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DISSERTATION

Glucose transport at the renal brush border membrane: GLUT2 and the involvement of PKC-βI

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Abstract

The facilitated glucose transporter GLUT2 is the main renal glucose transporter upregulated by hyperglycaemia when it becomes detectable at the brush border membrane (BBM). Previous studies in the laboratory of Dr Debnam have reported increased GLUT2 expression at the brush border membrane (BBM) of animals with streptozotocin (STZ)-induced Type 1 diabetes (Marks, Carvou et al. 2003).

In the present study it was the aim to establish whether subtle changes in ambient glucose concentrations influence GLUT2 expression at the BBM. Administration of nicotinamide to STZ-injected animals has been shown to partially abolish the effect of STZ on plasma glucose concentrations. Therefore, I modulated plasma glucose concentrations using various doses of nicotinamide in STZ-diabetic animals and measured GLUT2 expression at the BBM by Western blotting. Since glucose-induced PKC activation in the kidney is linked to diabetic nephropathy, I also investigated the effect of glycaemic status on the protein levels of PKC isoforms α, βI, βII, δ and ε at the proximal tubule, and their relationship to changes in GLUT2 protein at the BBM.

The results from this study demonstrate that hyperglycaemia significantly increases the protein levels of GLUT2 and PKC-βI at the BBM. The levels of both GLUT2 and PKC-βI correlated positively with plasma glucose concentration. Hyperglycaemia had no effect on BBM levels of α, βII, δ and ε isoforms of PKC. Thus, altered levels of GLUT2 and PKC-βI protein at the BBM may be important factors in the pathogenic processes underlying diabetic renal injury.

Zusammenfassung

Der Glukosetransporter GLUT2, der bei Hyperglykämie an der Bürstensaummembran (BSM) des proximalen Tubulus der Niere heraufreguliert wird, ist einer der wichtigsten Glukosetransporter der Niere. Studien im Labor von Dr. Debnam haben eine erhöhte Expression von GLUT2 an der BSM des proximalen Nierentubulus bei Tieren eines Streptozotocin (STZ)-induzierten Diabetes Typ 1 Tiermodells gezeigt (Marks, Carvou et al, 2003).

In der vorliegenden Arbeit war es mein Ziel, herauszufinden, ob bereits geringe Veränderungen der Glukosekonzentration die Expression von GLUT2 an der BSM beeinflussen. In Studien konnte gezeigt werden, dass der Blutzucker-steigernde Effekt des injizierten STZ bei Versuchstieren durch das vorherige Verabreichen von Nikotinamid teilweise wieder aufgehoben werden kann. Ich habe daher die Blutzucker-Konzentration bei Tieren eines STZ-induzierten Diabetes Tiermodells verändert, indem ich unterschiedliche Dosen von Nikotinamid verabreicht habe. Anschliessend wurde die Expression von GLUT2 an der BSM des proximalen Tubulus der Niere durch Western Blotting ermittelt. Da ein Zusammenhang zwischen der Aktivierung von Proteinkinase C durch Glukose und diabetischer Nephropathie existiert, habe ich zusätzlich den Effekt von Hyperglykämie auf die Expression der PKC Isoformen α, βI, βII, δ und ε am proximalen Nierentubulus bestimmt. Anschliessend wurde untersucht, ob ein möglicher Zusammenhang zwischen der Expression von PKC und der GLUT2 Expression existiert.

Die Ergebnisse der vorliegenden Arbeit zeigen, dass Hyperglykämie signifikant die Expression von GLUT2 und PKC-βI an der BSM des proximalen Nierentubulus steigert. Des weiteren konnte eine positive Korrelation zwischen GLUT2 und der Blutzucker-Konzentration sowie PKC-βI und der Blutzucker-Konzentration nachgewiesen werden. Hyperglykämie hatte keinen signifikanten Effekt auf die PKC Isoformen α, βII, δ und ε an der BSM des proximalen Tubulus. Die vorliegenden Ergebnisse zeigen, dass die Veränderungen der Expression von GLUT2 und PKC-βI an der BSM möglicherweise wichtige Faktoren in der Pathogenese der diabetischen Nephropathie darstellen.

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Abbreviation List

ADVANCE = Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation

AGE = advanced glycation end products

ADP = adenosine diphosphate

BBM = brush border membrane

BLM = basoleteral membrane

cAMP = cyclic adenosine monophosphate

DAG = diacylglycerol

db/db mouse = model of type 2 diabetes

DCCT = Diabetes Control and Complications Trial

DM = diabetes mellitus

ECM = extracellular matrix

EGTA = glycol-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid

ERK = extracellular signal-regulated kinase

ESRD = end stage renal disease

mGDH = FAD-glycerophosphate dehydrogenase

GFR = glomerular filtration rate

GK rats = Goto-Kakizaki rats

GLUT = facilitative glucose transporter

H_2O_2 = Hydrogen peroxide

IDDM = insulin dependant diabetes mellitus

i.p. = intraperitoneally

K_m = Michaelis constant, substrate concentration at half V_{max}

NAD^+ = nicotinamide adenine dinucleotide

NADH = nicotinamide adenine dinucleotide, reduced form

NEFA = non-esterified fatty acids

NIDDM = non insulin dependant diabetes mellitus

NO = nitric oxide

O_2 = Superoxide

OLETF = Otsuka Long-Evans Tokushima Fatty (rats)

PCT = proximal convoluted tubule

PBS = phosphate buffered saline (pH7.4)

