption across the BBM which was absent in T1R3 or Gα-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

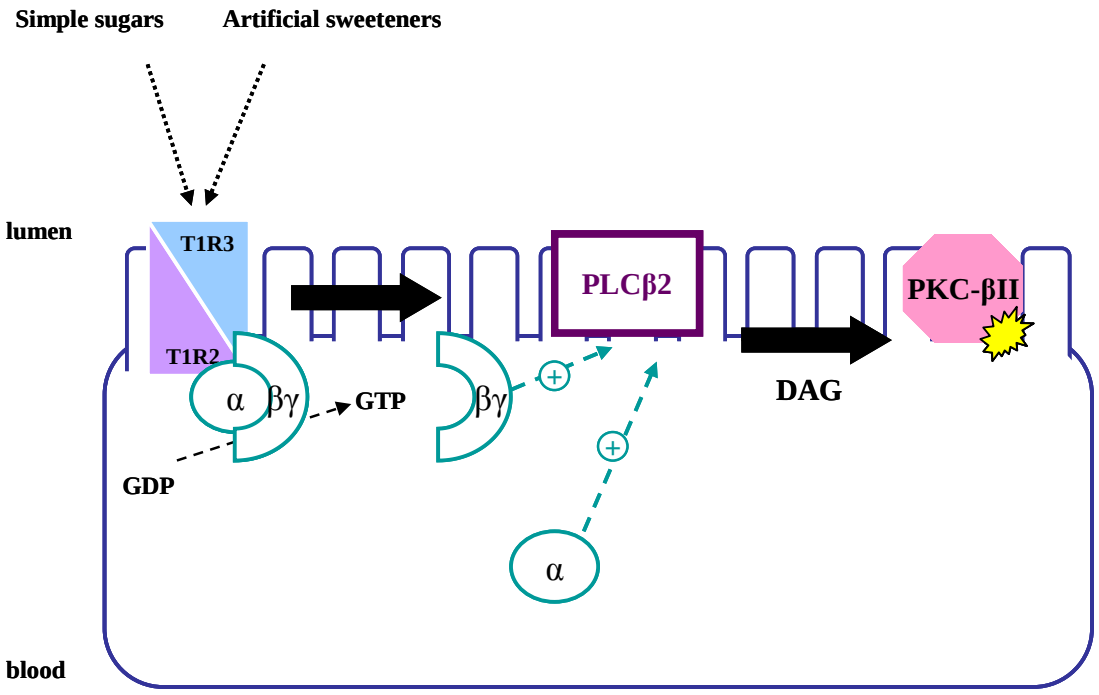
transporter being unable to transport fructose (Stearns *et al.*, 2010). Furthermore this effect was also seen using artificial sweetener saccharin, with the response occurring after 3 hours, similar to the time needed for SGLT1 synthesis (Stearns *et al.*, 2010).

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 IP_3-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 adenylyl 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))

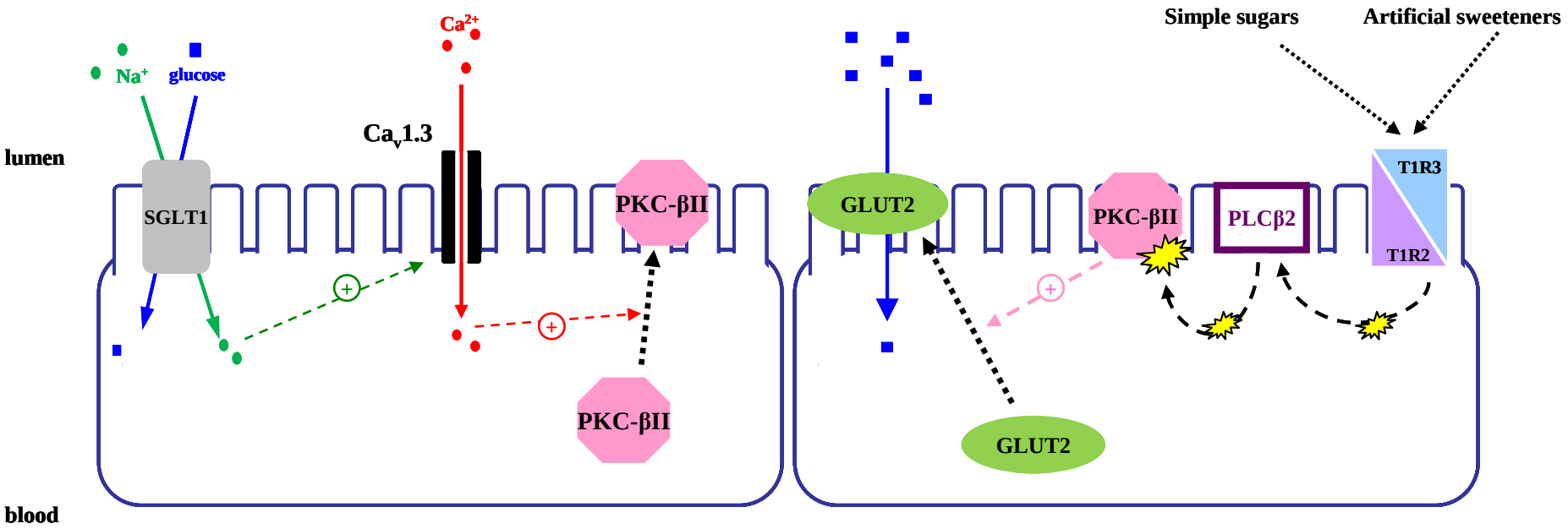


5.8.4 Revised model of glucose absorption across the enterocyte

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 dissacharidases 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 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.

5.8.5.1.6 Hormones

5.8.5.1.6.1 Incretin hormones

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-βII (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 moduliating 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^{o}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 libitium* 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

		Chow	**Junk-food**
Protein (%)		77.4	4.4
Carbohydrates (%)		3.7	35.9
	Of which sugars (%)	2.1	15.4
Fat (%)		0.8	19.6
	Of which saturates (%)	15.7	4.6
Fibre (%)		0.2	1.5
Sodium (%)		0	18.6

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 CO_2 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-PK_1 cell culture

Porcine renal epithelial LLC-PK_1 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% CO_2 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^{o}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^{o}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 $MgCl_2$ 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 $MgSO_4$ 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^{o}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:

$$\frac{\text{Glucose uptake (pmol.mg protein}^{-1})}{\text{[Glucose] (μM)}}$$

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}[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 ($DPM_{background}$). DPM of ^{3}[H] in the initial uptake buffer, with glucose, was also measured with scintillant ($DPM_{initial\ counts}$).

The counts were converted to uptake (pmol.mg protein^{-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.

$$\frac{\left[\text{DPM}_{\text{samples}} - \text{DPM}_{\text{background}}\right] \times [\text{glucose}]}{\text{DPM}_{\text{initial counts}} \times \left(2\,[\text{protein}]\right)}$$

The kinetic parameters for SGLT-mediated glucose transport between 30 μM to 960 μM, V_{max} (maximum transport capacity) and K_T (glucose concentration at half V_{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.

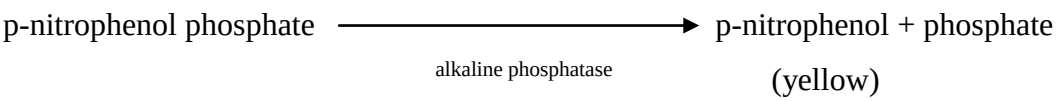
$$\text{protein} + \text{NaOH} \xrightarrow[\text{Coomassie Brilliant Blue (brown)}]{} \text{bound protein-dye complex (blue)}$$

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 phsophatase, into free p-nitrophenol and phosphate. The free p-nitrophenol can be measured spectrophotometrically at 400 nm wavelength (Forstner *et al.*, 1968).



Homogenate samples were diluted 1:5 and BBM vesicle at 1:50, in dH_2O, and 100 µl stock was used in the assay. 4-nitrophenol was used as a standard and prepared, in dH_2O, 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 phsophatase, using the equation below:

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

6.8 Determination of plasma and urine glucose concentration

6.8.1 Plasma

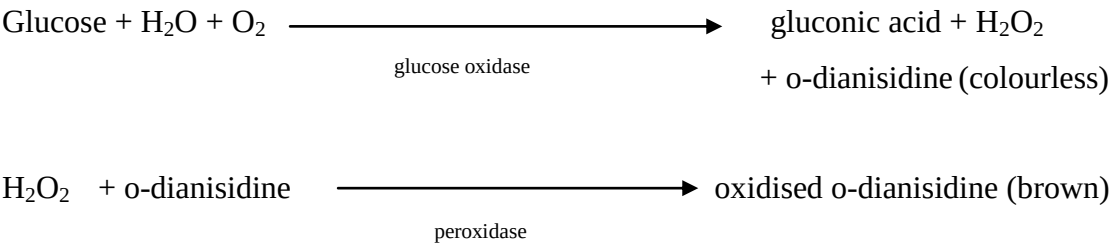
Blood from cardiac puncture, or collected via femoral artery cannulation, was placed into heparinised tubes, containing 7500 kallikrain inactivator units (KIU) of the broad range protease inhibitor Aprotonin. After centrifugation at 8000 rpm for 15 minutes, the resulting supernatant (plasma) was used in the glucose oxidase assay. Plasma from diabetic or glucose-infused rats was diluted 1:10, in dH_2O, 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_2O, 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 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 H_2O.

Glucose standards (25 µl) were used at 5, 10 and 15 mM, with 500 µl dH_2O and 4 ml GOR. Samples (25 µl) were also prepared in 500 µl dH_2O 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^{o}C.

6.11.2 Western blotting

BBM vesicles, LLC-PK_1 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^{o}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% H_2O_2). 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

Antibody	Species raised in	Concentration used at	Epitope antibody raised against		Company
			Species	Region	
GLUT2	Rabbit	1:1000	Rat	C-terminal	AbD Serotec
GLUT5	Rabbit	1:500	Rat	C-terminal (cytoplasmic domain)	Alpha Diagnostic International
SGLT1	Rabbit	1:1000	Human	N-terminal	Gift from G. Kellett (University of York)
PKC-βI	Rabbit	1:1000	Human	C-terminal	Santa Cruz Biotechnology
PKC-βII	Rabbit	1:1000	Human	C-terminal	Santa Cruz Biotechnology
T1R2	Rabbit	1:500	Mouse	N-terminal (extracellular domain)	Santa Cruz Biotechnology
T1R3	Rabbit	1:500	Mouse	N-terminal (extracellular domain)	Santa Cruz Biotechnology
β-actin	Mouse	1:5000	*Xenopus laevis*	C-terminal	Abcam

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

Antibody	Species raised in	Concentration used at	Species antibody raised against	Company
Anti-mouse-HRP	Goat	1:5000	Mouse	Sigma
Anti-rabbit-HRP	Donkey	1:1000	Rabbit	GE Healthcare

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_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 $\mu g.ul^{-1}$. 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™ 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™ 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

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

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

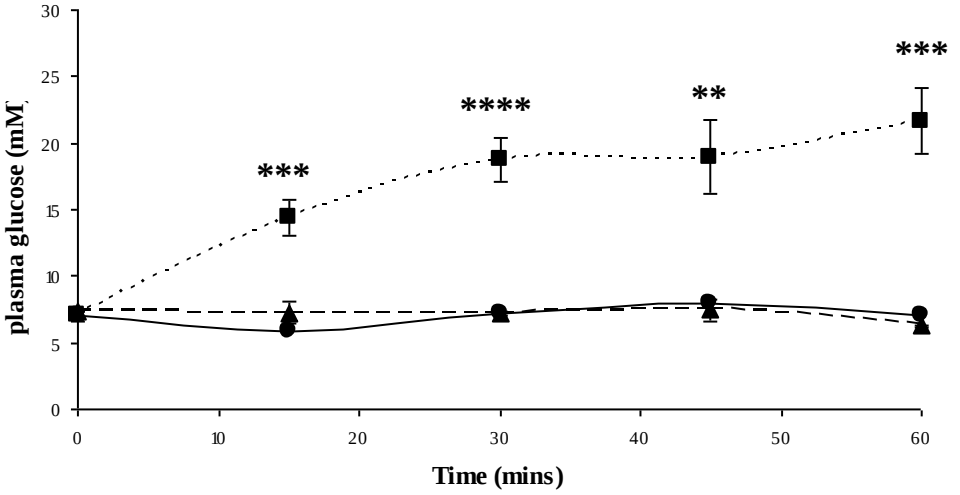


Figure 7.2 – Effect of 2 hour infusion of 1.1 M glucose (dotted line), with initial bolus, on plasma glucose concentrations, compared with 154 mM saline (broken line) and 0.08 M mannitol (solid line) control infusions. Values are expressed as mean ± SEM, n=4-6, ****p<0.001 compared with both controls at each time point

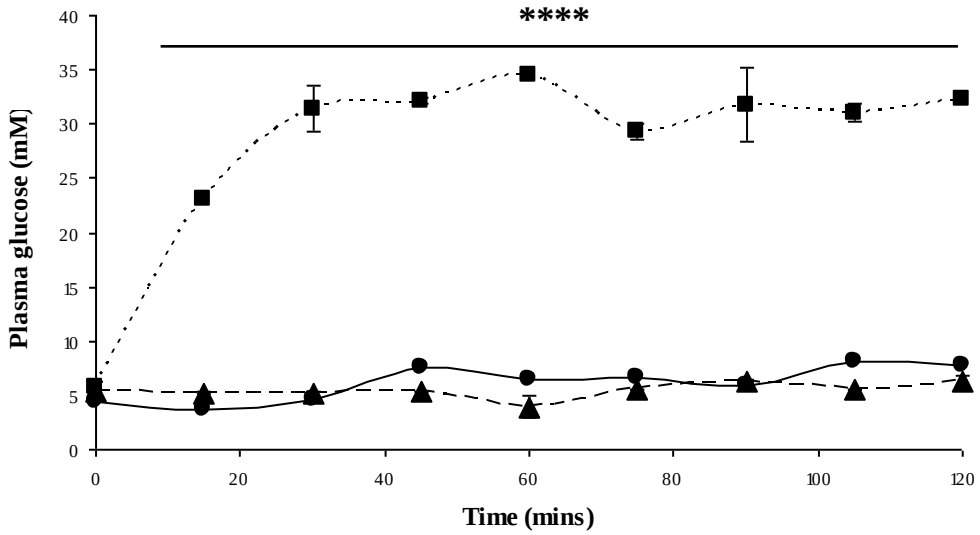


Table 7.1 – Body and kidney weights and renal BBM vesicle enrichment of alkaline phosphatase marker enzyme, in rats infused with 154 mM saline, 1.1 M glucose, 0.08 M and 1.09 M mannitol. Values are expressed as mean ± SEM for 1 and 2 hour time period, n = 3-6, no significant change between infusions, p>0.5.

	154 mM Saline	**0.08 M Mannitol**	**1.1 M Glucose**	**1.09 M Mannitol**
Body weight (g)	291.4 ± 21.8	255.6 ± 3.07	301.6 ± 23	263.7 ± 12.7
Kidney weight (g)	1.04 ± 0.05	0.99 ± 0.03	1.06 ± 0.08	1.02 ± 0.09
BBMV enrichment	6.02 ± 0.29	5.82 ± 0.44	6.12 ± 0.36	6.19 ± 0.37

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

	154 mM Saline	**0.08 M Mannitol**	**1.1 M Glucose**	**1.09 M Mannitol**
Infusate osmolality	312 ± 7 mOsM	388 ± 10 mOsM	1706 ± 26 mOsM	1675 ± 18 mOsM
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.

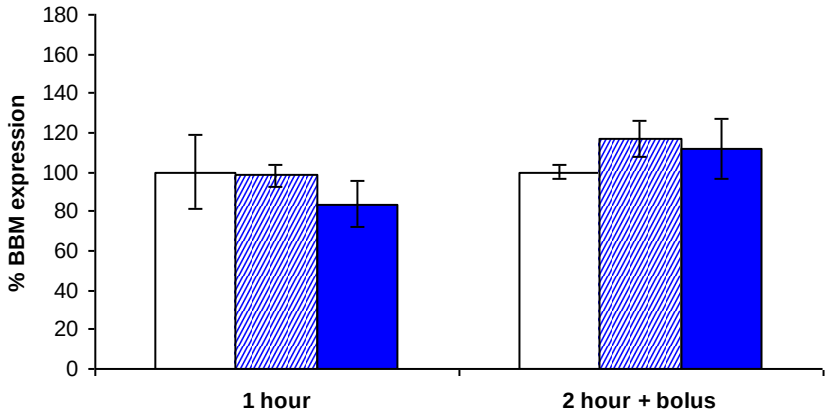
Glycosuria was significantly higher in the 2 hour glucose infusion with bolus group, with an increase from 0.157 ± 0.037 mM pre-infusion, to 306 ± 6 mM after 30 minutes. After 2 hours infusion, the glucose concentration in the urine had dropped to 191 ± 3 mM, suggesting the initial peak was due to the 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

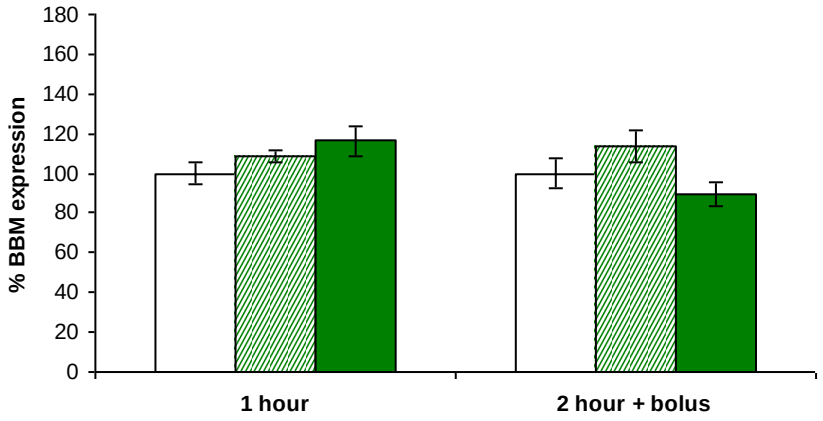
Despite the hyperglycaemic and glycosuric effects of the glucose infusions described above, there was no effect on renal GLUT2 expression in BBM vesicles prepared from both the 1 and 2 hour infused animals (Figure 7.3). There was also no significant effect on expression of the GLUT2 effector, PKC-βI 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.

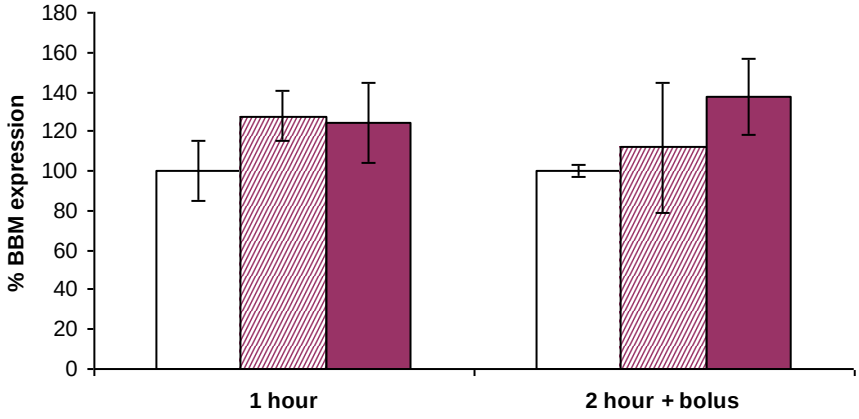
1 – GLUT2



2 – PKC-βI



3 – SGLT1

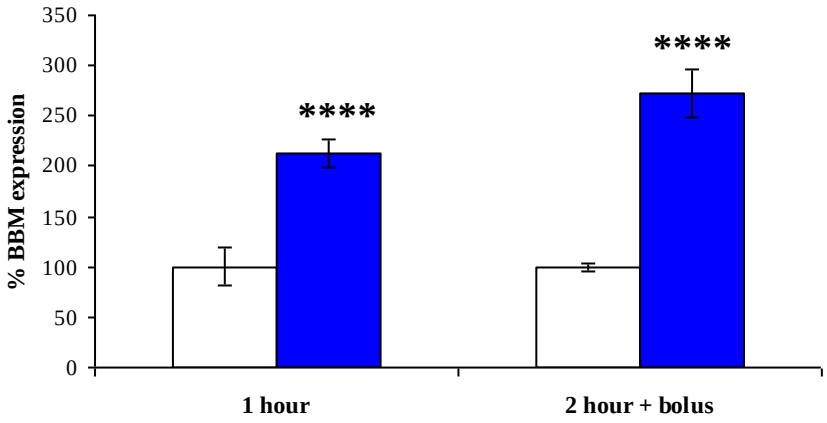


7.3.1.4 Effect of plasma osmolality on renal GLUT2 expression

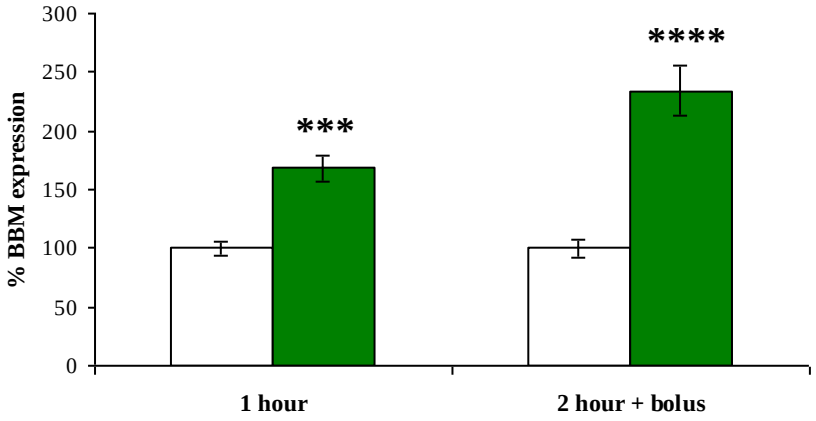
As an osmotic control for the short-term infusion studies, 1.09 M mannitol was also used as an infusate since its osmolality is similar to 1.1 M glucose (1675 ± 18 mOsM compared to 1706 ± 26 mOsM, (Table 7.2). The 1.09 M mannitol infusion had a dramatic effect on GLUT2 expression at the proximal tubule 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.