PBS-T = phosphate buffered saline (pH7.4) containing 0.1% Tween 20

PKC = protein kinase C

PKA = protein kinase A

PLA_2 = phospholipase A_2

PLC = phospholipase C

PMA = phorbol 12-myristate 13-acetate

RBX = ruboxistaurin

mRNA = messenger ribonucleic acid

ROS = reactive oxygen species

rpm = rotations per minute

SDH = sorbitol dehydrogenase

SDS = sodium dodecyl sulphate

SGLT = sodium glucose linked transporter

SEM = standard error of the mean

STZ = streptozotocin

T1R = gene of taste receptor family

TGF-β = transforming growth factor-β

UKPDS = United Kingdom Prospective Diabetes Study

VEGF = vascular endothelial growth factor

V_{max} = maximum transport capacity

Chapter 1:
Background

1.1 Introduction

Our kidneys play a major role in the maintenance of glucose homoeostasis. Under normal conditions, glucose in the blood is completely filtered and reabsorbed. However, under conditions of hyperglycaemia, the renal absorptive mechanism is saturated and glucose appears in the urine. It has been hypothesised that in poorly controlled diabetes, the high concentrations of glucose filtered by the kidneys induce changes in tubular glucose transport, which may contribute to the pathophysiological aspects associated with diabetic nephropathy.

1.2 Mechanisms of epithelial glucose transport: SGLT and facilitative glucose transport

The conventional model of renal and intestinal glucose transport displays striking similarities. Glucose moves across the proximal tubule cell and enterocyte via specific glucose transporters. A Na^+-dependent glucose transporter (SGLT), which is located at the brush border membrane (BBM), couples glucose transport to the inwardly directed Na^+ electrochemical gradient. This gradient is maintained by the basolaterally located Na^+/K^+-ATPase. GLUT5 is also expressed at the BBM, where its primary function is fructose transport. The exit of glucose from the cell occurs via facilitative diffusion across the basolateral membrane and is mediated by the glucose transporter isoforms, GLUT1 and GLUT2 (Debnam and Unwin 1996; Thorens 1996) (Fig. 1.1).

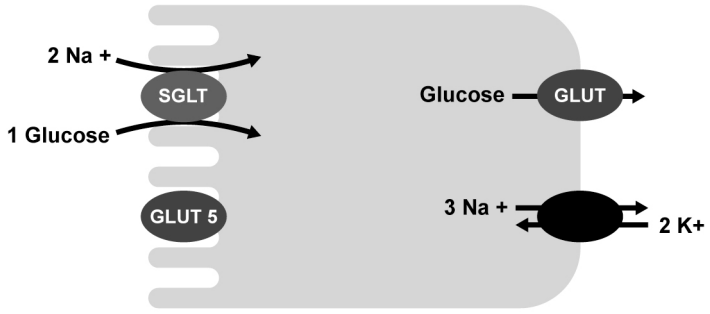

Figure 1.1: conventional model of glucose transport in the renal proximal tubule and small intestine

In the small intestine there is only one isoform of SGLT present at the BBM, which is SGLT1. At the basolateral membrane the efflux occurs via the facilitative glucose transporters GLUT1 and GLUT2. In contrast, in the kidney both active and facilitative glucose transporters have distinct distribution profiles along the proximal tubule that relate to their specific kinetic characteristics (Dominguez, Camp et al. 1992). This provides a proximal tubule environment in which the bulk of filtered glucose is reabsorbed in the early S1 segment by the low affinity/high capacity glucose transporters, SGLT2 (Lee, Kanai et al. 1994) and GLUT2 (Chin, Zhou et al. 1993); whereas the high affinity/low capacity transporters, SGLT1, (Kanai, Lee et al. 1994) and GLUT1 (Chin, Zhou et al. 1993), scavenge the remaining glucose that is presented to the distal regions of the proximal tubule (Fig. 1.2).