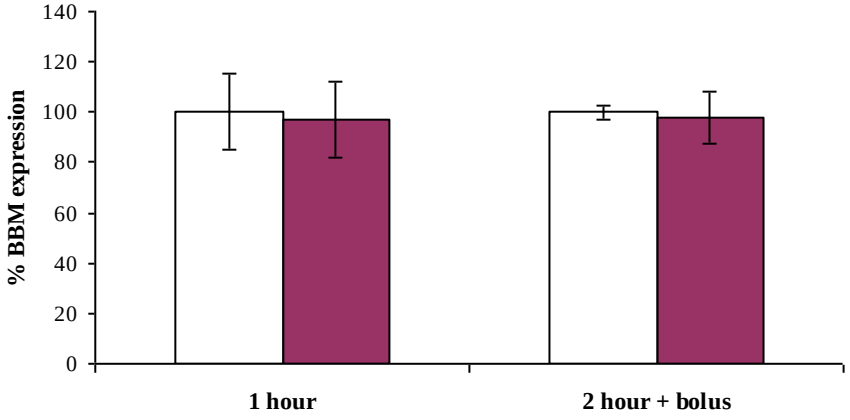
1 – GLUT2



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

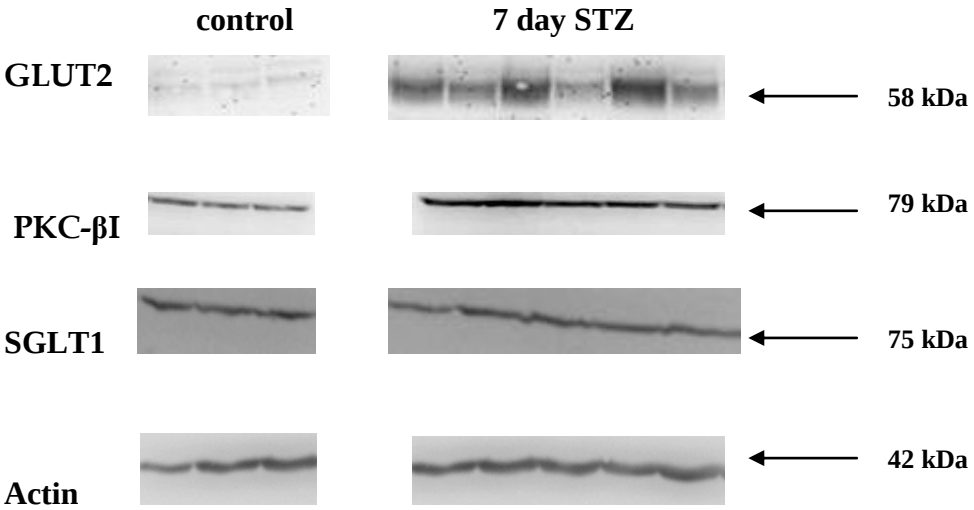
kidney weight in 7-day STZ diabetic animals compared with those from control animals; however no difference in BBM enrichment was detected in membranes prepared from control and 7-day STZ diabetic animals. There was also a decrease in body weight in the 7-day STZ diabetic rats compared to the controls, 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

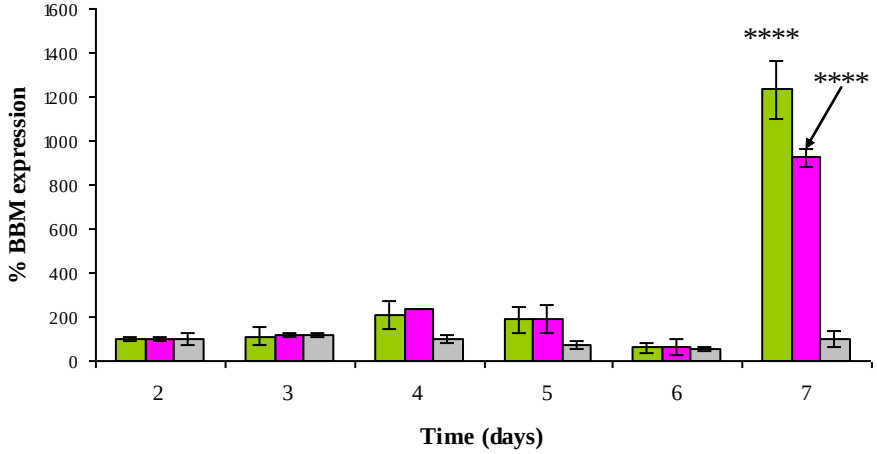
	Control	**STZ**
Body weight (g)	268.5 ± 11.3	244.4 ± 7.1
Kidney weight (g)	0.95 ± 0.11	1.17 ± 0.04*
BBMV enrichment	5.86 ± 0.31	5.79 ± 0.22

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



2

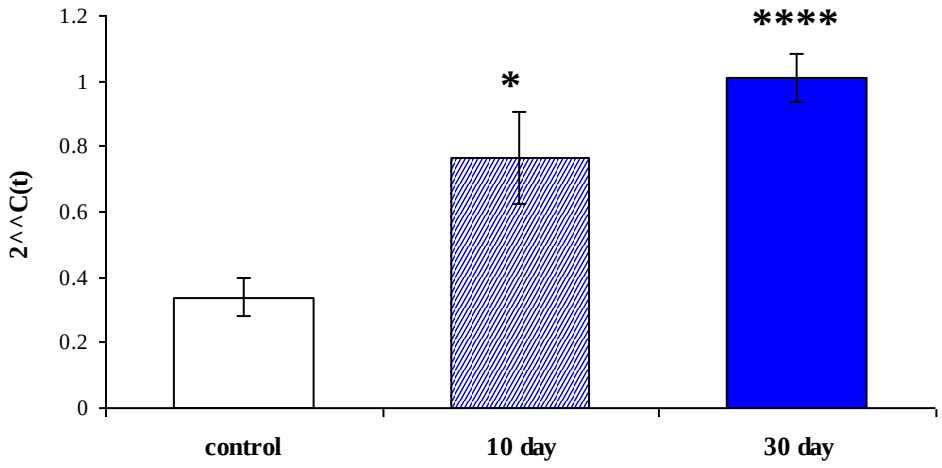


7.3.3 Effect of STZ-induced diabetes on GLUT2 intracellular pool in proximal tubule cells

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* jejunual 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 adequeately 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-βI 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 libitium* 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 libitium* access to 60% fat-as-calories chow (Research Diets, New Brunswick, USA) largely comprised of soybean oil and lard for 5 weeks.

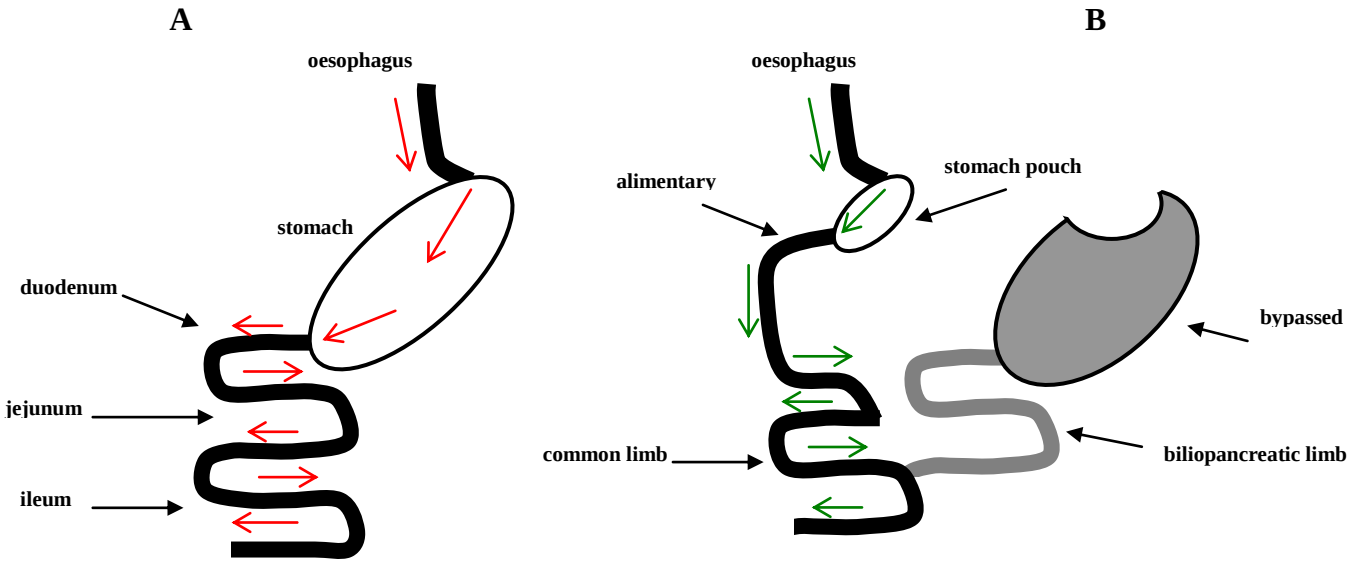
8.1.2.3 RYGB surgery

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 gastroectomy, 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

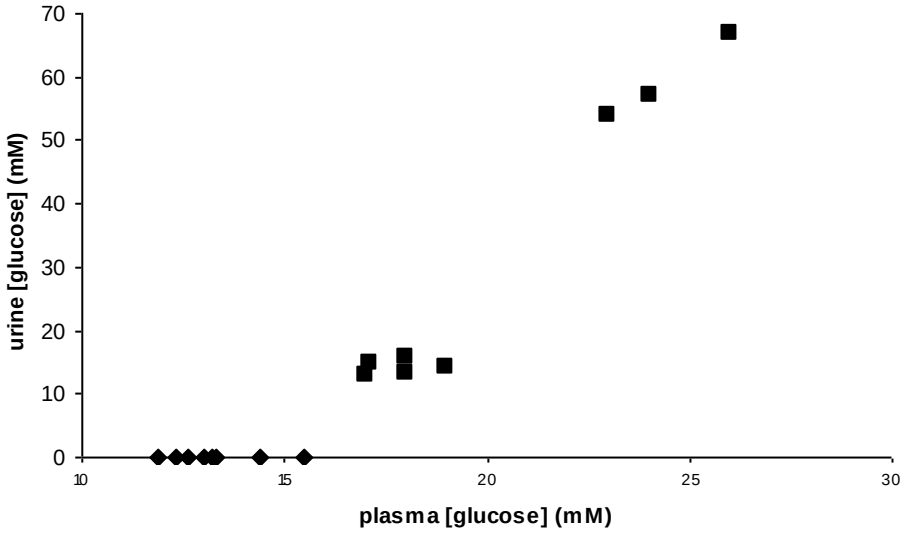


Table 8.1 – 1) Body and kidney weight, and kidney-to-body weight ratio and 2) Plasma glucose, osmolality and basal circulating insulin measurements in 8-9 week lean and type II diabetic GK rats. Values are expressed as mean ± SEM, n=8, ***p<0.005, ****p<0.001 compared with lean.

1

	Lean	**GK**
Body weight (g)	397 ± 7.4	316.6 ± 5.2 ****
Kidney weight (g)	2.51 ± 0.42	2.38 ±1.05
Kidney:body weight ratio	6.33 ± 0.1	7.53 ± 0.35 ***

2

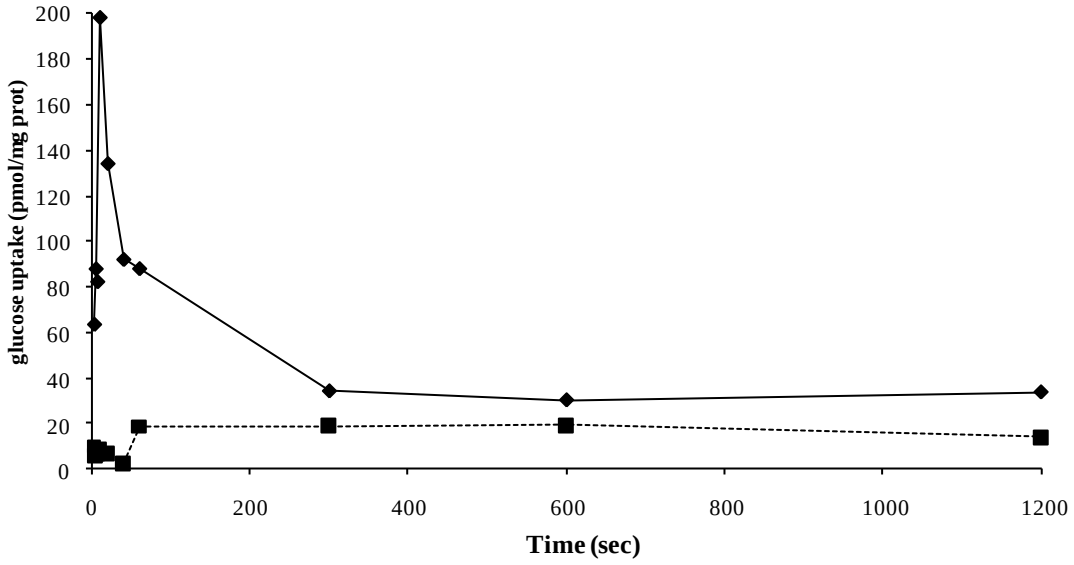
	Lean	**GK**
Plasma glucose (mM)	13.28 ± 0.41	20.26 ± 1.25 ****
Plasma osmolality (mOsM)	329 ± 2	353 ± 3 ****
Basal plasma insulin (ng.ml^{-1})	4.83 ± 1.12	6.35 ± 1.38

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).

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)

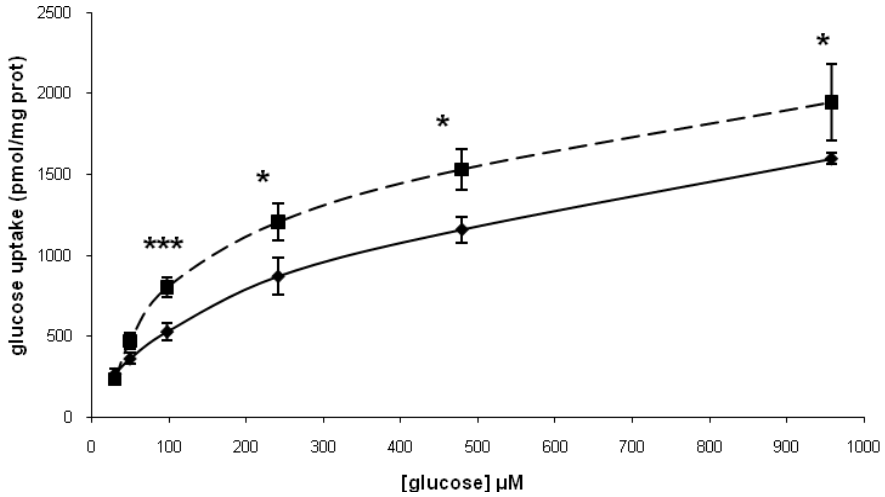


8.3.1.2.2 Renal BBM glucose transport in GK type II diabetic rats

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_{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 ± 21.6 μM compared with GK K_m 264.3 ± 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 ± 380.8 pmoles.mg protein^{-1} in lean animals to 3809.5 ± 426.4 pmoles.mg protein^{-1} in type II diabetic animals.

Figure 8.4 – Effect of type II diabetes in GK rats (broken line) on SGLT-mediated glucose uptake (1) Glucose concentration curve (2) V_{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



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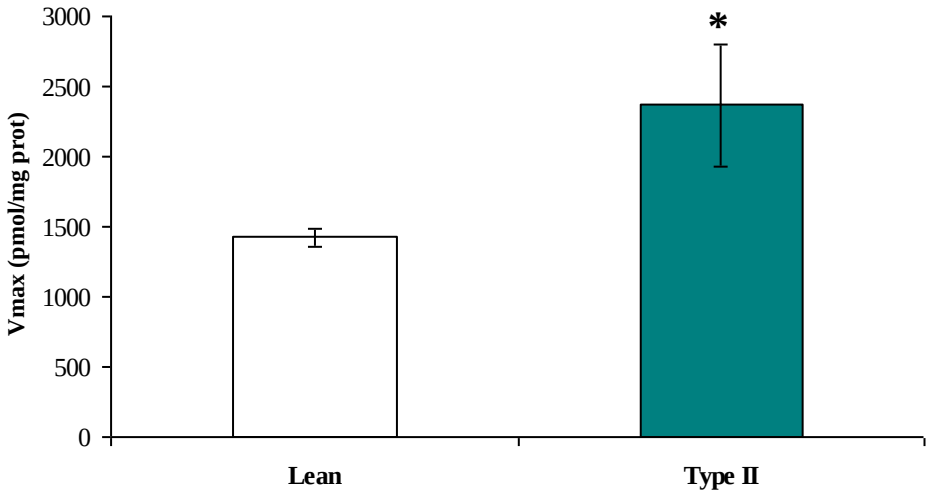

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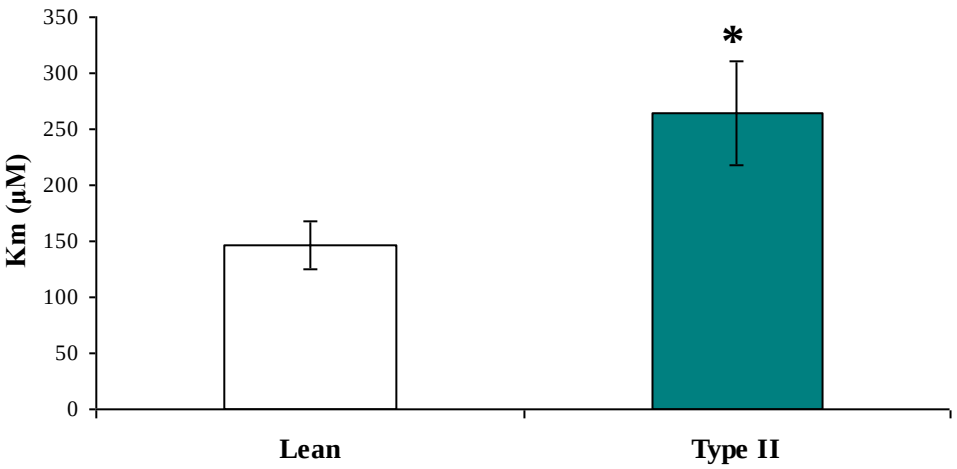
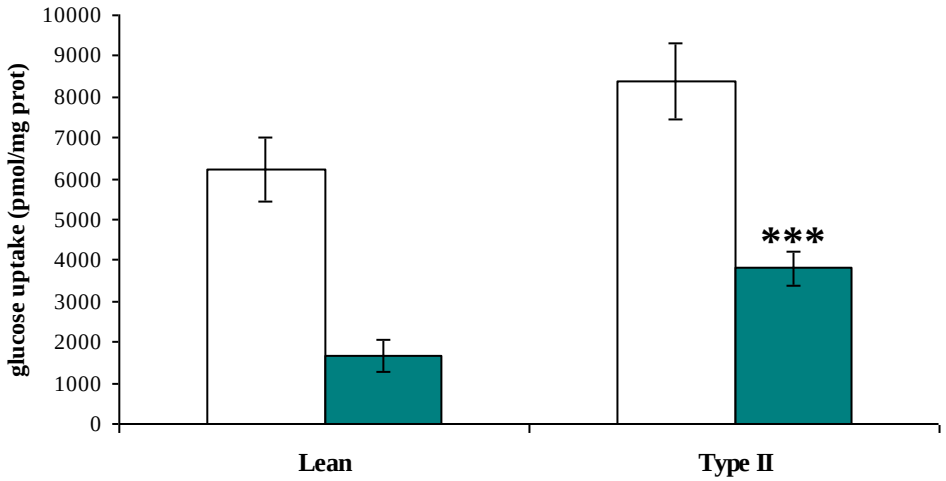


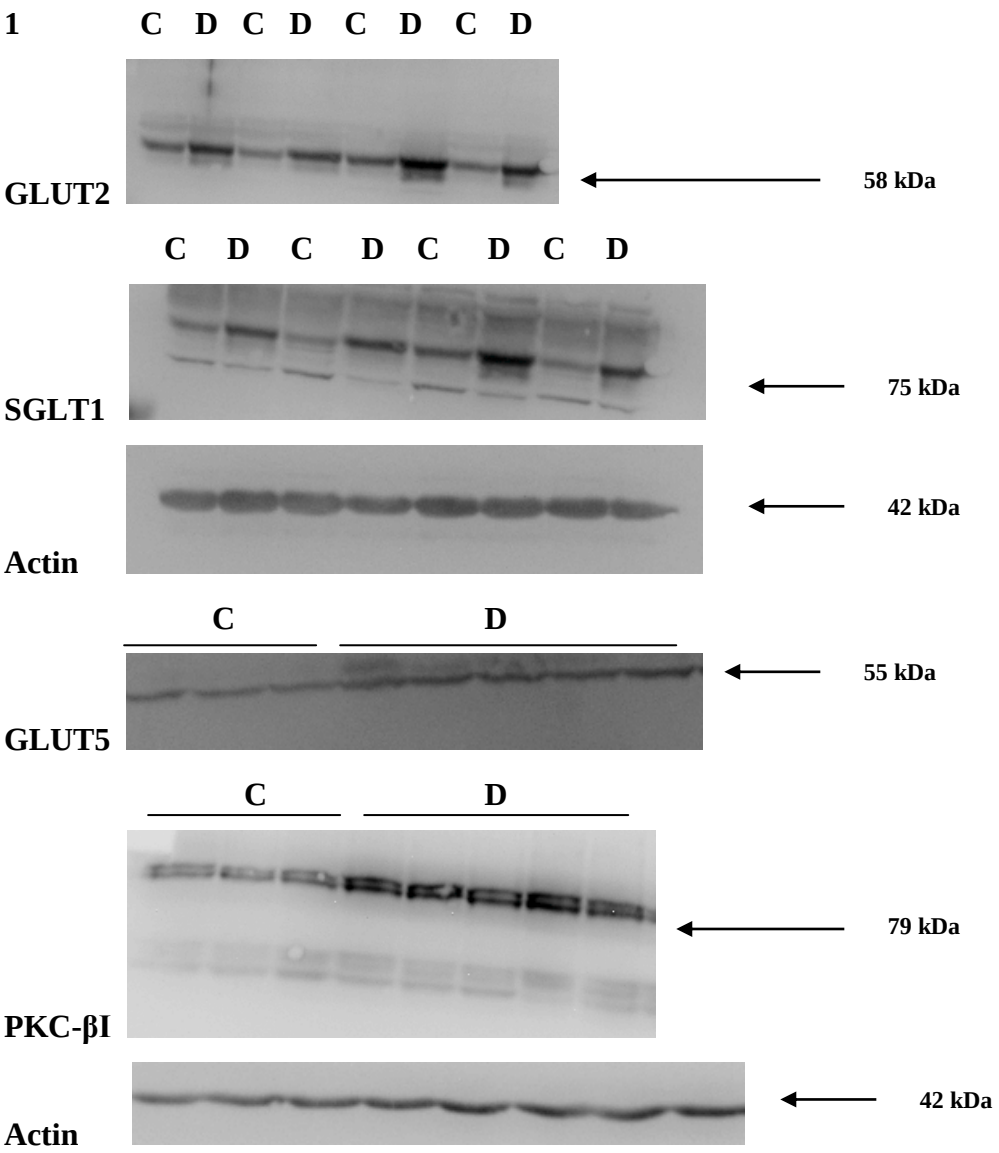
Figure 8.5 – Effect of type II diabetes on total glucose uptake (open bars) and GLUT-mediated glucose uptake, in the presence of phloridzin (closed bars) using renal BBM vesicles incubated in 20 mM glucose for 4 seconds. Mean ± SEM, n=6-8, ***p<0.005 compared with lean control rats.



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)



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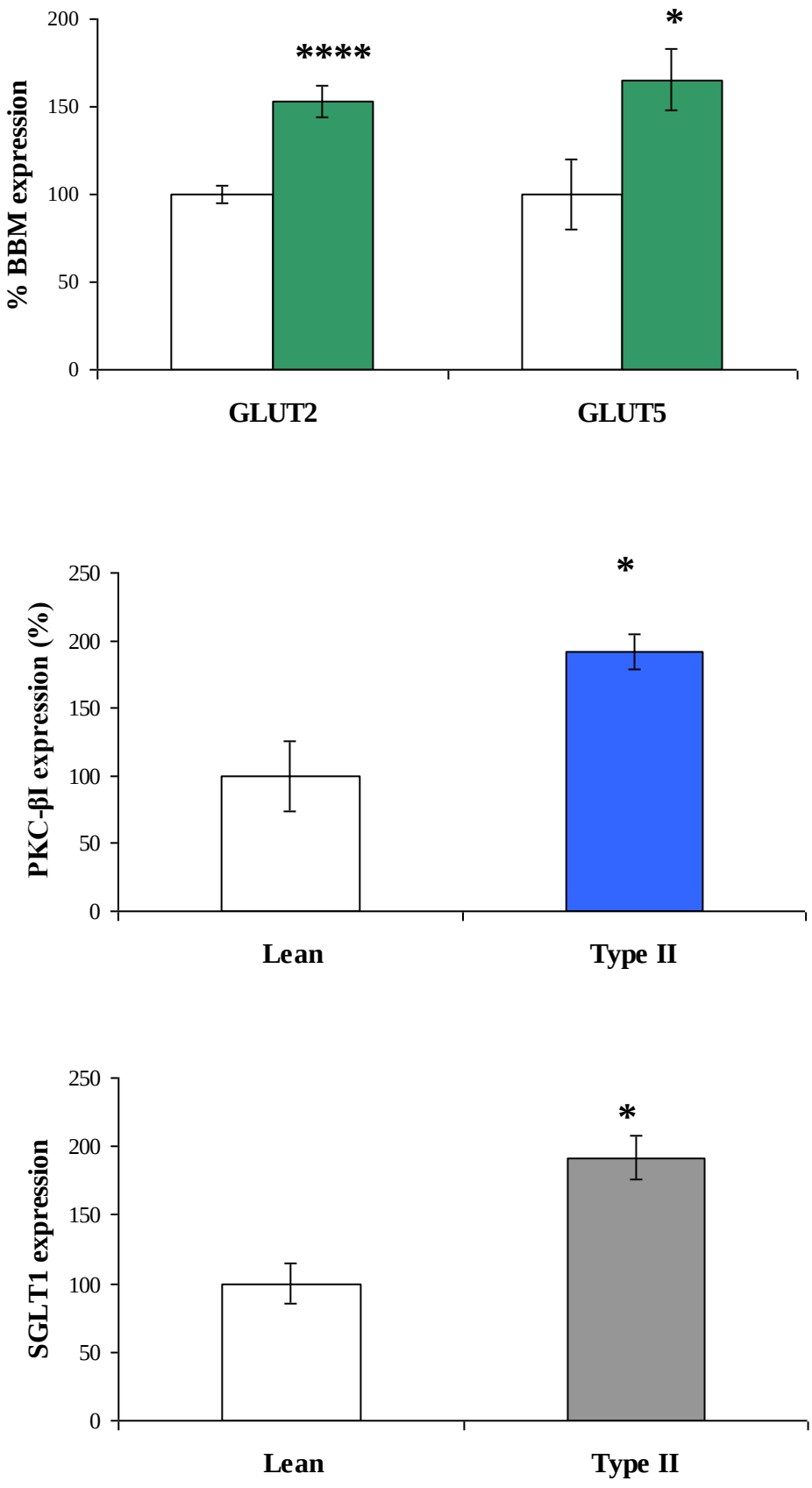

% BBM expression
200
150
100
50
0
****
*
GLUT2
GLUT5
PKC-βI expression (%)
250
200
150
100
50
0
*
Lean
Type II
SGLT1 expression
250
200
150
100
50
0
*
Lean
Type II

8.3.2 Junk-food model

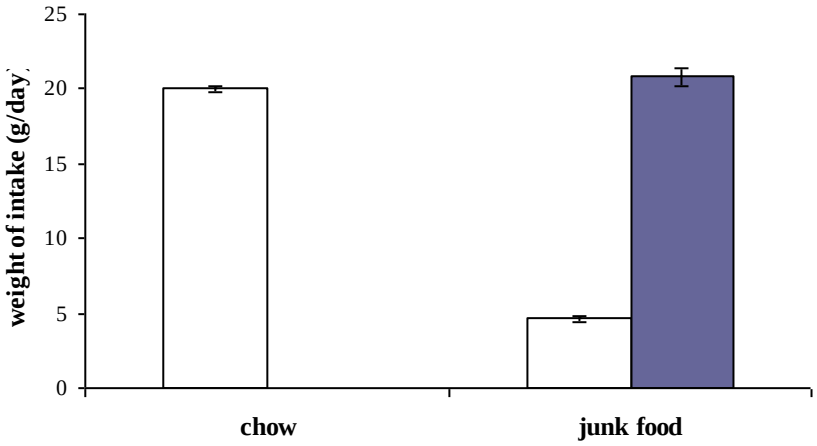
The augmentation of glucose transport in both STZ-induced type I diabetes and the non-obese GK model of type II diabetes, raised the interesting question of whether a pre-diabetic metabolic model would also be associated with changes in expression of renal BBM glucose transporters. The junk-food model 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.

1



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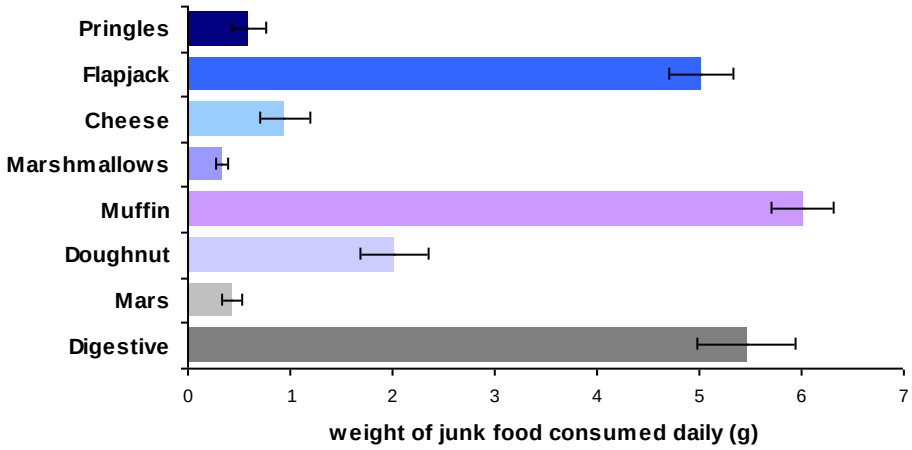
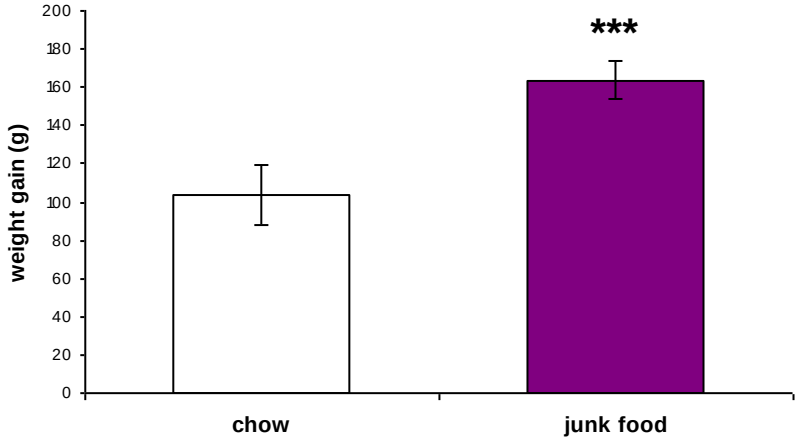


Figure 8.8 - Body weight increase in response to 8 weeks junk-food diet 1) Total weight gain in control chow-fed animals and experimental junk-food-fed animals at time of cull 2) Progressive weight gain in chow-fed (diamond points) and junk-food-fed (square points) animals over 8 week feeding period. Values are expressed as mean ± SEM, n=6-12, *p<0.05, ***p<0.005 compared with chow-fed animals

1



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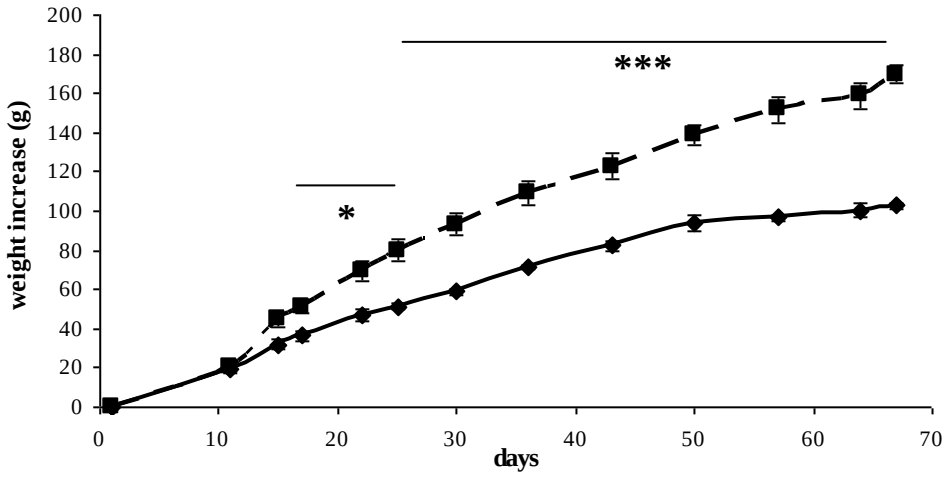
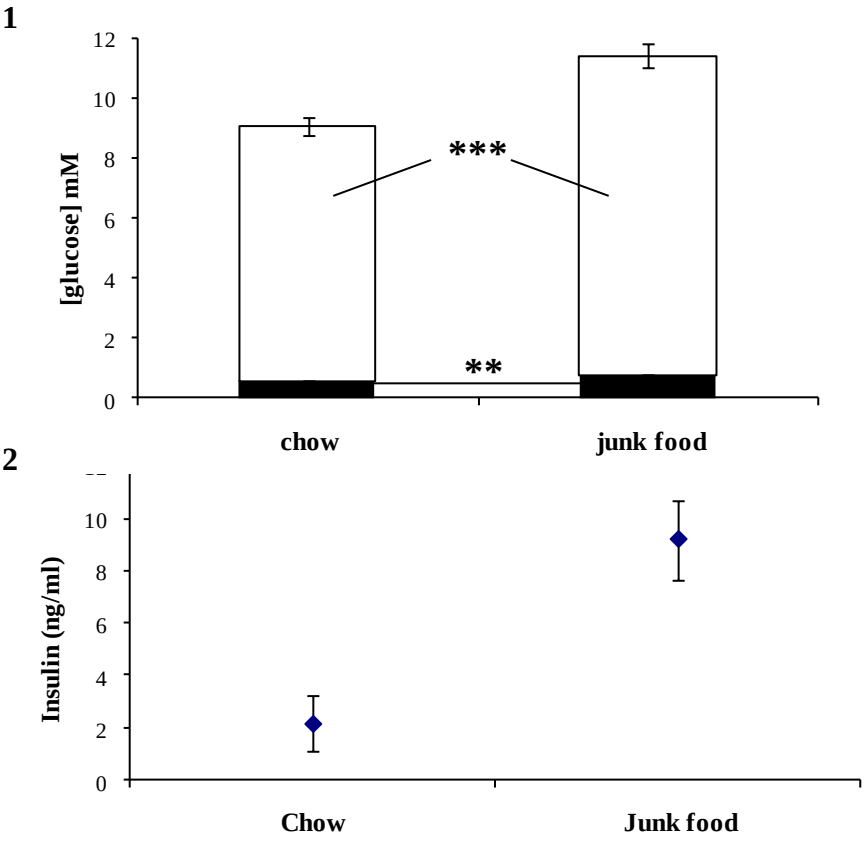


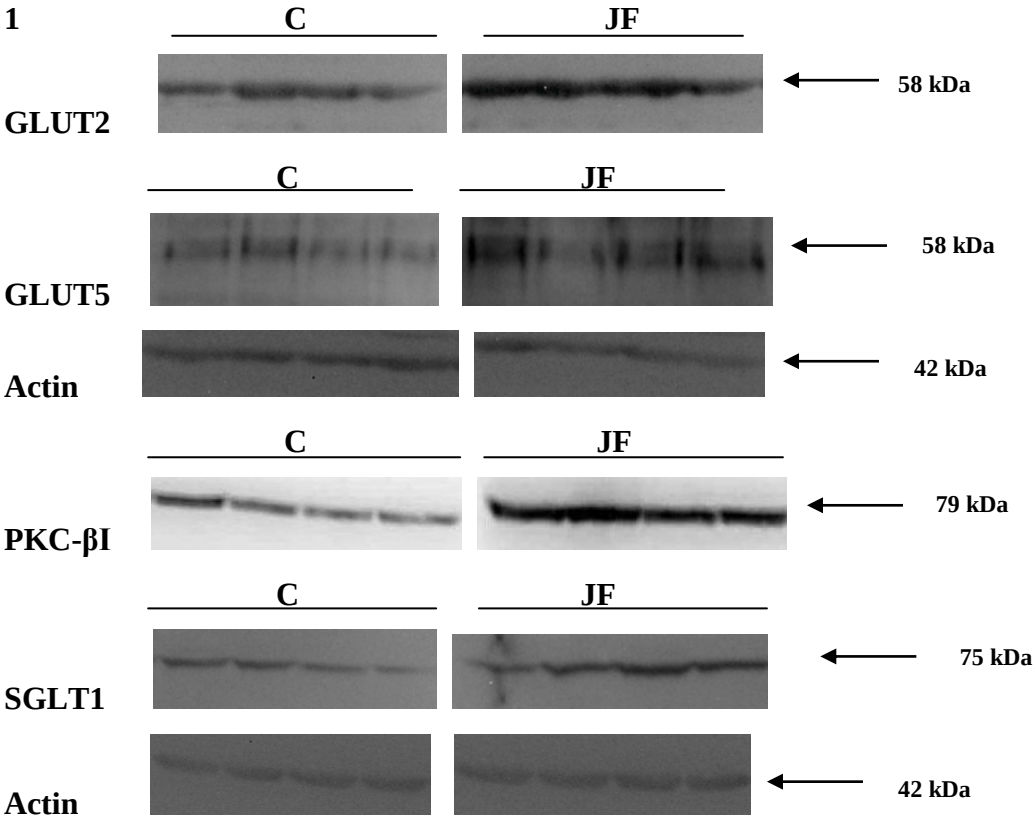
Figure 8.9 - 1) Effect of chow and junk-food diet on plasma (open bar) and urine (closed bar) glucose concentration and 2) plasma insulin levels. Values are expressed as mean ± SEM, n = 6-12, **p<0.01, ***p<0.005 compared with chow-fed controls



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)