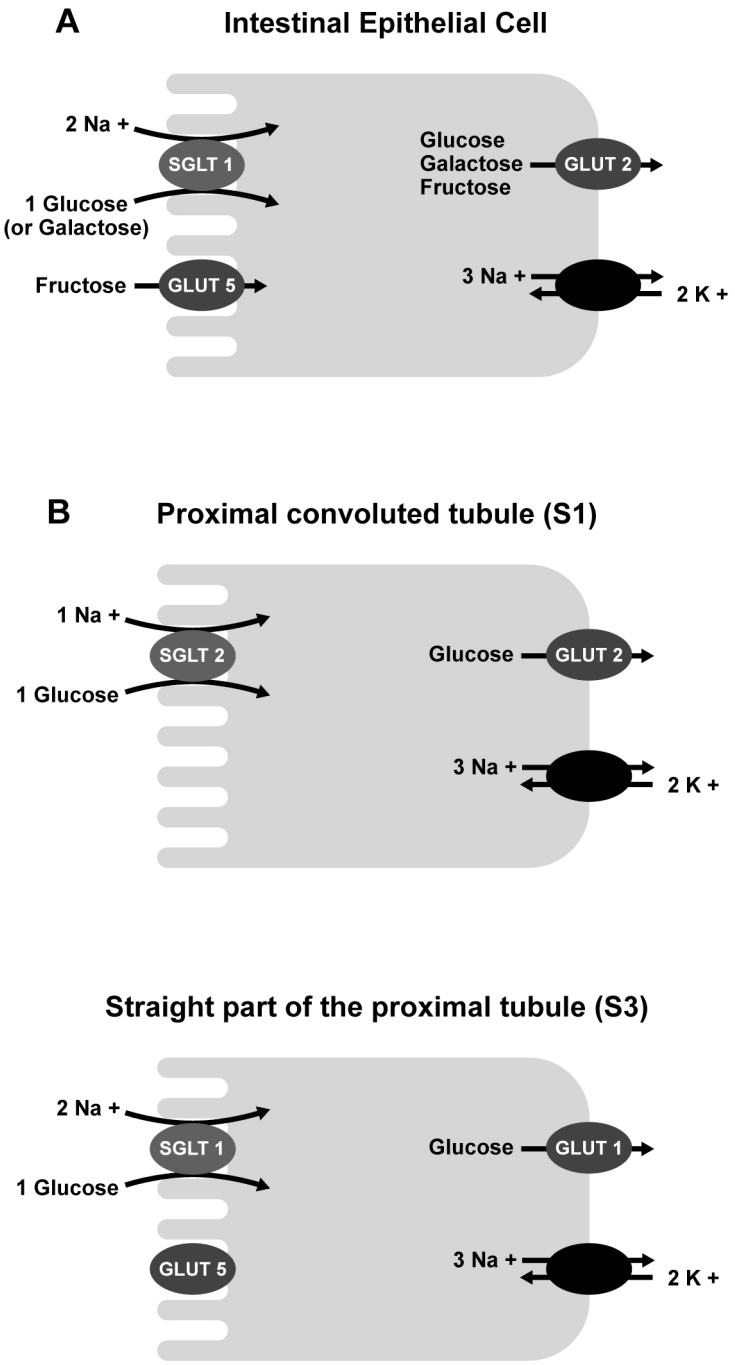

Figure 1.2: glucose transport in the small intestine and different regions of the renal proximal tubule

The classic model of intestinal glucose transport was challenged in 2000 by Kellett *et al.* who demonstrated that GLUT2 contributes significantly to the uptake of sugars across the BBM during assimilation of a meal and that GLUT2 levels are lower during periods of fasting. At low glucose concentrations, SGLT1 is the primary route of glucose absorption at the apical membrane. However, under conditions of high luminal glucose concentrations SGLT1 becomes saturated and promotes GLUT2 recruitment to the apical membrane where it can scavenge more glucose from the lumen (Kellett and Helliwell 2000; Kellett 2001).

Recent studies have shown two mechanisms by which GLUT2 is recruited to the apical membrane of enterocytes. First, saturation of SGLT under conditions of high luminal glucose depolarises the BBM, which induces a rapid influx of luminal calcium ions (Ca^{2+}) through the apical voltage-dependant L-type calcium channel, Ca_v 1.3. The increase in luminal Ca^{2+} concentration activates protein kinase C-βII (PKC-βII) and causes phosphorylation of myosin II in the terminal web. This, in turn, leads to movement of the intracellular GLUT2-containing storage vesicles to the apical membrane via cytoskeletal rearrangement within the cell (Mace, Morgan et al. 2007; Morgan, Mace et al. 2007). Second, GLUT2 recruitment to the apical membrane is mediated by a sweet taste receptor which is located at the apical membrane. This receptor is controlled by the G-protein coupled receptor T1R taste receptor family. The heterodimer T1R2+T1R3 senses simple sugars (glucose, fructose, sucrose). At high luminal sugar concentrations the receptor activates the PLC-β2-dependent pathway via α-gustducin to stimulate PKC-βII (Le Gall, Tobin et al. 2007; Mace, Lister et al. 2009) (Fig. 1.3). A similar mechanism occurs for the amino acids; L-glutamate acts via apical amino acid receptors which are controlled by T1R1+T1R3/α-gustducin to regulate the H+/oligopeptide transporter PepT1 via activation of PKC-βII. In summary, this suggests the existence of a wider transport network which acts via stimulation of a common enterocytic pool of PKC-βII to control nutrient absorption (Mace, Lister et al. 2009).

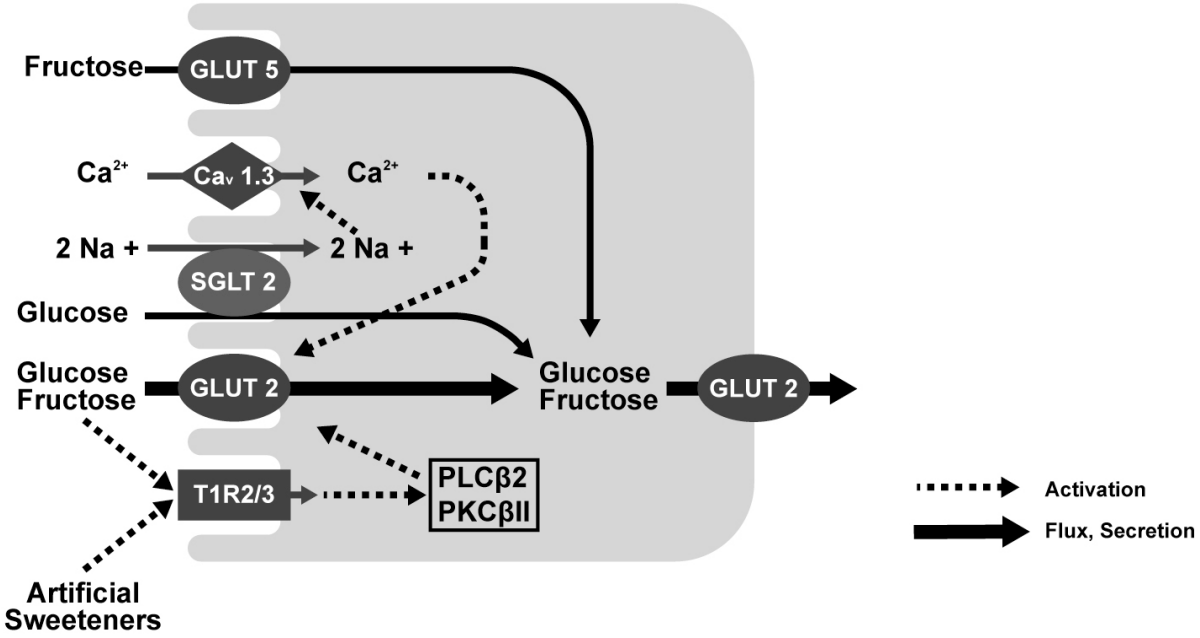

Figure 1.3: Regulation of apical GLUT2 in enterocytes by calcium and taste receptors

There is less evidence to support a similar process occurring in the renal proximal tubule BBM, but because the basic glucose transport mechanisms are similar in both tissues, it seems likely that a similar model may apply. Indeed, it has been demonstrated that GLUT2 is translocated to the proximal tubule BBM in a model of STZ-induced diabetes. Overnight fasting, which reduces plasma glucose concentrations to normal levels, abolishes this pattern of expression (Marks, Carvou et al. 2003) (Fig. 1.4).

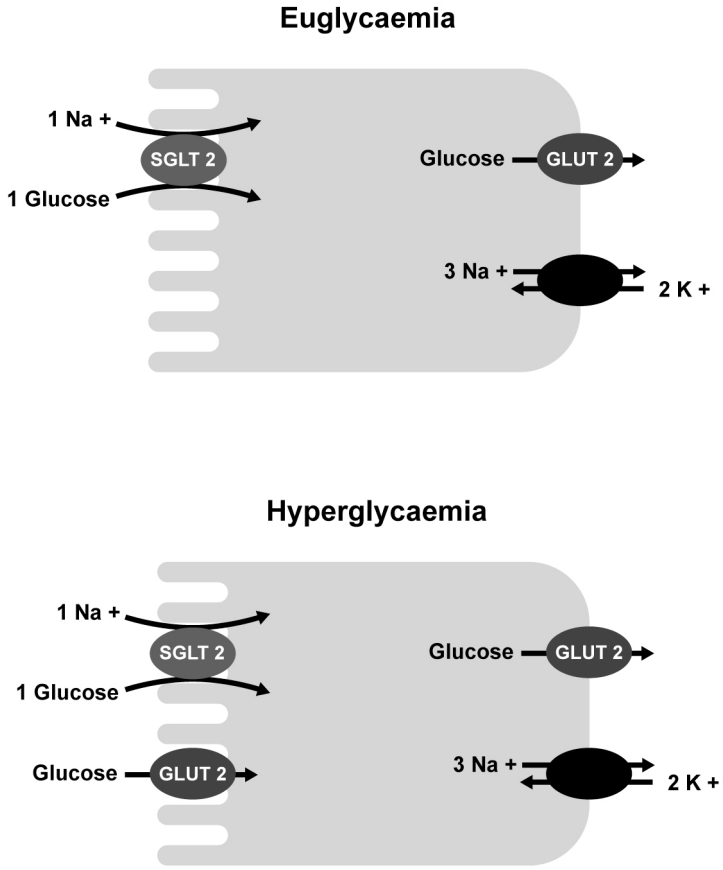

Figure 1.4: Regulation of apical GLUT2 at the renal proximal tubule BBM

There are 14 different facilitative glucose transporters, most of which function to allow glucose to diffuse down its concentration gradient across the plasma membrane. These transporters have different profiles of expression not only in the different tissues of the body but also in the different parts of the kidney. They are divided into 3 families or subclasses (class I-III); GLUT2 is a member of class I (Manolescu, Witkowska et al. 2007).

There have recently been advances in evaluating the localization and role of new members of the GLUT family in renal glucose handling. It has been shown that GLUT8 is expressed in podocytes and tubular cells in the distal portion of the nephron; expression of this transporter is influenced by plasma glucose levels *in vivo* (Schiffer, Susztak et al. 2005). Furthermore, mouse GLUT9 splice variants have shown to be expressed in adult liver and the outer cortex of the kidney. Expression of GLUT9 is significantly increased in the kidney and liver from STZ-induced diabetic mice compared with nondiabetic animals (Keembiyehetty, Augustin et al. 2006). Another glucose transporter, GLUT12, has been detected in the distal tubules and collecting ducts of the kidney. GLUT12 expression in the liver and kidney has been shown to be significantly increased in Ren-2 diabetic rats, an animal model of diabetic nephropathy (Linden, DeHaan et al. 2006). These findings indicate that in addition to GLUT2 other GLUT transporters in the kidney may also be regulated by glucose.

1.3 Diabetes

Diabetes mellitus (DM) is a common chronic metabolic disease resulting from an absolute (type I) or relative (type II) lack of insulin. The disease is characterised by hyperglycaemia and the typical long-term complications in both forms of diabetes are diseases of the eye (retinopathy), nerves (neuropathy) and kidney (nephropathy).

Type I diabetes, formerly termed juvenile onset or insulin-dependent diabetes mellitus (IDDM), is characterised by an absolute lack of insulin. It predominantly occurs in children and young adults and accounts for about 5-10% of the cases of DM (Olefsky 2001). The basic cause of the insulin deficiency is an auto-immune destruction of the insulin producing

β-cells of the pancreas which ultimately leads to failure of the pancreas to secrete insulin. Genetic factors play a predisposing role in the genesis of the disease. Treatment of type I diabetes involves the administration of insulin by injection together with diet and exercise to aid glycaemic control.

Type II diabetes is the most common form of this disease accounting for 90-95% of all cases of DM and is characterised by a relative lack of insulin. Formerly known as adult onset or non insulin-dependent diabetes mellitus (NIDDM), it is mainly based upon insulin-resistance and disturbed insulin secretion. In type II diabetes many patients develop a "metabolic syndrome", which is defined by obesity, dyslipoproteinaemia, hypertension and dysfunction of glucose tolerance. Based on the severity of the metabolic syndrome the number of insulin receptors in the insulin-sensitive cells of the body is downregulated. This leads to reduced response to insulin even if it is abundant, a status called insulin resistance. Treatment of type II diabetes involves changes of dietary habits, weight loss and exercise. When dietary treatment has failed, oral hypoglycaemic drugs, such as sulphonamides or biguanides can be given. If therapy with oral hypoglycaemic drugs fails to normalise plasma glucose levels, the administration of insulin by injection is indicated.