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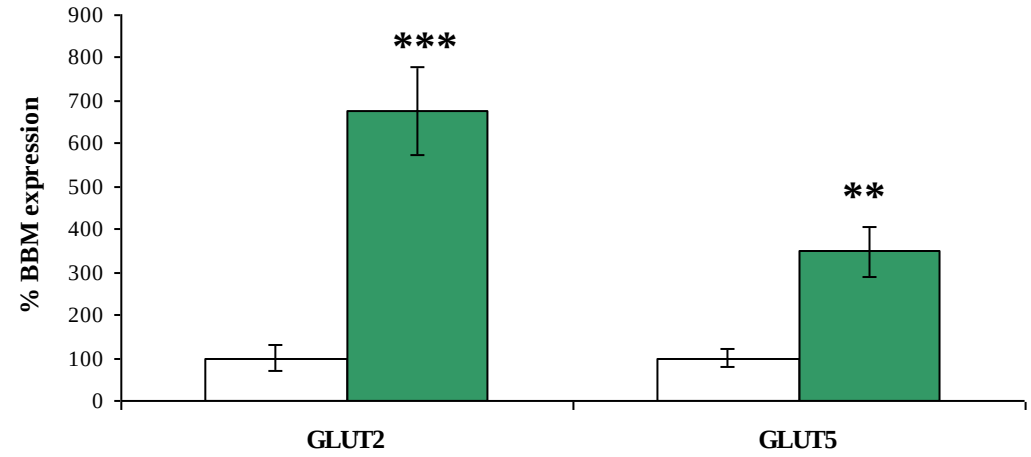
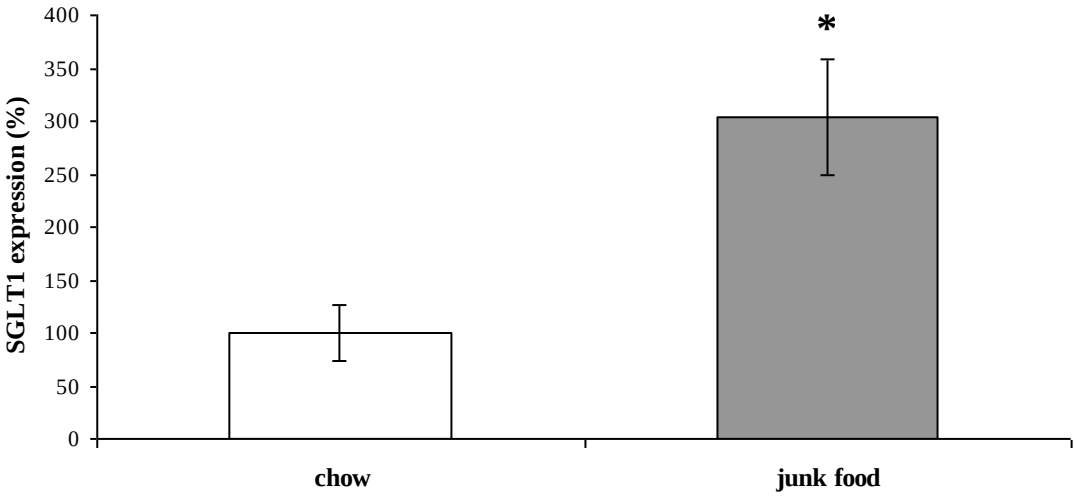
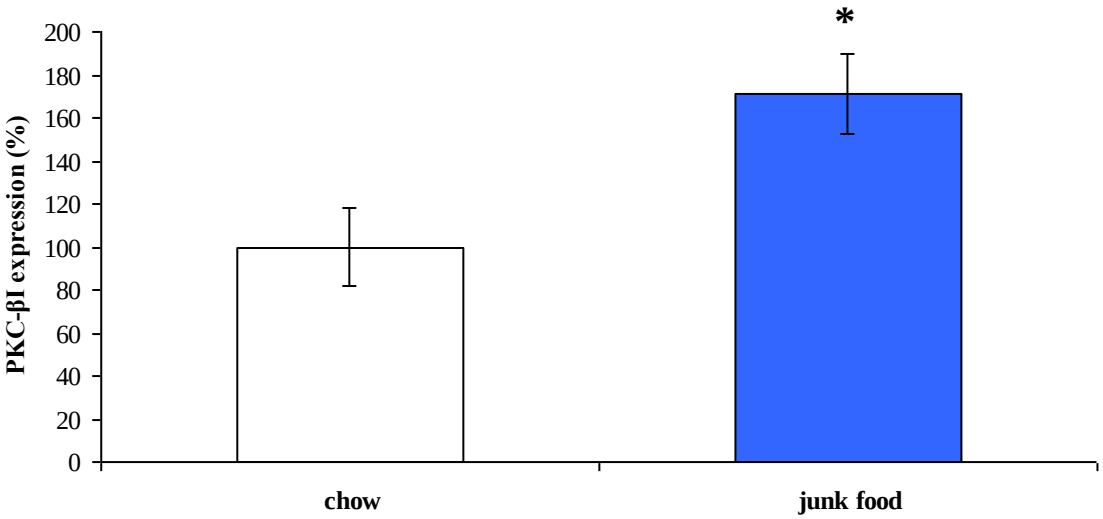

% BBM expression
900
800
700
600
500
400
300
200
100
0
***
**
GLUT2
GLUT5


SGLT1 expression (%)
400
350
300
250
200
150
100
50
0
*
chow
junk food


PKC-βI expression (%)
200
180
160
140
120
100
80
60
40
20
0
*
chow
junk food

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.

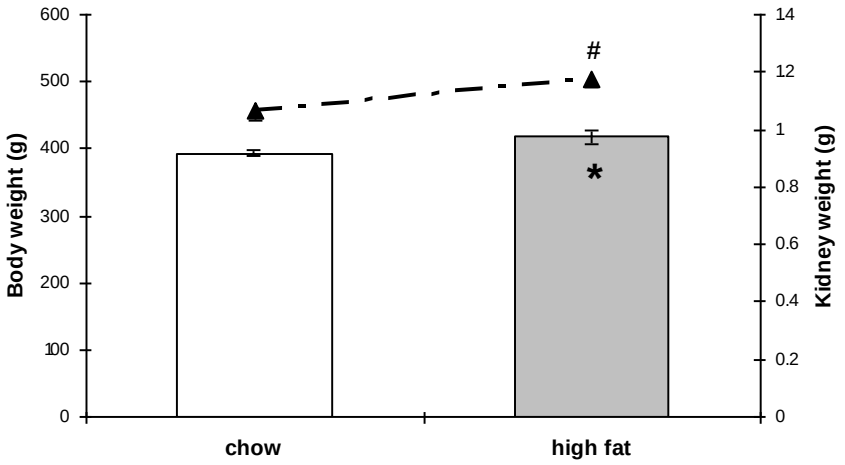


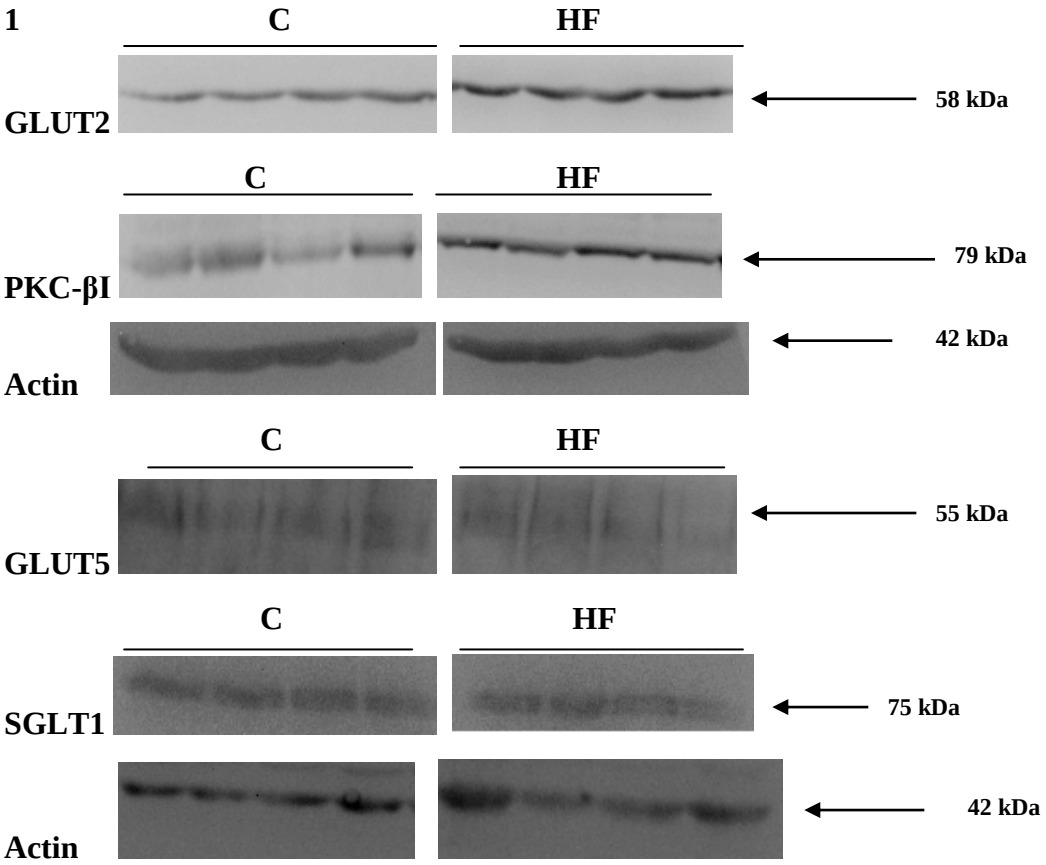
Table 8.2 – Fasted plasma concentrations of glucose, basal insulin and insulin concentration following the insulin tolerance test. Values are expressed as mean ± SEM, n = 6-8, *p<0.05, ***p<0.005 compared with chow-fed controls

	Chow	**High-fat**
Fasting plasma glucose (mM)	4.17 ± 0.14	4.67 ± 0.17 *
Basal plasma insulin (ng.ml^{-1})	4.91 ± 0.87	10.07 ± 1.76 *
Plasma insulin post-tolerance test (ng.ml^{-1})	15.5 ± 1.93	23.8 ± 1.94 ***

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)



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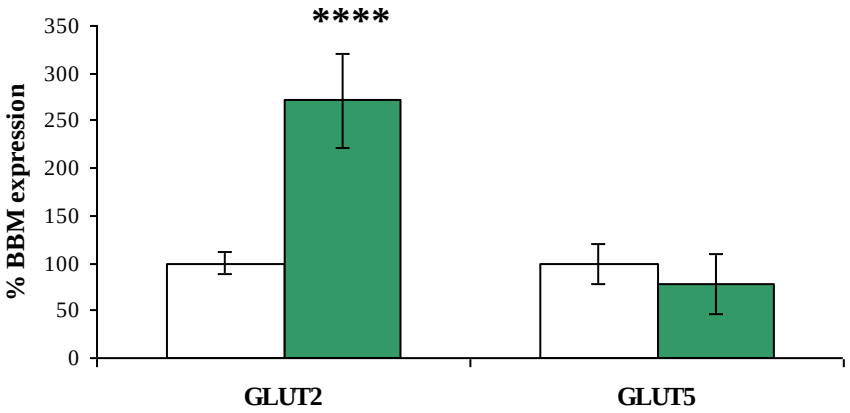
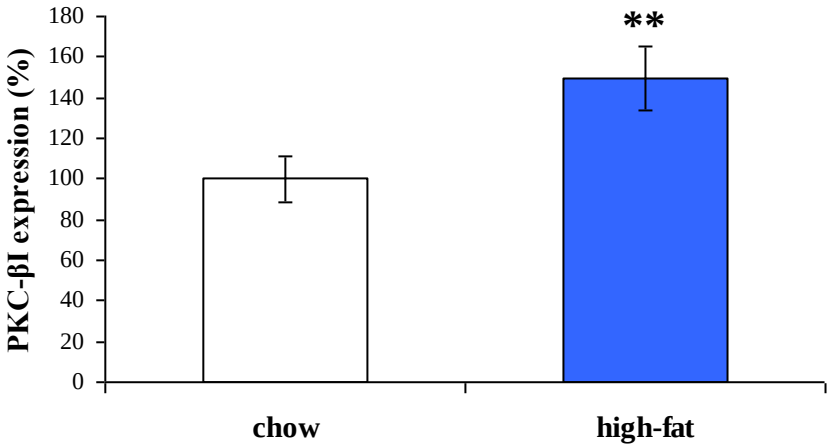
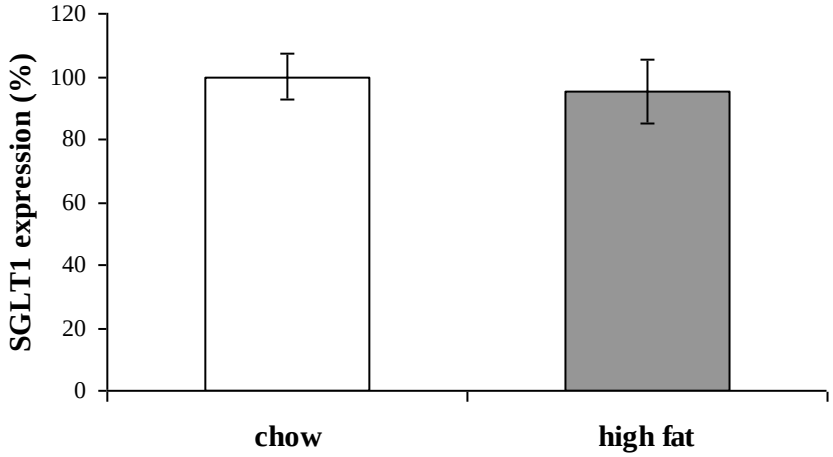

****
350
300
250
200
150
100
50
0
% BBM expression
GLUT2
GLUT5


**
180
160
140
120
100
80
60
40
20
0
PKC-βI expression (%)
chow
high-fat


120
100
80
60
40
20
0
SGLT1 expression (%)
chow
high fat

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

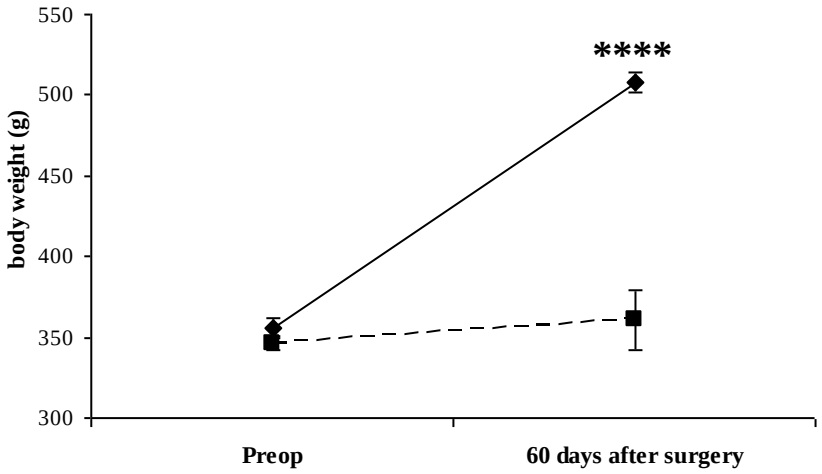
The increase in GLUT2 transporter in the outlined models of diabetes and obesity was investigated further in a rodent model of RYGB surgery. As well as an effective treatment for weight loss in obesity, gastric bypass surgery also results in the rapid resolution of hyperglycaemia in type I and II diabetes.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

1



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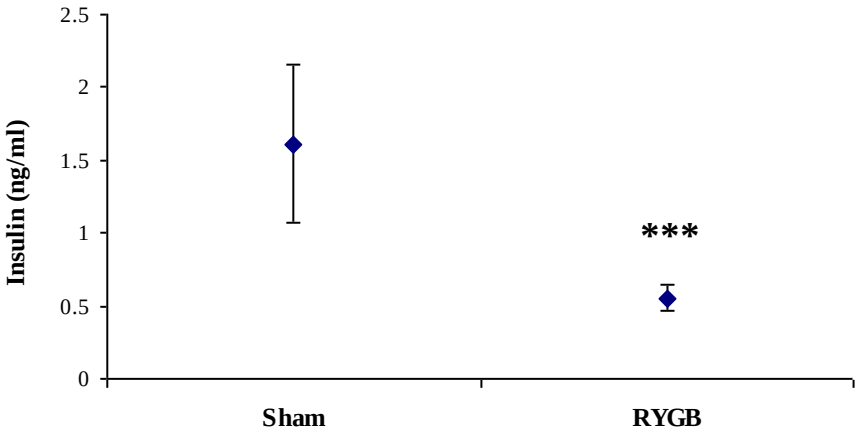
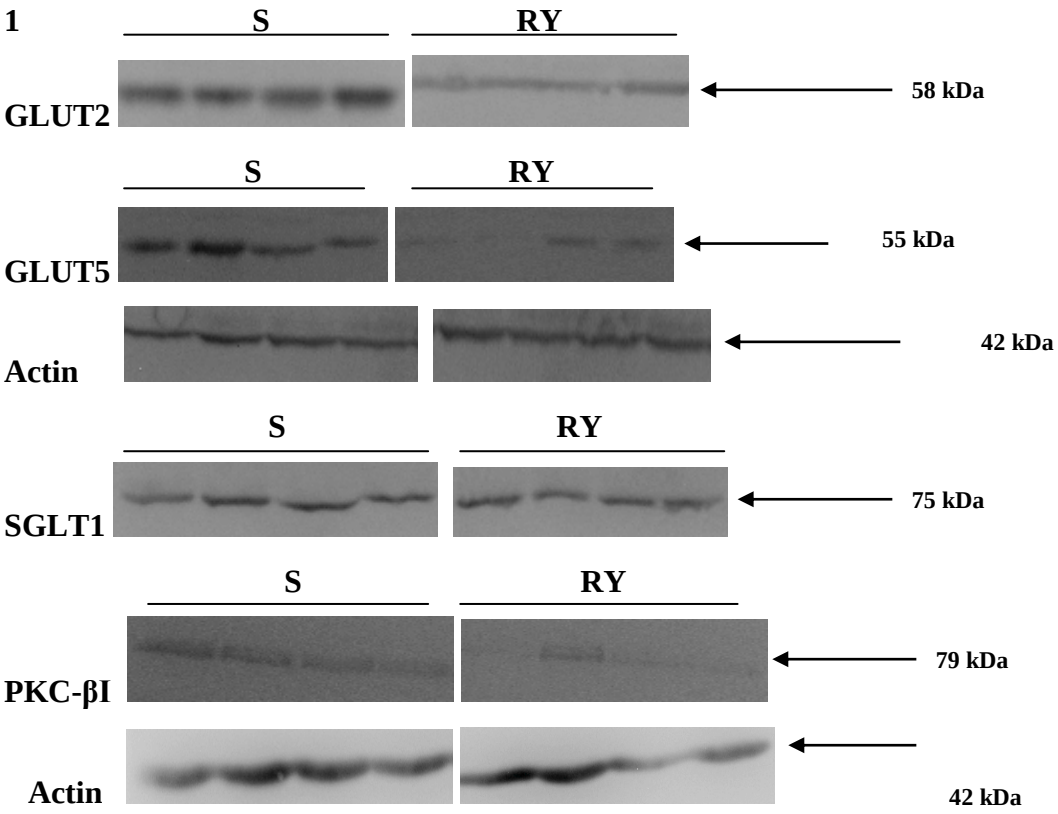


8.3.4.2 Effect of RYGB surgery on glucose transporter expression at the proximal tubule BBM

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)