Due to its complications, the rate of morbidity of patients with diabetes is significantly increased. For example, diabetic nephropathy accounts for 40% of all new cases of end-stage renal disease (ESRD) (Ritz and Orth 1999). Heart diseases and strokes occur 2-4 times more frequently in diabetic patients than in healthy individuals (Olefsky 2001).

The Diabetes Control and Complications Trial (DCCT, 1993) demonstrated that strict control of blood glucose levels prevents and retards the development of the typical diabetic complications mentioned above and has been confirmed by the United Kingdom Prospective Diabetes Study (UKPDS) Group (UKPDS, 1998) and the ADVANCE Trial (Patel, MacMahon et al. 2008). Hypertension is also known to be a major risk factor in the progression of diabetic complications. Various studies have demonstrated that stringent control of blood pressure is beneficial in decelarating the development and progression of

diabetic nephropathy and reduces the risk of coronary heart disease in diabetic patients (UKPDS, 1998).

1.4 Diabetic nephropathy

Diabetes is the most common cause of ESRD. Data suggest that the renal risk of developing ESRD is currently equal in the two types of diabetes. It has been reported that the time to proteinuria from the onset of diabetes and the time to ESRD from the onset of proteinuria were similar in type I and type II diabetes (Ritz and Stefanski 1996).

Approximately 20-30% of patients with type I diabetes will have microalbuminuria after a mean duration of diabetes of 15 years (Orchard, Dorman et al. 1990). The overall incidence of end-stage renal disease was reported to be 16 percent at 30 years from time of initial diagnosis (Krolewski, Eggers et al. 1996). However, in the last several decades, the renal prognosis of type I diabetes has dramatically improved mainly due to better glycaemic control, more aggressive blood pressure reduction and the use of angiotensin converting enzyme inhibitors (ACE inhibitors) (Bojestig, Arnqvist et al. 1994). In type II diabetes the prevalence of microalbuminuria at 10 years following diagnosis was 25% according to the United Kingdom Prospective Diabetes Study (UKPDS, 1998).

In patients with long-standing diabetes mellitus various pathologic abnormalities are noted before the onset of microalbuminuria. As glomerulopathy is the characteristic clinical and histological feature of diabetic renal disease, most studies have concentrated on the effect of high glucose concentrations on mesangial cells. In the glomerulus, there are three major histological changes that occur in diabetic nephropathy: mesangial expansion, glomerular basement membrane thickening and glomerular sclerosis (Adler 2004).

However, diabetes also increases overall kidney size. Since the tubulointerstitium comprises the bulk of the kidney, thickening of the tubular basement membrane and renal interstitium is quantitatively responsible for most of this renal enlargement leading to eventual cell loss and declining renal function (Nath 1992). Furthermore, hypertrophy of the

glomerulus and proximal tubule cells may contribute to the renal abnormalities that occur at the later stages of diabetes, such as glomerulosclerosis, tubular atrophy and interstitial fibrosis (Ziyadeh 1993). These occur partly because the network of cytokines and growth factors that induce cellular hypertrophy can also stimulate extracellular matrix synthesis and deposition (Wolf and Ziyadeh 1997).

Glomerulosclerosis, which is defined by fibrotic scarring of the glomerulus leading to proteinuria, is a consequence of the accumulation of fibronectin, laminin and collagen leading to mesangial matrix expansion and thickening of the glomerular basement membrane (Ziyadeh 1993). Tubulointerstitial fibrosis is characterised by the accumulation of extracellular matrix (ECM) proteins, such as fibronectin and collagen, within the tubular interstitium leading to tubulointerstitial scaring (Norman and Fine 1999).

It is now well established that hyperglycaemia is a key factor involved in the pathogenesis of diabetic nephropathy. Four major hypotheses as to how cellular damage occurs have been proposed (Fig. 1.5):

1. Formation of reactive oxygen species (ROS)
2. Increased activation of aldose reductase (polyol pathway)
3. Increased formation of advanced glycation end products (AGEs)
4. Activation of protein kinase C (PKC) isoforms

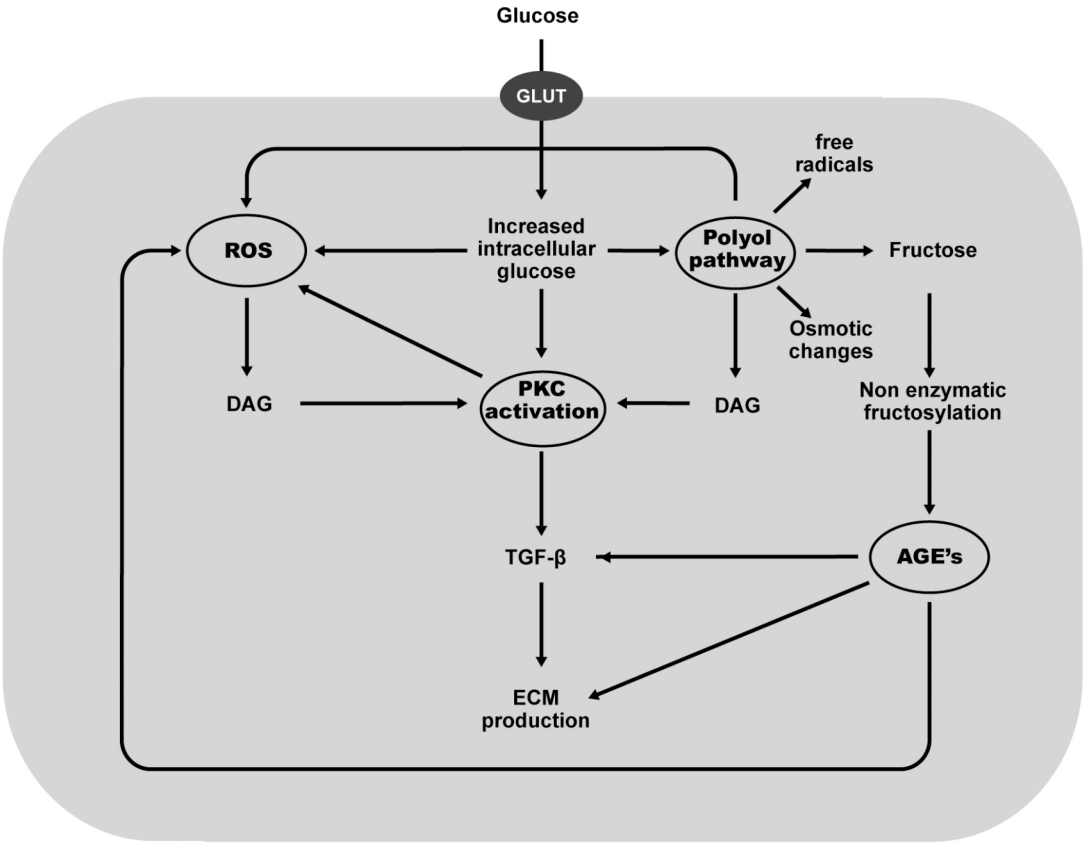

Figure 1.5: Potential mechanisms of cellular pathways of glucose-induced cellular damage

1. Reactive oxygen species (ROS) are ions that include oxygen ions, peroxides and free radicals. Free radicals can be produced by a number of factors. Many of them, like hydrogen peroxide (H_2O_2) or superoxide formation (O_2) are by-products of various metabolic processes, e.g. mitochondrial oxidation of NADH to NAD^+. Others are generated as secondary messengers during signal transduction. There have been a number of studies that support a role of ROS in the pathogenesis of diabetic nephropathy. Glomerular and tubule cells have been shown to generate ROS with the potential to cause oxidative stress (Baud, Fouqueray et al. 1992; Gwinner, Deters-Evers et al. 1998). Additionally, glucose induced synthesis of tumor growth factor β (TGF-β) and subsequent ECM

production can be suppressed by antioxidant administration, thereby preventing glomerular and tubular hypertrophy.

It appears that under conditions of hyperglycaemia, there is an interaction between PKC and ROS. PKC activation can enhance ROS production and ROS have also been shown to increase PKC activity, through increased production of diacylglycerol (DAG) (Nishikawa, Edelstein et al. 2000). Inhibition of PKC has been shown to block fibronectin accumulation in mesangial cells resulting from both H_2O_2 and high glucose environments (Ha and Lee 2000).

2. The metabolism of glucose by the polyol pathway consists of two reactions, using the enzymes aldose reductase and sorbitol dehydrogenase. The enzyme aldose reductase has a low affinity for glucose; therefore, at normal glucose concentrations this pathway only represents a small proportion of glucose metabolism. However, the involvement of this pathway during hyperglycaemia increases dramatically. Experiments using human proximal tubule cells exposed to 25 mM D-glucose have demonstrated increased glucose metabolism by the polyol pathway resulting in accumulation of intracellular sorbitol and production of fibronectin (Phillips, Morrisey et al. 1999).

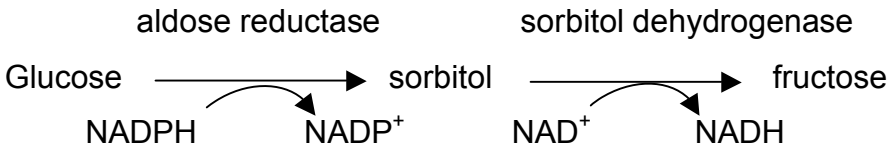

Fig. 1.6: The polyol pathway

Four mechanisms have been proposed by which increased glucose metabolism by the polyol pathway may contribute to the complications of diabetic nephropathy (Larkins and Dunlop 1992; Dunlop 2000):

-osmotic changes due to the accumulation of sorbitol
-PKC activation as a result of increased polyol pathway activity
-free radical production as a result of increased polyol pathway activity
-increased fructose formation due to polyol pathway activation

3. Glucose has been shown to form glycation products with extra and intracellular proteins to produce Schiff bases. This occurs by a non-enzymatic process whereby N-terminal amino acids are glycosylated. These Schiff bases can then spontaneously transform to form more stable Amadori products. These early glycation products can undergo an irreversible, complex series of biochemical events to form advanced glycation end products (AGEs).

It has been shown that the process of AGE accumulation is greatly accelerated under conditions of hyperglycaemia (Larkins and Dunlop 1992). The damage to the target cells is caused by three mechanisms:
- altered protein function via AGEs
- modifying extra cellular matrix (ECM) by AGE precursors
- binding of precursors to AGE receptors on macrophages, which in turn results in ROS production and subsequent release of cytokines and hormones

4. Activation of Protein kinase C (PKC) is another factor that is involved in the pathogenesis of diabetic nephropathy. In summary, in the setting of high glucose activation PKC has been shown to result in increased production of cytokines, extracellular matrix proteins and other factors that are involved in the pathophysiology of diabetic vascular complications (Meier, Menne et al. 2009).
In the following section, this is described in depth.