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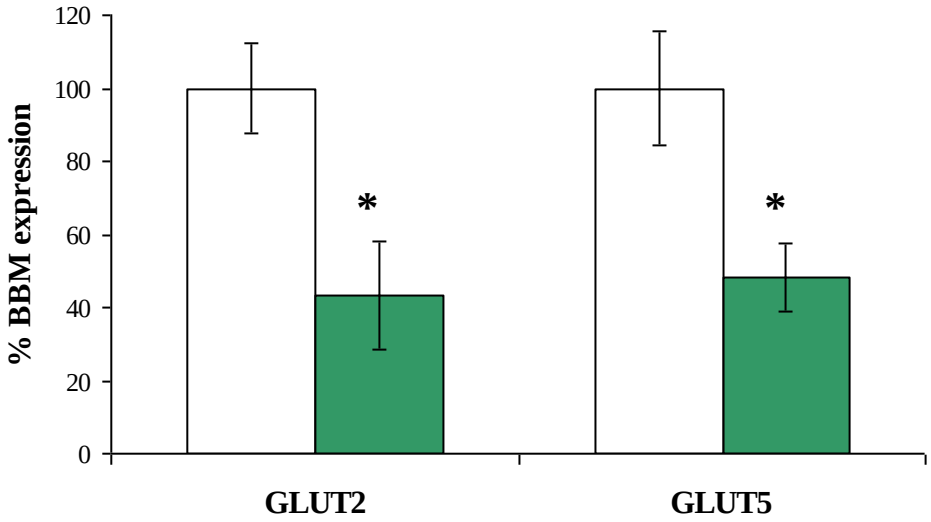
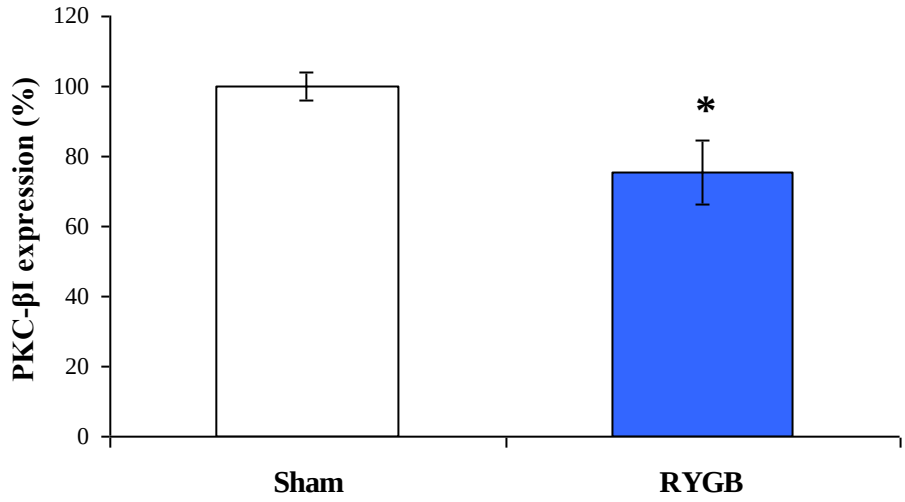
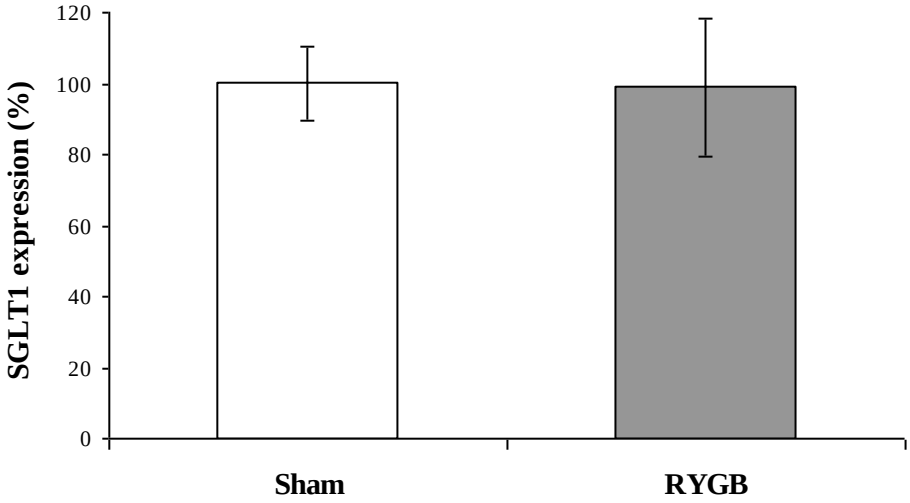

% BBM expression
120
100
80
60
40
20
0
*
*
GLUT2
GLUT5

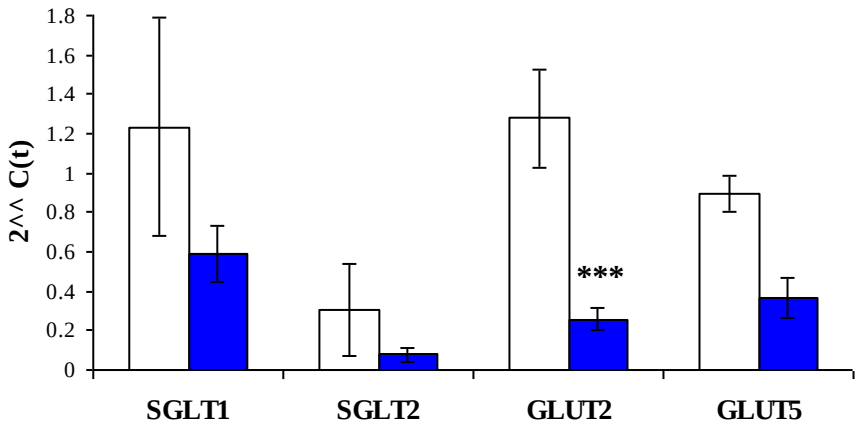

PKC-βI expression (%)
120
100
80
60
40
20
0
*
Sham
RYGB


SGLT1 expression (%)
120
100
80
60
40
20
0
Sham
RYGB

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).

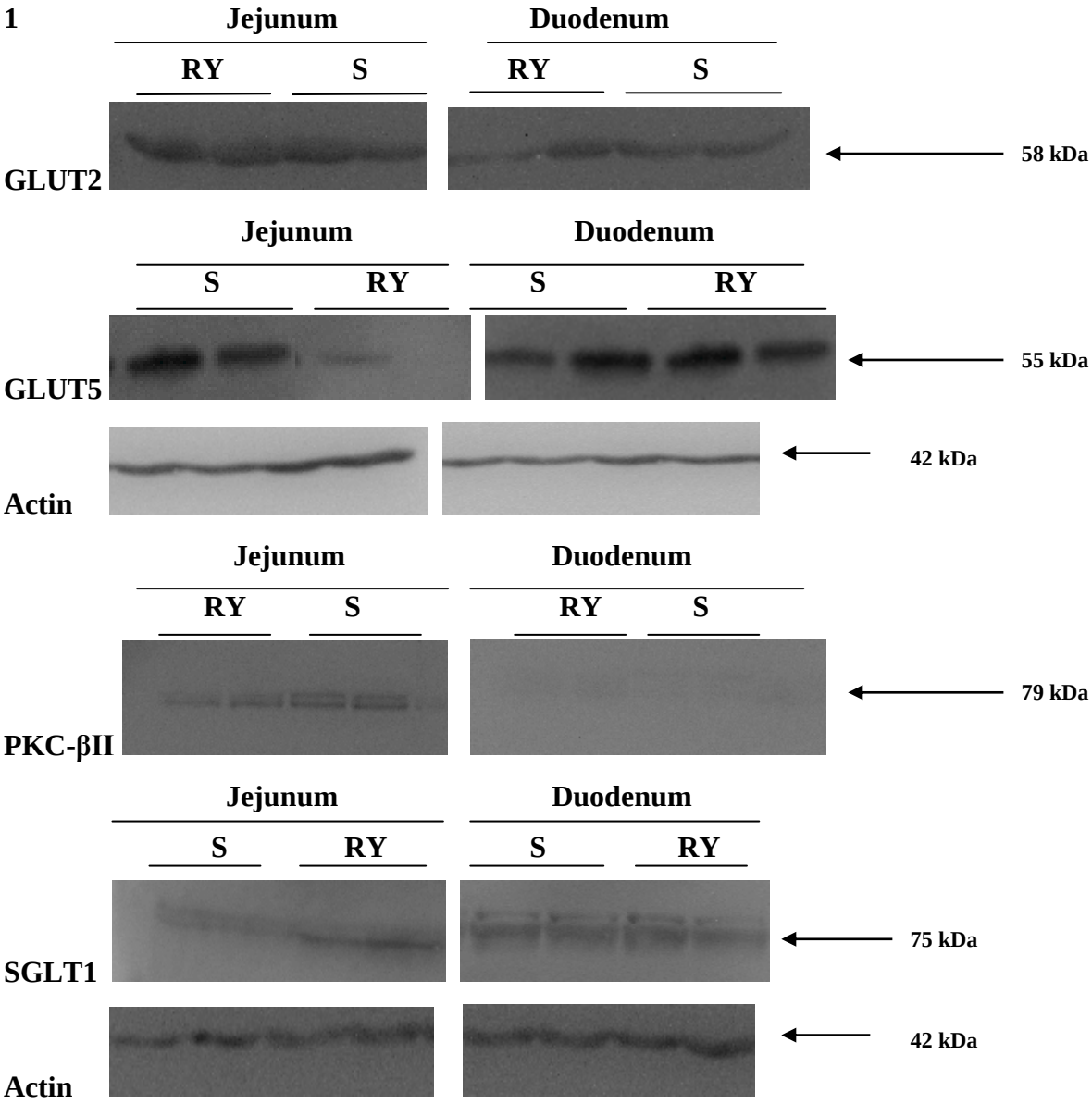
Figure 8.15 – Effects, after 60 day sham (open bars) or RYGB surgery (closed bars), on SGLT1, SGLT2, GLUT2 and GLUT5 mRNA expression in renal cortex. Values are expressed as mean ± SEM standardised to β-actin, n=6-8, ***p<0.005 compared with sham surgery control animals



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)



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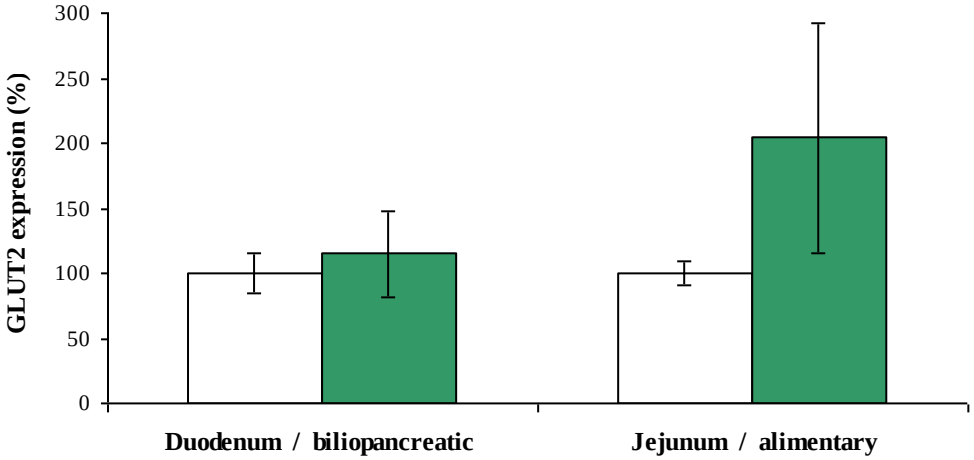
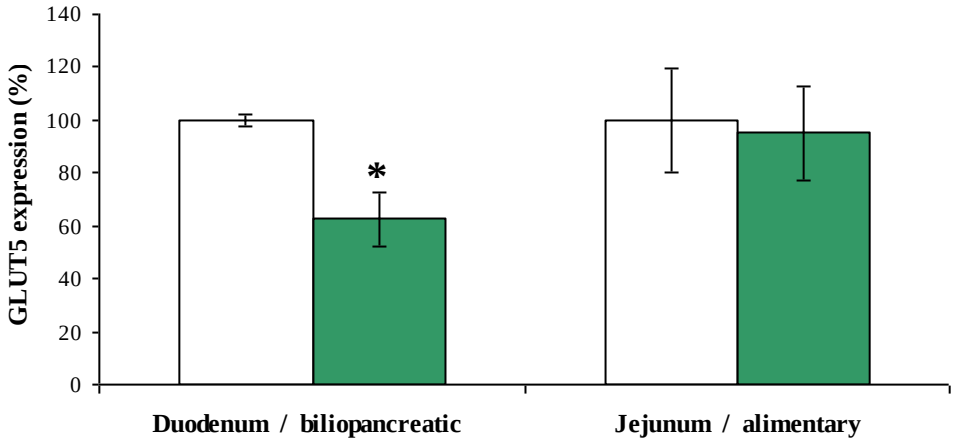
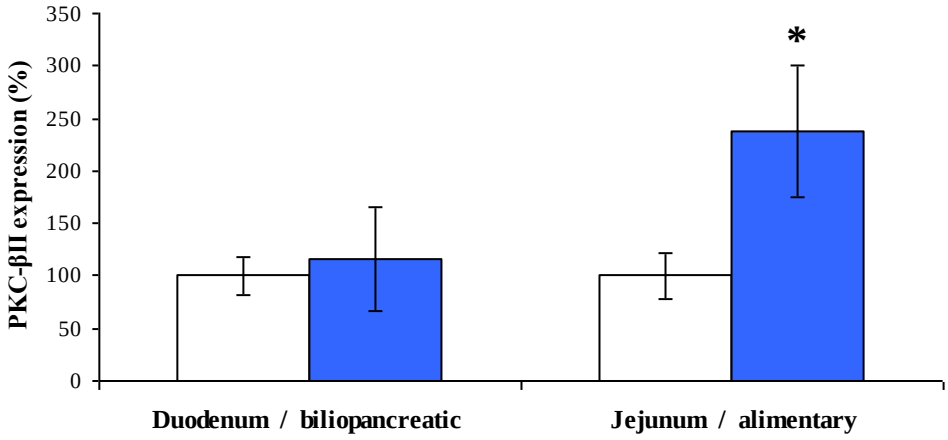
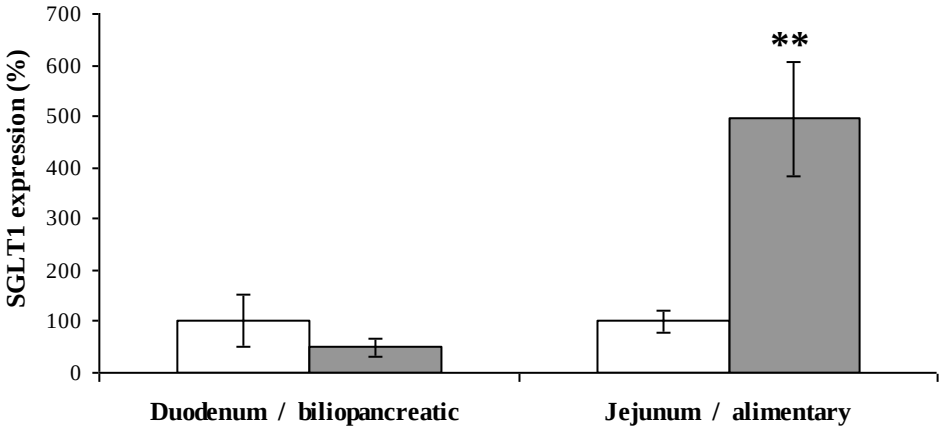

GLUT2 expression (%)
300
250
200
150
100
50
0
Duodenum / biliopancreatic
Jejunum / alimentary


GLUT5 expression (%)
140
120
100
80
60
40
20
0
*
Duodenum / biliopancreatic
Jejunum / alimentary


PKC-βII expression (%)
350
300
250
200
150
100
50
0
*
Duodenum / biliopancreatic
Jejunum / alimentary

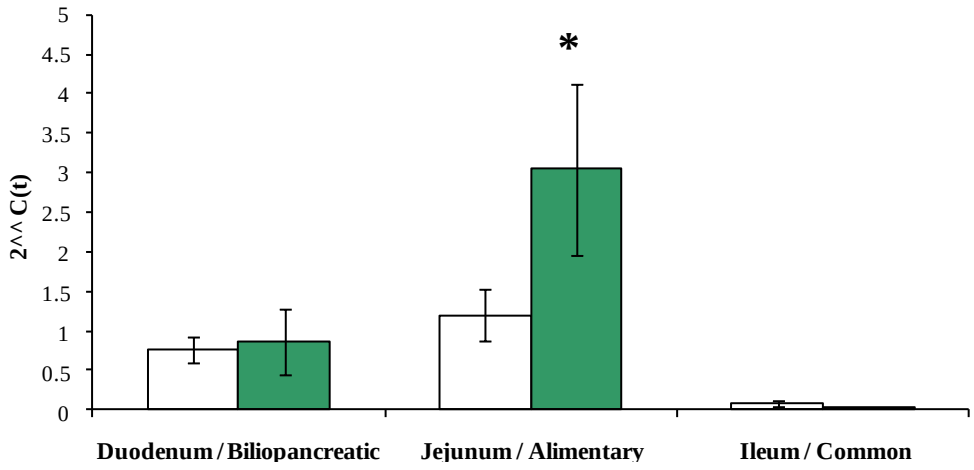

SGLT1 expression (%)
700
600
500
400
300
200
100
0
**
Duodenum / biliopancreatic
Jejunum / alimentary

8.3.4.5 Effect of RYGB surgery on gene expression of glucose transporters in the small intestine

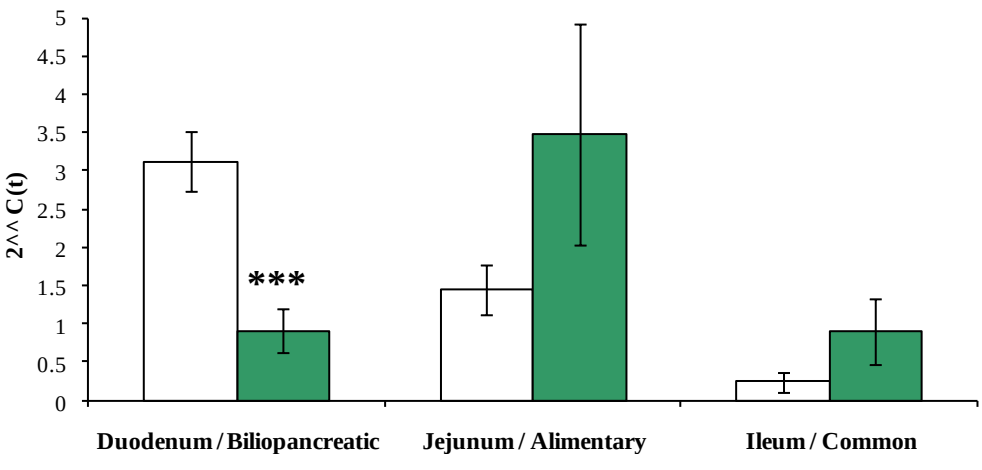
The mRNA expression profile of intestinal glucose transporters from sham and RYGB surgery animals was also studied (Figure 8.17). mRNA expression of GLUT2 was increased significantly by 266%, in the alimentary limb from RYGB rats (Figure 8.17 - 1). No change in GLUT2 mRNA was noted in the duodenum or 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

1 GLUT2



2 GLUT5



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.

	Body weight	Kidney weight	Plasma glucose	Urine glucose	Plasma insulin	Proximal tubule BBM expression of:			
						GLUT2	PKC-βI	GLUT5	SGLT1
Type II diabetes	↓	↔	↑	↑	↔	↑	↑	↑	↑
Junk food diet	↑	↔	↑	↑	↑	↑	↑	↑	↑
High-fat diet#	↑	↑	↑	↔	↑	↑	↑	↔	↔
RYGB#	↓	↓	↔	↔	↓	↑	↓	↓	↔

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 insulinaemia 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_{max} of 83 pmol/min per mm) than SGLT1 (V_{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_{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, the

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;Bezencon *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 stereo-selective 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.

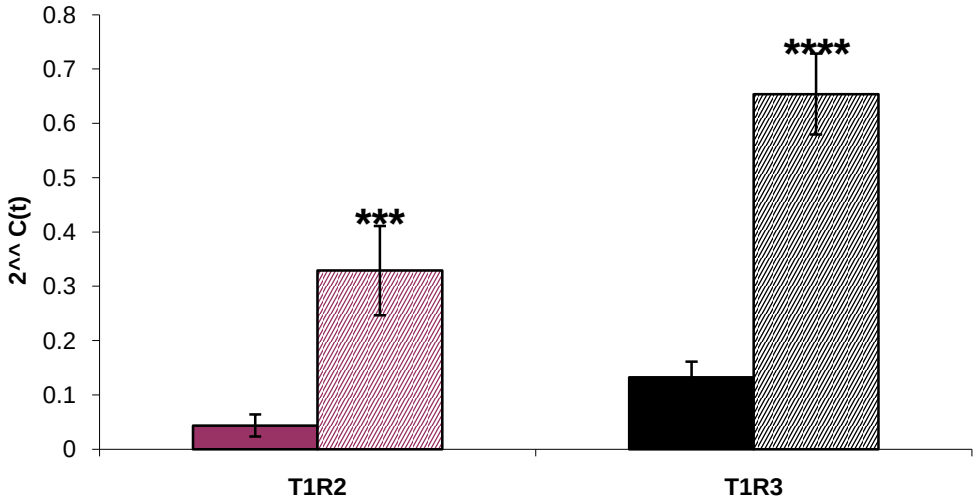
9.3 Results

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.