1.5 PKC isoforms and the involvement of PKC activation in diabetic nephropathy

PKC comprises a family of serine threonine kinases which can be divided into 3 subfamilies based on both sequence homology and mechanism of activation: conventional or classical PKCs (cPKCs: PKC-α, PKC-βI, PKC-βII, PKC -γ), novel PKCs (nPKCs: PKC-δ, PKC-ε, PKC-η and PKC-θ) and atypical PKCs (aPKCs: PKC-ζ and PKC-λ/ι) (Parker and Murray-Rust 2004). Under normal cellular conditions activation of PKC plays an important role in transducing biological events including cell growth, differentiation, apoptosis and cellular responses to environmental stress. Diacylglycerol (DAG), which is synthesised under conditions of hyperglycaemia, is the main stimulus of PKC activation. The cPKCs and nPKCs are DAG sensitive, whilst the aPKCs are not sensitive to activation by DAG. Other stimulants of PKC are vasoactive peptides (angiotensin II, vascular endothelial growth factor (VEGF), endothelin), phorbol esters, phosphatidylserine and non-esterified fatty acids (NEFAs) (Meier, Menne et al. 2009).

The direct synthesis of DAG has been demonstrated in a variety of tissue including glomeruli isolated from diabetic rats (Craven and DeRubertis 1989). DAG is the regulatory stimulator of PKC and its elevation is the presumed mechanism for the increased PKC activity observed in several tissues obtained from diabetic animals (Larkins and Dunlop 1992). In elucidating the cellular mechanisms involved in PKC activation, Haneda *et al.* have demonstrated *in vivo* and *in vitro* that the DAG-PKC-ERK (extracellular regulatory protein kinase) pathway is involved in the cellular damage that occurs during hyperglycaemic conditions (Haneda, Koya et al. 2001). Incubation of mesangial cells under conditions of high glucose in the presence of PD98059, a specific inhibitor of MAP/ERK kinase (MEK), which is an upstream kinase activator of ERK, has been shown to inhibit the glucose induced production of TGF-βI and the ECM proteins collagen and fibronectin (Isono, Cruz et al. 2000). Thiazolidinedione compounds, such as troglitazone and pioglitazone (oral hypoglycaemic drugs), administered to STZ-diabetic animals prevented the activation of PKC by reducing DAG content (Haneda, Koya et al. 2001). This reduction in the cellular content of DAG prevents the activation of PKC and ERK. Furthermore, these compounds prevent glomerular hyperfiltration and normalise gene expression of TGF-β

mRNA and that of ECM proteins (Isshiki, Haneda et al. 2000). Furthermore, administration of LY333531, a PKC-inhibitor, to db/db mice (model of type II diabetes) resulted in a decrease in PKC-β activation and, using histological methods, was shown to reduce fractional mesangial volume (Koya, Haneda et al. 2000).

Previous studies have shown that many PKC isoforms are expressed in rat and mouse kidneys. Immunohistochemistry has localized different isoforms to specific nephron segments and there are also distinct species differences. In the proximal tubule of rat kidney the PKC isoforms α, βI, βII, δ and ε have been detected (Karim, Defontaine et al. 1995; Kang, Alexander et al. 1999), with prominent staining for PKC-α (Dong, Stevens et al. 1991; Pfaff, Wagner et al. 1999) and -βI (Pfaff, Wagner et al. 1999) at the BBM. In contrast, PKC-α and -β isoforms are undetectable in mouse proximal tubules with PKC-ε being the predominant isoform in this species (Redling, Pfaff et al. 2004). Studies have also spotted altered levels of PKC isoforms in proximal tubule cells following exposure to high glucose concentrations: specifically, PKC-α, -ε (Kang, Alexander et al. 1999) and -βI (Hsieh, Chen et al. 2006) are increased. These findings suggest that diabetes-induced alterations in the level and activity of PKC isoforms may affect proximal tubular function, as well as having their already established effects in glomerular cells.

PKC activation has been shown to occur via a number of pathways. Activation can occur as a consequence of the polyol pathway, whereby inhibition of aldose reductase *in vitro* has been demonstrated to reduce cellular DAG content and inhibit the translocation of PKC-δ and -ε (Kapor-Drezgic, Zhou et al. 1999). There is increasing evidence that ROS can directly and indirectly activate PKC during conditions of hyperglycaemia (Ha and Kim 1999). Ha and co-workers have demonstrated that the antioxidant taurine can inhibit the alterations in PKC-δ and -ε in cortical tubules and glomeruli isolated from STZ-diabetic animals (Ha, Yu et al. 2001). Indirect activation of PKC-β, -δ, -ε, and -ζ has been shown to occur by activation of vasoactive peptides, specifically angiotensin II and endothelin 1 (Whiteside and Dlugosz 2002). Proteins rich in AGEs have also been shown to selectively activate mesangial cell PKC-βII through a mechanism involving oxidative stress without the involvement of DAG (Scivittaro, Ganz et al. 2000). In this context glycated albumin

stimulates PKC-β, which leads to an increase in collagen IV protein expression (Cohen, Ziyadeh et al. 1999).

1.6 Effect of high glucose concentrations on epithelial transport and PKC

The effects of streptozotocin (STZ)-induced diabetes, a model of type I diabetes, on intestinal glucose transport have been extensively studied. It has been shown that chronic STZ treatment results in the induction of SGLT transporter mRNA and protein (Debnam, Smith et al. 1995) in the enterocyte. Additionally, in enterocytes diabetes increases levels of GLUT1, GLUT2 (Corpe, Basaleh et al. 1996) and GLUT5 (Corpe, Basaleh et al. 1996) at the BBM. Taken together, these enterocyte adaptions during diabetes provide a situation that allows enhanced glucose absorption. Furthermore, it has been demonstrated that rapid insertion of GLUT2 into the BBM occurs via luminal glucose-induced activation of apical Ca^{2+} channels and taste receptors which in turn activate PKC-βII from an intracellular pool (Mace, Morgan et al. 2007; Morgan, Mace et al. 2007; Kellett, Brot-Laroche et al. 2008). Since the process of glucose transfer across the enterocyte and proximal tubule cell displays striking similarities, insights into the diabetes-induced and luminal glucose-induced changes in intestinal glucose transport may be of relevance to the understanding of the effects of the disease on renal glucose transport.