1



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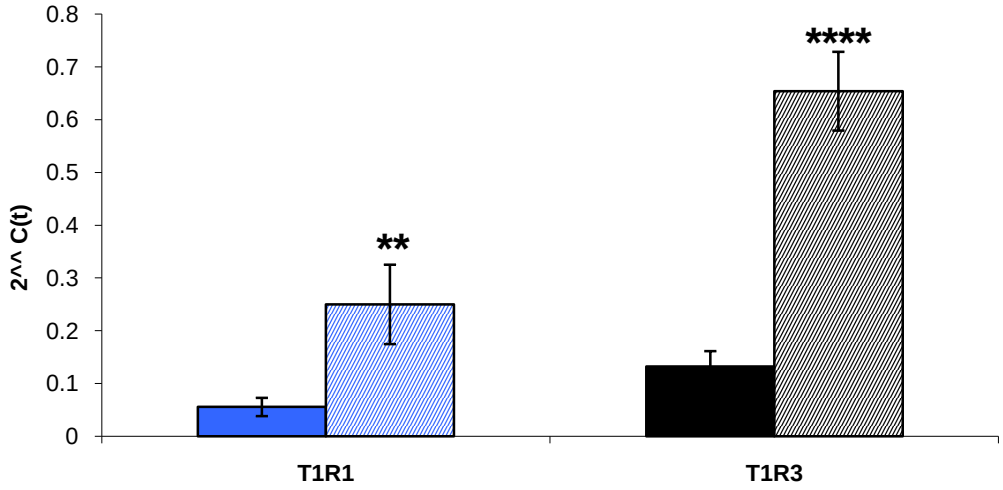


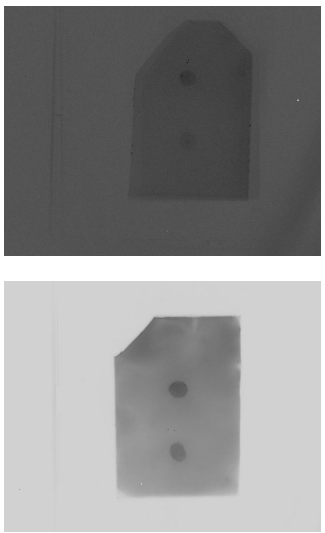
9.3.2 Detection of sweet taste dimer component proteins in the kidney

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).

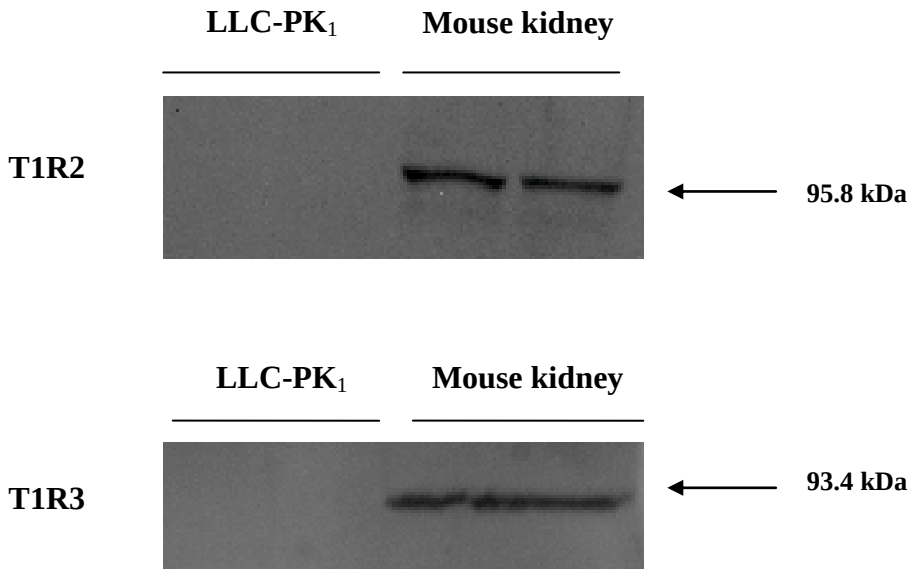
Figure 9.2 – Dot blot detection of sweet taste heterodimer components T1R2 and T1R3 in whole membrane prepared from control rat kidney cortex and jejunal mucosa



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-PK_1) 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-PK_1 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-PK_1)

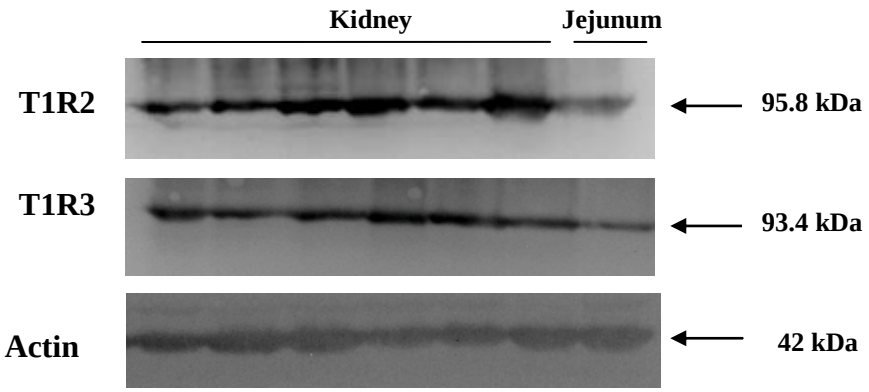


9.3.2.3 Detection of renal sweet taste receptors at the proximal tubule BBM

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.

1



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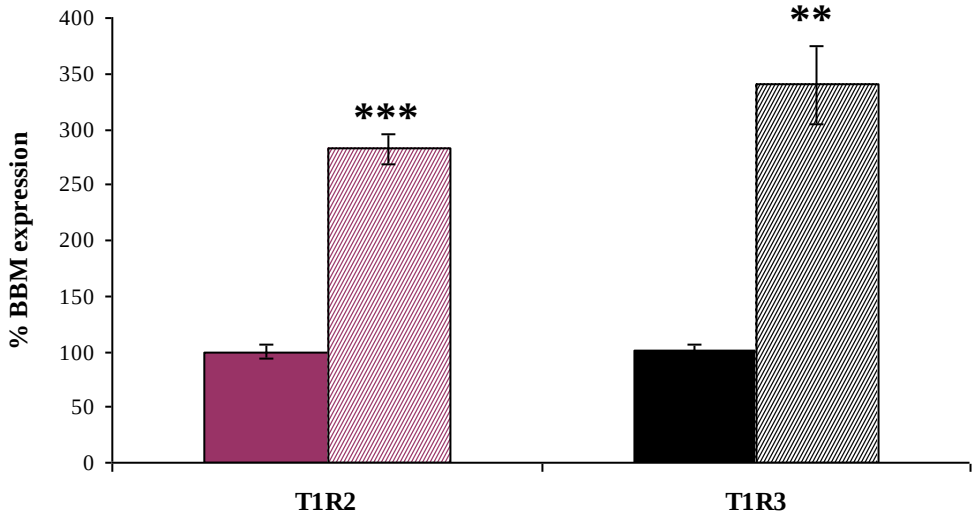
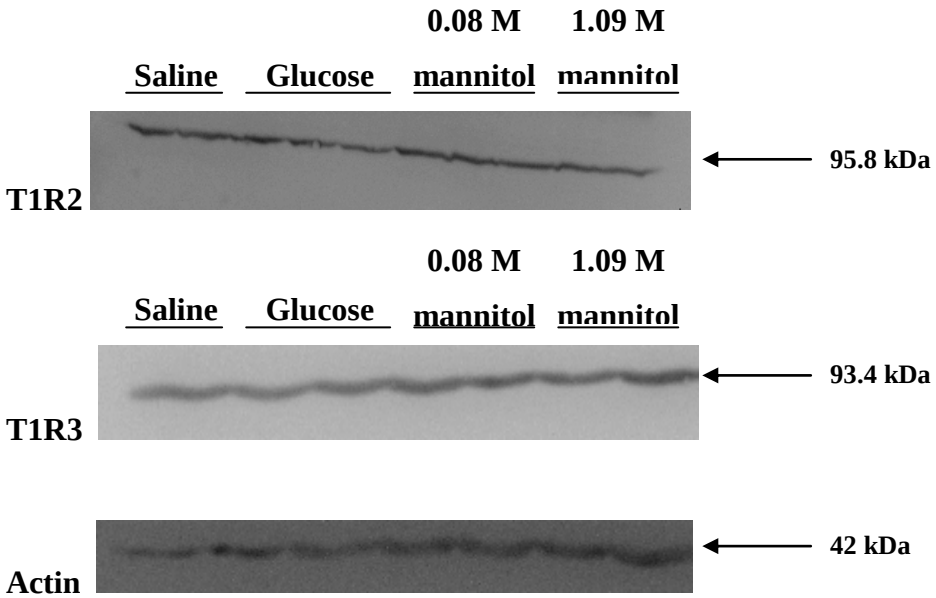


9.3.3 Effects of acute high plasma glucose and osmolality on expression of sweet taste receptors at the proximal tubule BBM

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



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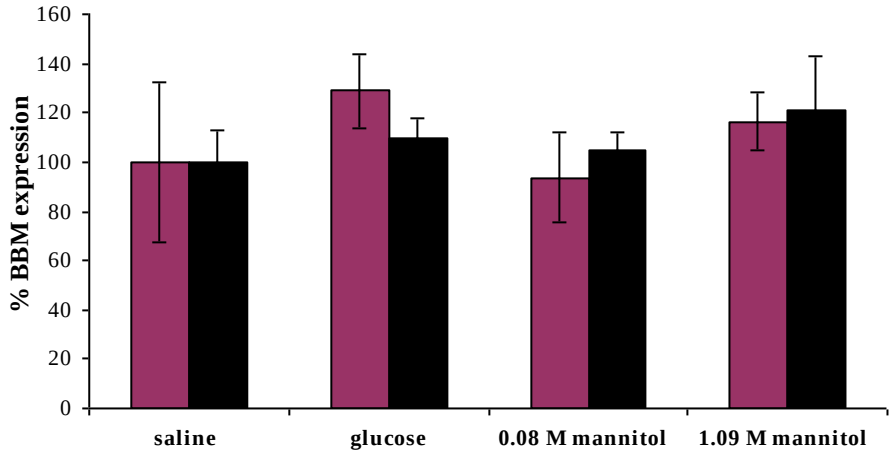


9.3.4 Effect of chronic hyperglycaemia on sweet taste receptors at the proximal tubule BBM

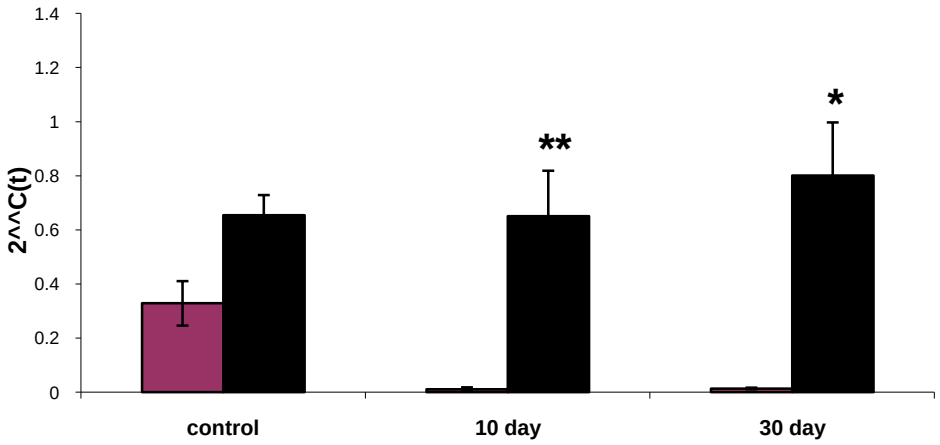
To assess further the response of the renal sweet taste sensing heterodimer to simple sugars, chronic hyperglycaemia was used to assess the effects of long-term, high-glucose tubular fluid luminal concentration. As was the case in Chapter 7, chronic hyperglycaemia was studied using the STZ-induced model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

1



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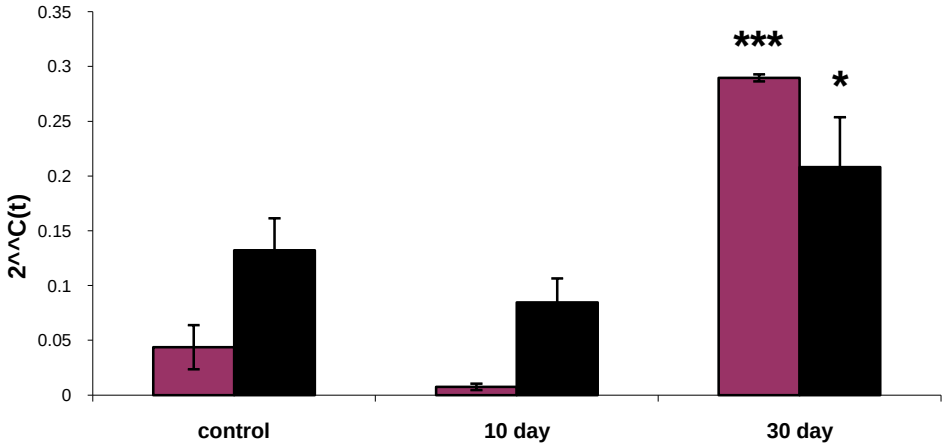
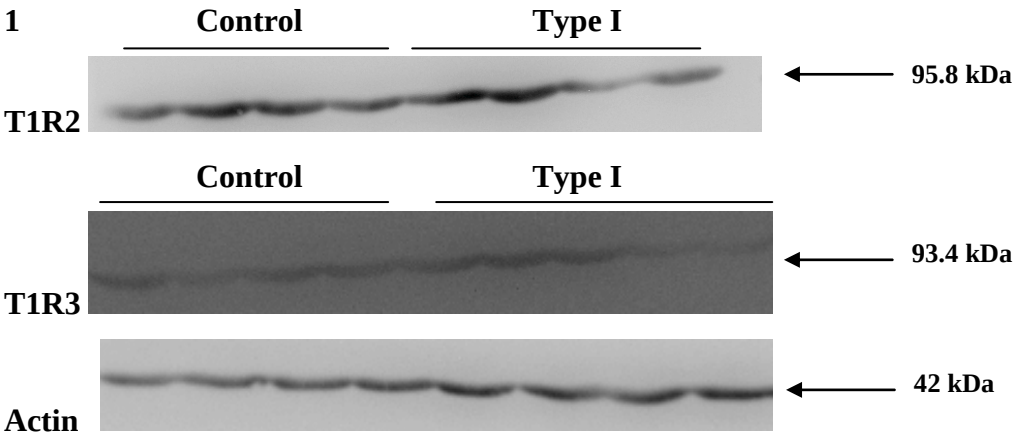
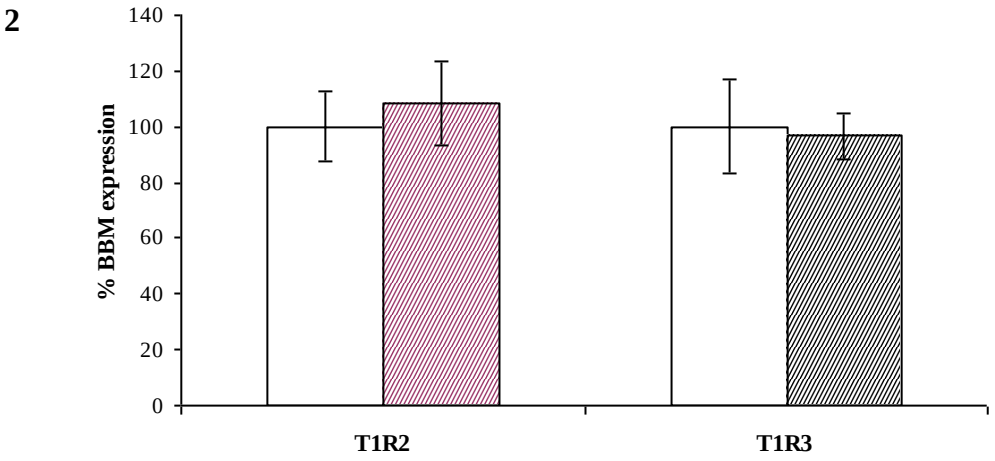


9.3.4.2 Effect of type I diabetes on the expression of sweet taste receptor proteins, T1R2 and T1R3, at the proximal tubule BBM

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

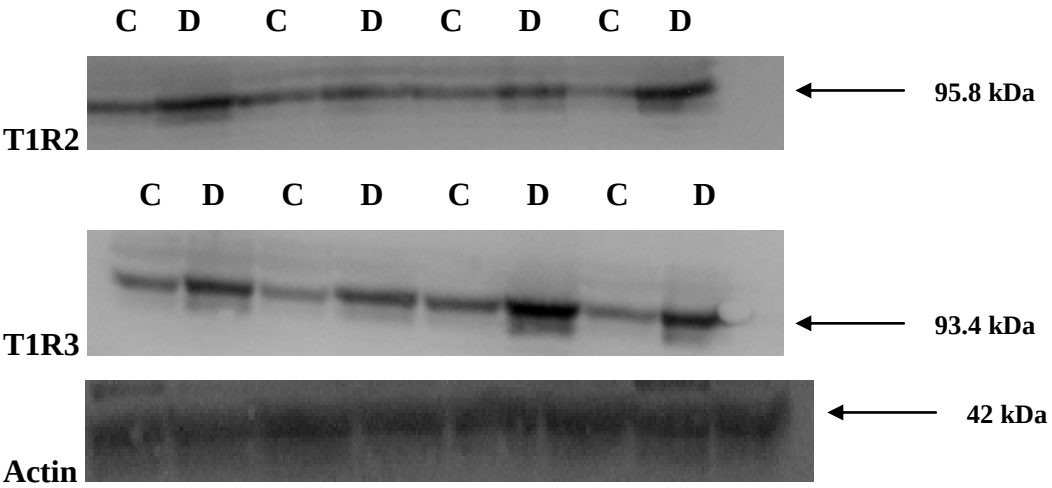
Studies presented in this thesis have established the influence of different metabolic conditions on glucose transport at the proximal tubule BBM (Chapter 8). Therefore to further understand the renal sweet taste heterodimer, it was of interest to study whether the expression of T1R2 and T1R3 at the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

1



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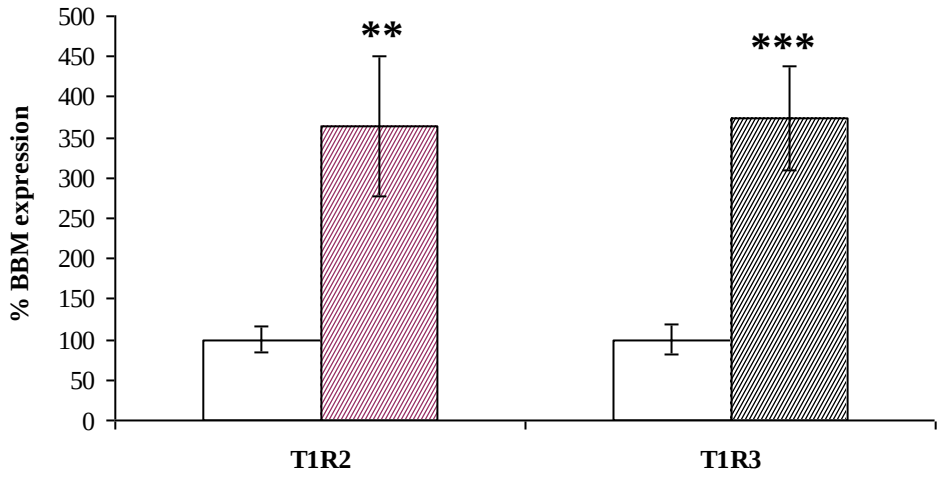
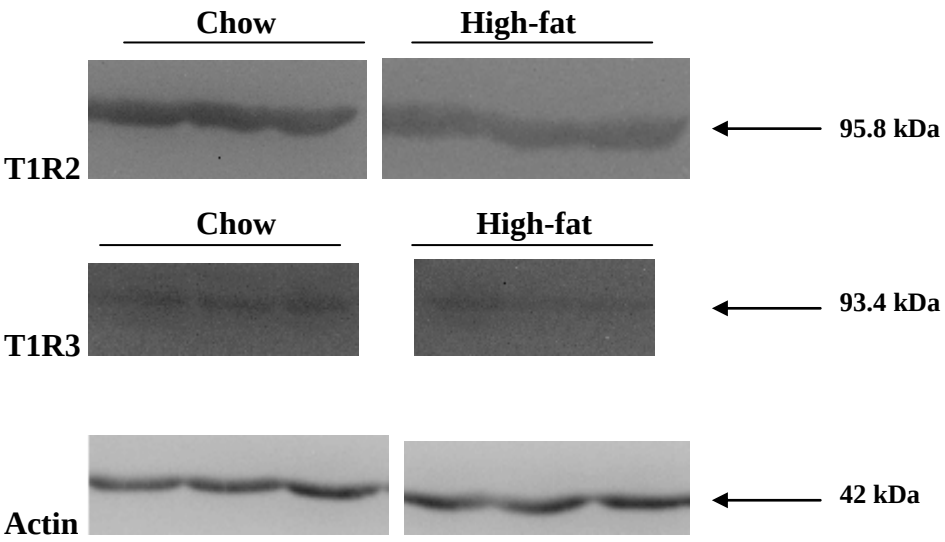


9.3.5.2 High-fat diet-induced metabolic syndrome model

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)

1



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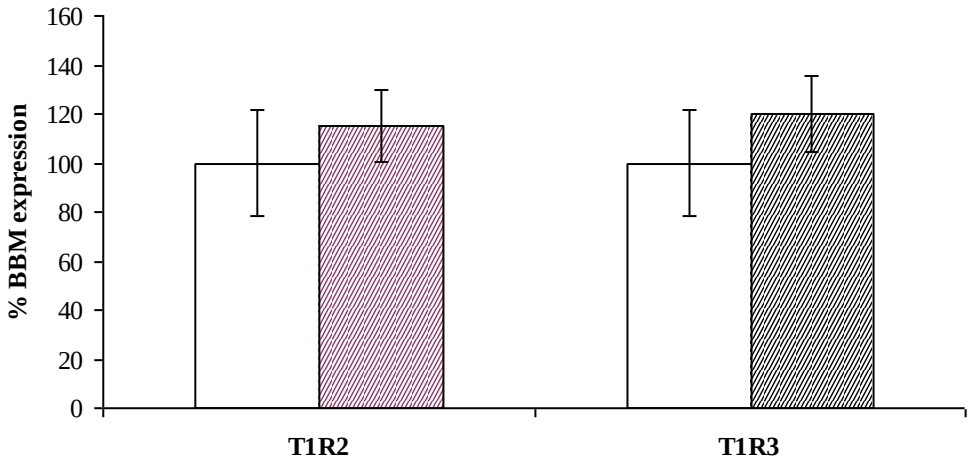
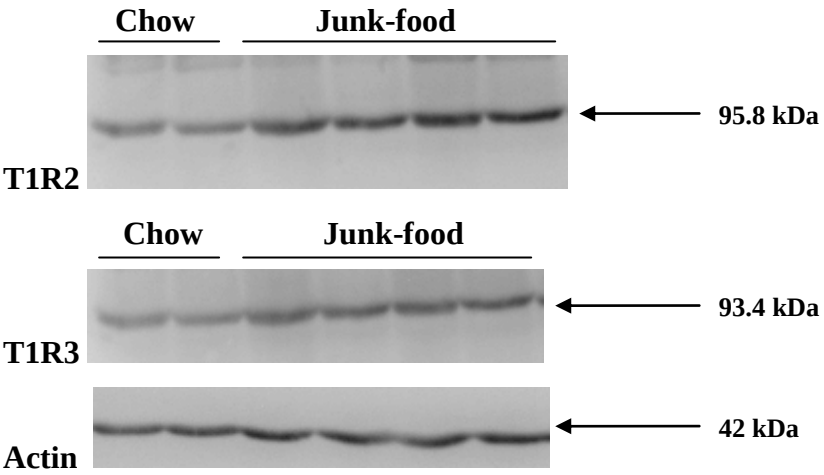


9.3.5.3 Junk-food diet-induced metabolic syndrome model

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)

1



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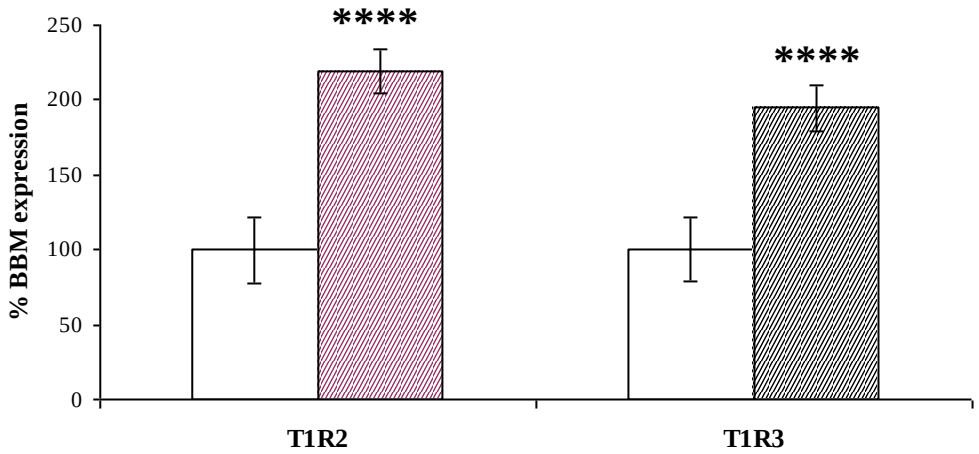
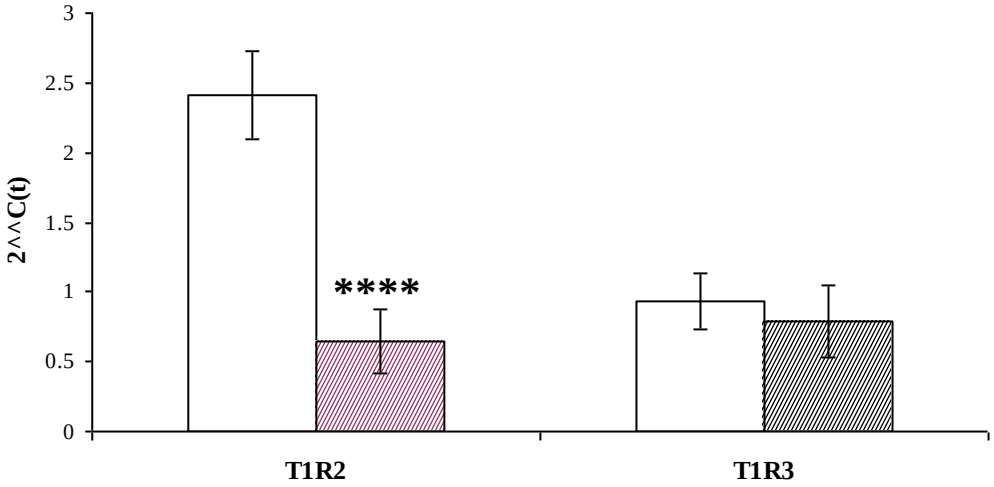


9.3.5.4 Roux-en-Y gastric bypass (RYGB) model

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).

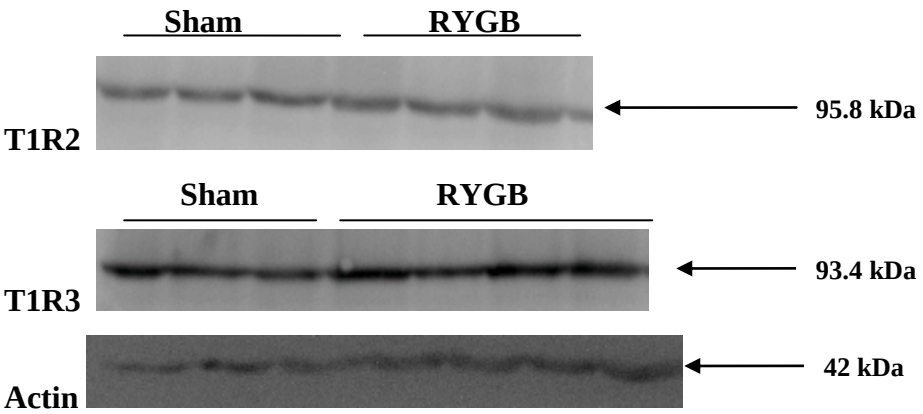


9.3.5.4.2 Effect of RYGB surgery on expression of renal sweet taste receptor proteins at the proximal tubule BBM

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



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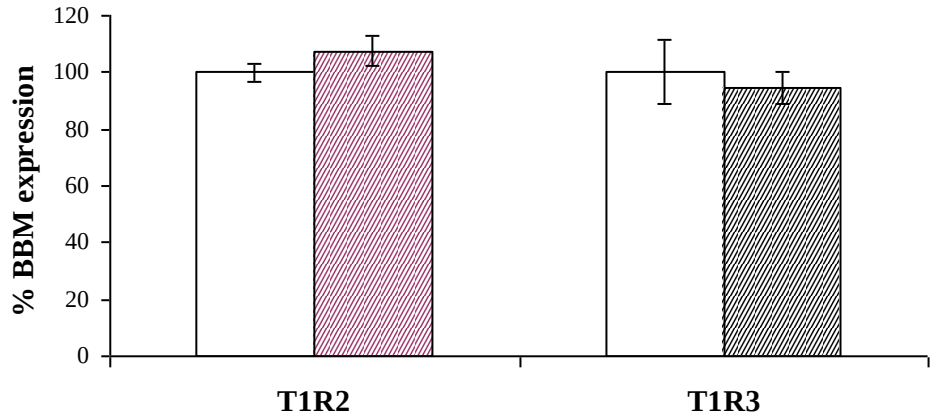


9.3.5.4.3 Effect of RYGB on expression of sweet taste receptor mRNA expression in specific regions of the intestine

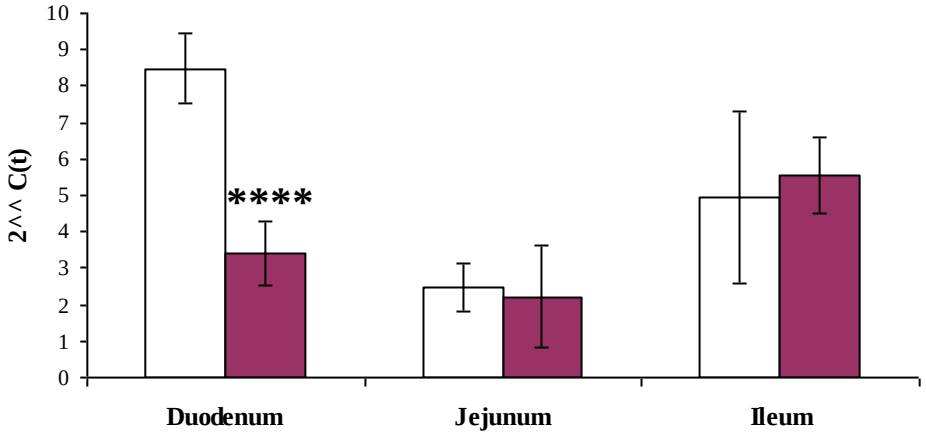
The gene expression profile of sweet taste receptors T1R2 and T1R3 in mucosal scrapes from three specific regions of the small intestine was studied 60 days after RYGB surgery (Figure 9.13).

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).

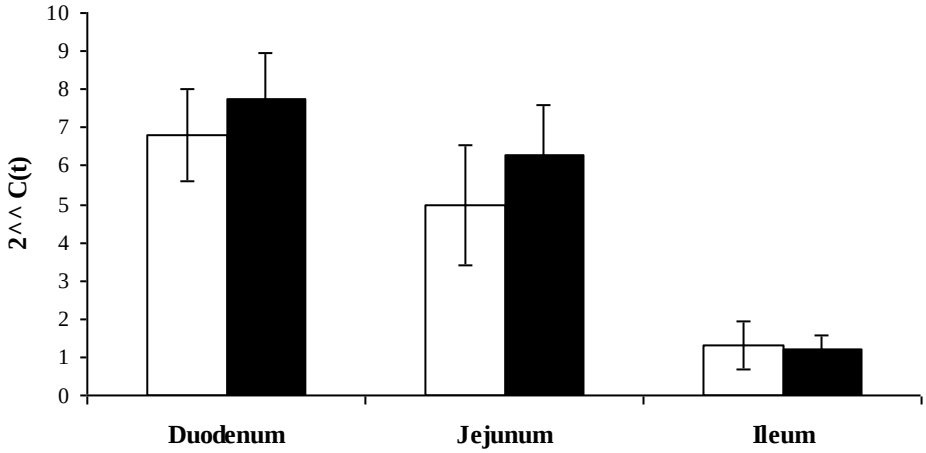
The expression profile of T1R3 in all three regions of the small intestine was unaffected by RYGB surgery. Although a 25% increase in T1R3 expression was seen in the alimentary limb compared wiith the jejunum from sham-operated control animals, this did not reach statistical significance (Figure 9.13 - 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



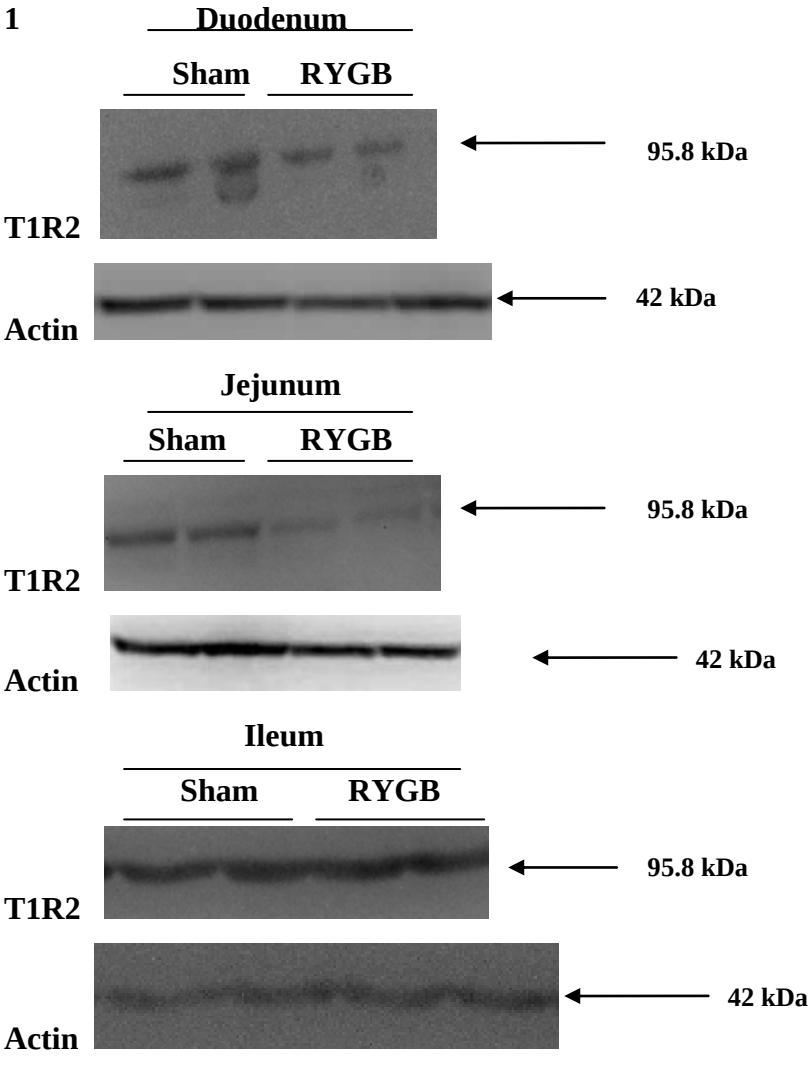
2 T1R3



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).



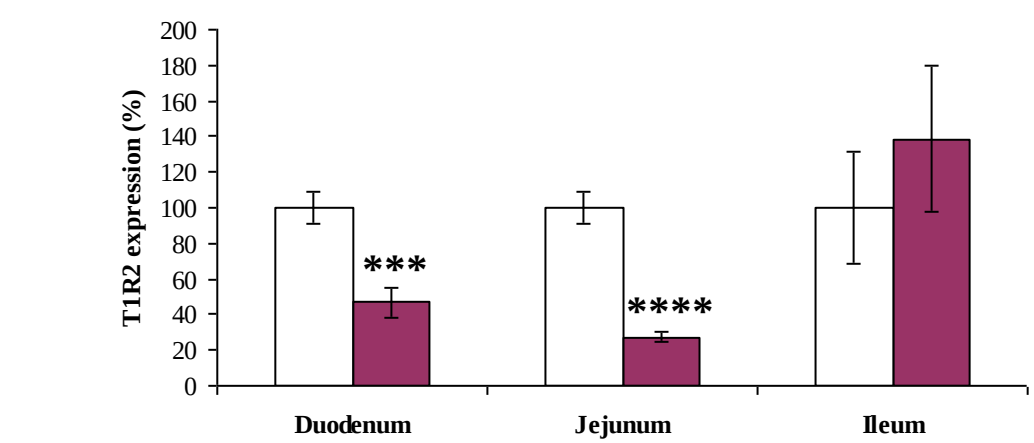
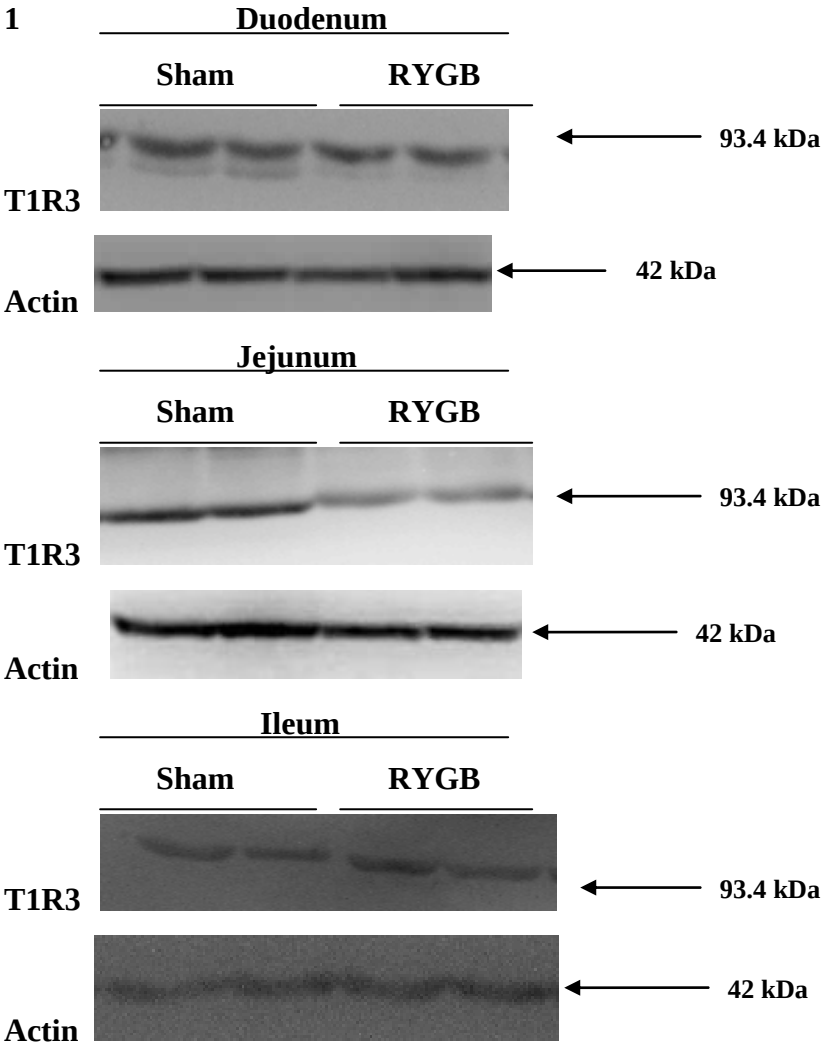
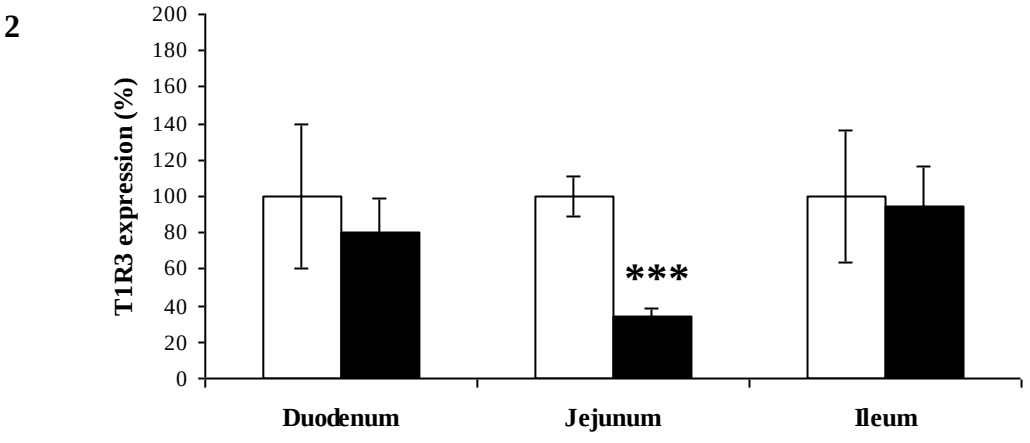


Figure 9.15 – Effects of RYGB surgery (closed bars) on expression of sweet taste receptor T1R3 protein in specific regions of the small intestine. Following RYGB, the duodenum becomes the biliopancreatic limb, the jejunum becomes the alimentary limb and the ileum becomes the common limb: 1) Western blots 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.





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, $p>0.5$.

1

	Saline	**Saccharin**
Plasma glucose (mM)	5.39 ± 0.2	6.27 ± 0.8
Infusate osmolality (mOsM)	327 ± 4	329 ± 8
Plasma osmolality (mOsM)	309 ± 0.9	310 ± 0.9

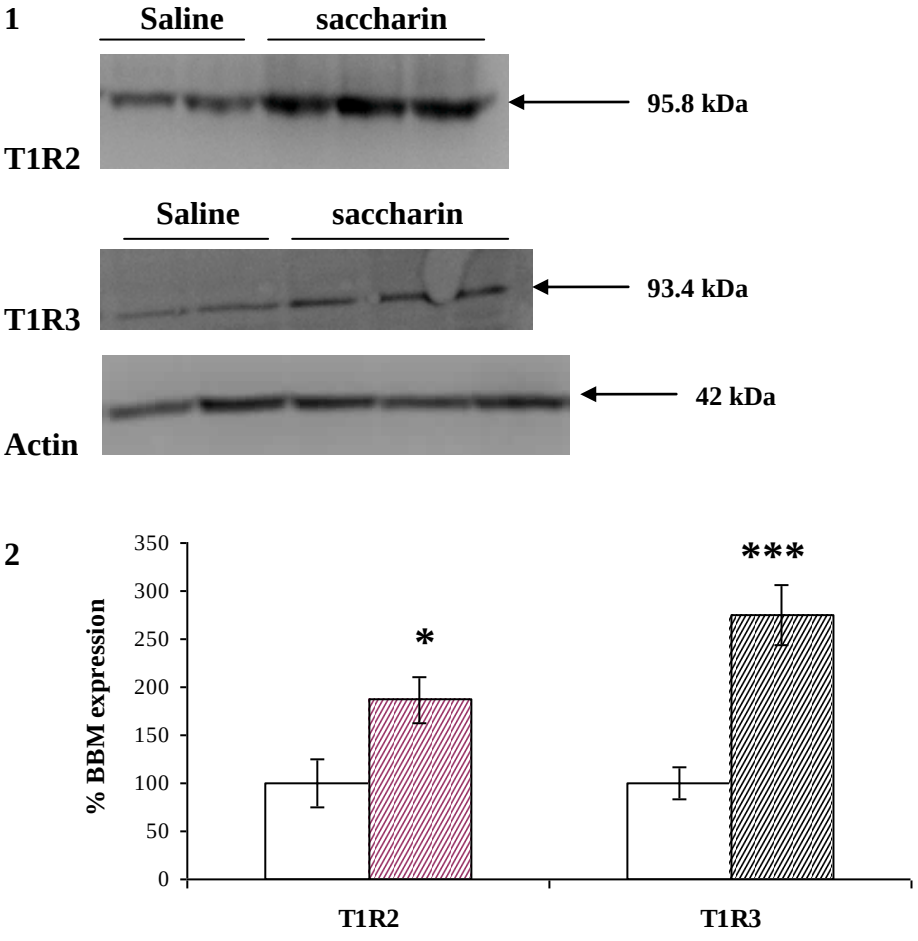
2

	Saline	**Saccharin**
Body weight (g)	398 ± 11.8	409.7 ± 15.3
Kidney weight (g)	1.04 ± 0.08	1.01 ± 0.02
BBM vesicle enrichment	6.81 ± 0.61	6.64 ± 0.57

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_{max} of SGLT-mediated glucose transport, as measured by glucose uptake between 30-960 μM concentrations, from 959.4 ± 90.5 pmol.mg $prot^{-1}$ in saline-infused control animals to 2333.2 ± 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_{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)

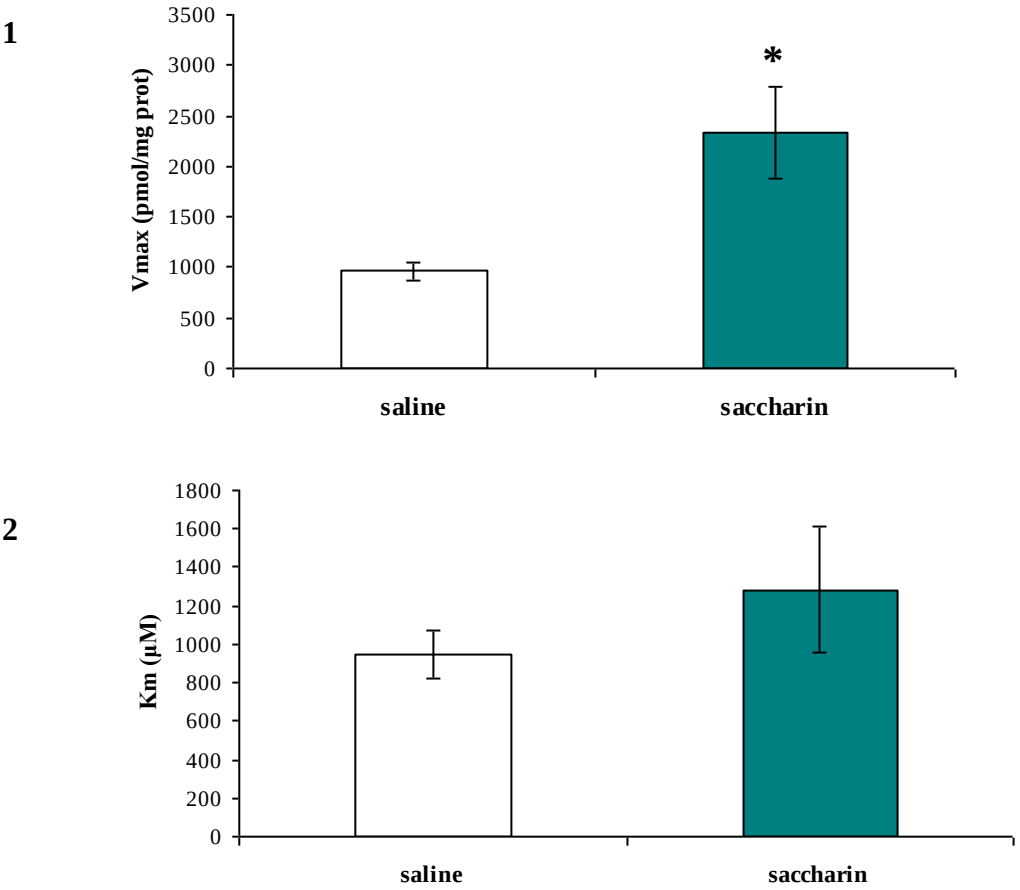
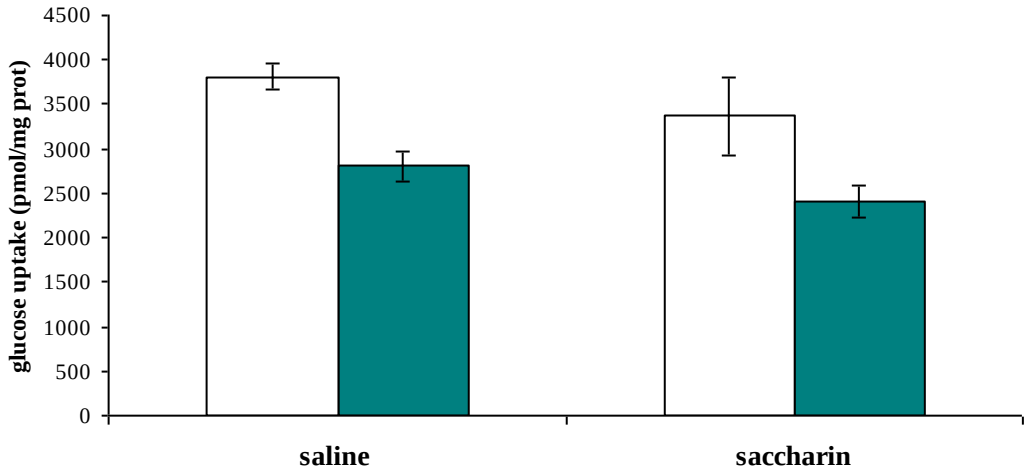


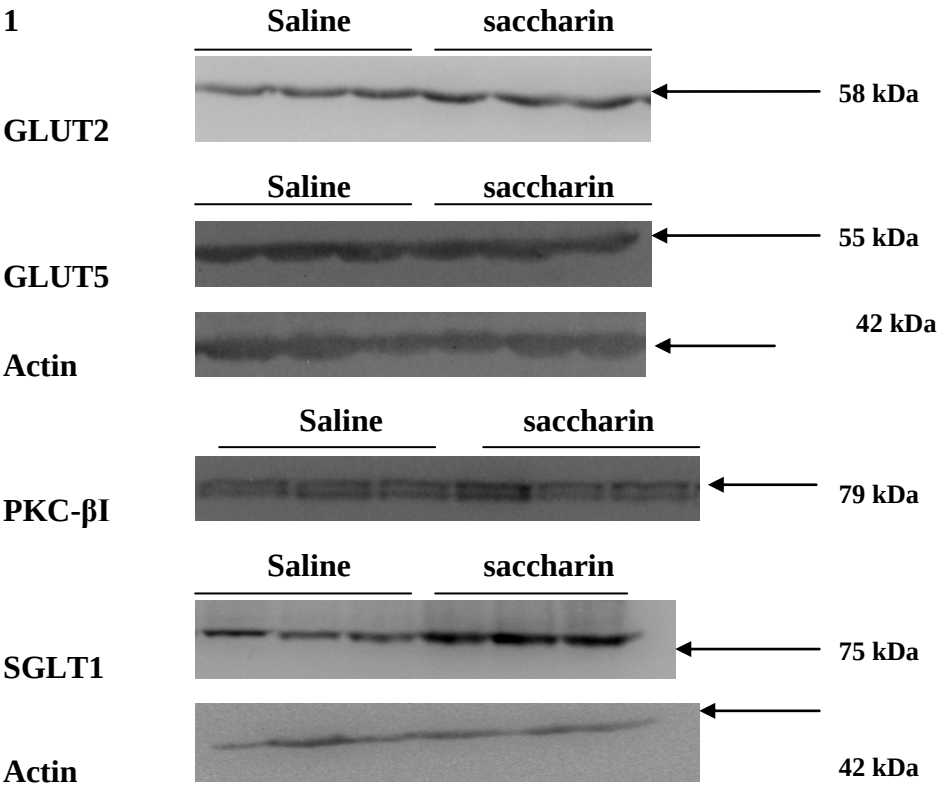
Figure 9.18 – Effect of artificial sweetener on total glucose uptake (open bars) and GLUT-mediated glucose uptake (closed bars) studied by IV infusion of 1.37 mM saccharin in 154 mM for 2 hours, using renal BBM vesicles incubated in 20 mM glucose for 4 seconds. Mean ± SEM, n=6-8, p>0.5 compared with 154 mM saline infusion.

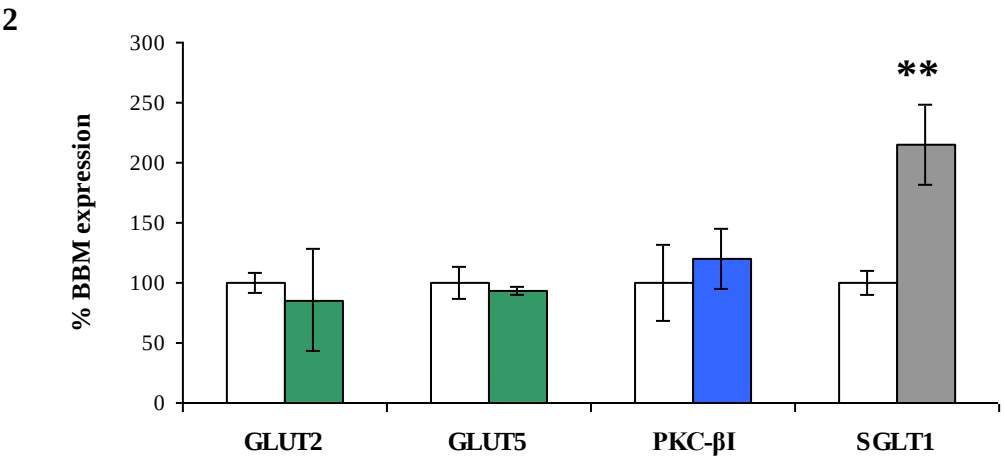


9.3.6.4 Effect of saccharin infusion on the expression of renal glucose transporters

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)

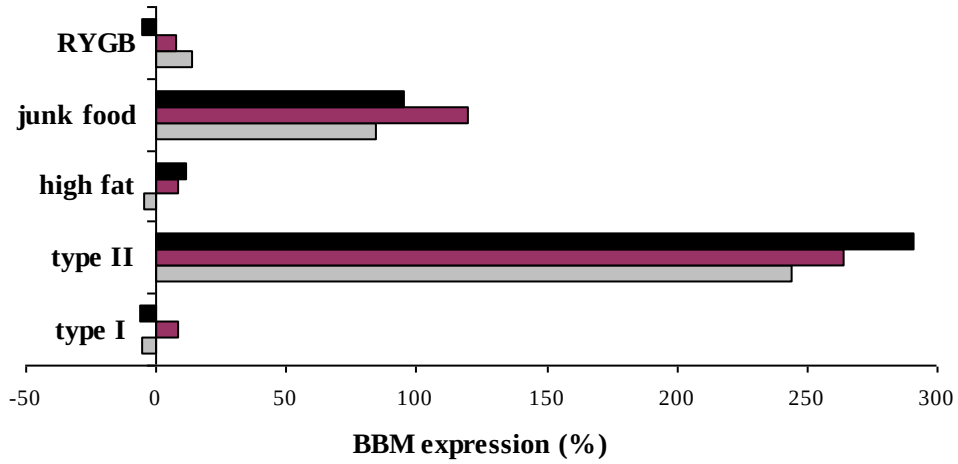




9.3.7 Relationship between T1R2 and T1R3, and SGLT1 expression at the proximal tubule BBM

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.

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

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.

	T1R2	T1R3	SGLT1
Type I diabetes (7 day)	↔	↔	↔
Type II diabetes	↑	↑	↑
Junk-food diet	↑	↑	↑
High-fat diet*	↔	↔	↔
RYGB*	↔	↔	↔

The heterodimer of T1R2 and T1R3 is a broadly-acting sweet taste receptor that acts through α-gustducin, to activate adenylyl 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 G_{olf} 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-PK_1 cells. Interestingly, the cell line originates from pigs which exhibit close homology to humans (Valentin *et al.*,

1999) 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 hypogeusia, 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

(Moestrup and Verroust, 2001;Wolf *et al.*, 2002). 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 blood stream 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 G_s-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/IP_3/Ca^{2+} 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.

9.4.5 Artificial sweeteners

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 dissacharide 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_{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 moduliating 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-βI-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-PK_1 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-Lily 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

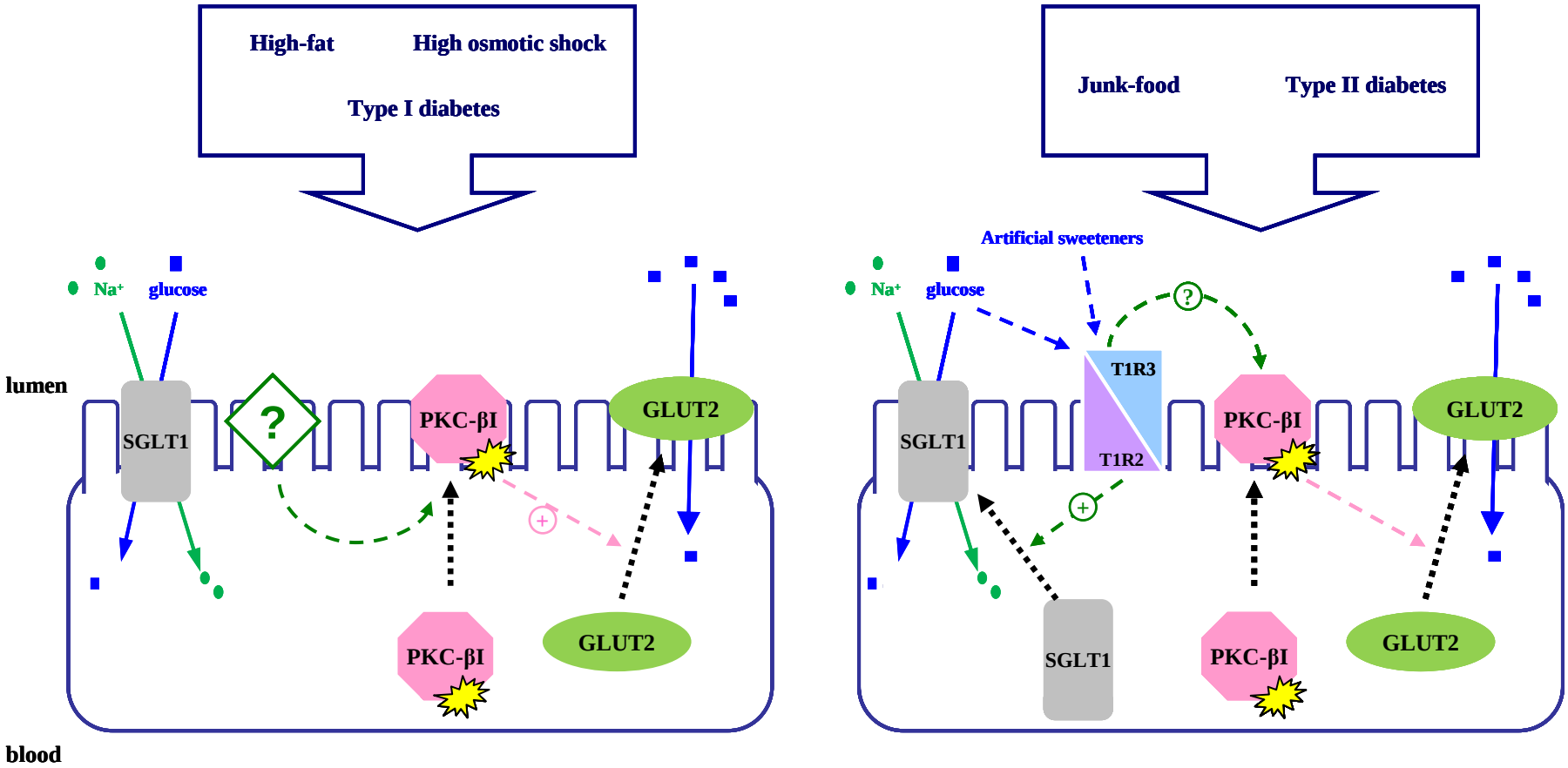
occurs in the kidney. In the studies presented in this thesis, components of the sweet taste heterodimer sweet taste receptors T1R2 and T1R3 were identified in the kidney. Although the localisation of these sweet taste receptors has not been studied, the presence of mRNA in the kidney cortex and protein 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_{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_{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 concommitantly with up-regulated SGLT1 and T1R2/3, in response to a junk-food diet and type II diabetes



A: T1R2/3 and SGLT1 independent pathway

B: T1R2/3 and SGLT1 dependent pathway

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.

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