Since the initial pathological changes of diabetic nephropathy occur in the mesangium of the kidney, the attention has been focused on changes in the mesangial cell glucose transporter GLUT1. Evidence suggests that transport of glucose into the cells by this transporter regulates the activity of the downstream pathways that culminate in cellular damage. It has been shown that an increase in GLUT1 protein expression and glucose uptake increases cellular sorbitol content and collagen, fibronectin and laminin synthesis (Heilig, Concepcion et al. 1995). Furthermore, cells overexpressing GLUT1 have been shown to display elevated levels of native GLUT1 transcripts, aldose reductase and PKC protein (Henry, Busik et al. 1999). From these data it was hypothesised that a positive

feedback mechanism exists which is pathological during hyperglycaemia and predisposes the mesangial cells to glucose induced damage. Thus, it has been hypothesised that increased GLUT1 expression leads to enhanced polyol pathway activity and production of PKC-βI, which in turn enhances the synthesis of fibronectin and other ECM proteins (Brosius and Heilig 2005).

The effect of STZ-induced diabetes on sodium-dependant glucose transport of the proximal tubule has yielded conflicting results. Reports have shown both increased (Blank, Bode et al. 1985) and decreased (Harris, Brenner et al. 1986; Yasuda, Kurokawa et al. 1990) SGLT-mediated glucose transport. Even quantification of SGLT mRNA and protein levels has proved inconclusive, with levels reported to decrease (Yasuda, Kurokawa et al. 1990), increase (Vestri, Okamoto et al. 2001) and remain unchanged (Dominguez, Song et al. 1994). In the kidney, it has been hypothesised that expression of the sodium-dependent glucose transport, SGLT2, at the BBM of the proximal convoluted tubule (PCT) quantitatively reabsorbs the bulk of the glucose filtered by the glomerulus (Kanai, Lee et al. 1994). Levels of SGLT2 mRNA have been reported to increase during experimentally induced diabetes (Vestri, Okamoto et al. 2001).

In contrast, the effect of STZ-induced diabetes on facilitative glucose transporters seems to be more consistent. Levels of GLUT5 protein are increased at the BBM, which is accompanied by corresponding increases in mRNA expression levels (Asada, Ogawa et al. 1997; Chin, Zamah et al. 1997). It has been consistently demonstrated that levels of GLUT2 protein (Dominguez, Camp et al. 1992; Asada, Ogawa et al. 1997) and mRNA (Chin, Zamah et al. 1997; Vestri, Okamoto et al. 2001) undergo the greatest degree of adaptation during experimental diabetes, with increases of this transporter being linked to the maintenance of increased glucose efflux in the presence of hyperglycaemia (Dominguez, Song et al. 1994). Whilst all these studies were carried out on whole cells of the proximal tubule, in recent studies the distinct expression levels of GLUT2 at the basolateral membrane and BBM were studied. It was shown that STZ-induced diabetes causes the insertion of GLUT2 into the BBM providing a low affinity/high capacity route of entry into the proximal tubule cells during hyperglycaemia. The effect of increased GLUT2

expression under conditions of hyperglycaemia was abolished by overnight fasting (Marks, Carvou et al. 2003).

Interestingly, levels of tubular GLUT1 protein and its mRNA have been shown to decrease in diabetes (Chin, Zamah et al. 1997; Vestri, Okamoto et al. 2001). This adaptation in tubular epithelial cells appears to contrast with the effects of diabetes on mesangial cell expression of GLUT1, where increased expression of GLUT1 has been strongly correlated to hyperglycaemia induced mesangial cell damage (Heilig, Liu et al. 1997; Mogyorosi and Ziyadeh 1999).

1.7 Aims of the thesis

It is evident from the literature that there is a direct correlation between hyperglycaemia and the progression of diabetic nephropathy. Experiments focusing on mesangial cells have linked overexpression of the facilitative glucose transporter, GLUT1, to the hyperglycaemia-induced damage that occurs in this cell type. It has been hypothesised that this process is triggered via production of PKC-βI, which in turn enhances the synthesis of fibronectin and other ECM proteins (Brosius and Heilig 2005).

Additionally, in the small intestine, recent studies have shown that a high level of luminal glucose leads to rapid insertion of GLUT2 into the BBM via activation of apical Ca^{2+} channels and taste receptors which in turn activate PKC-βII (Kellett, Brot-Laroche et al. 2008).

Therefore, since GLUT1 and PKC-βI may influence mesangial cell size in diabetes and PKC-βII regulates GLUT2 protein levels at the enterocyte BBM, I have investigated the relationship between changes in circulating glucose concentrations, protein levels of PKC-βI and PKC-βII, and other PKC isoforms, together with GLUT2 at the proximal tubule BBM.

Chapter 2:

Changes in plasma glucose levels and GLUT2 expression at the proximal tubule brush border membrane (BBM)

2.1 Introduction

In the classic model of glucose transport it is assumed that GLUT2 only occurs at the basolateral membrane (Dominguez, Camp et al. 1992) (chapter 1 Fig.1.1). However, numerous studies have provided convincing evidence that GLUT2 has a functional role at the BBM of enterocytes (Kellett and Helliwell 2000; Morgan, Mace et al. 2007). In the proximal tubule the basic mechanisms of glucose transport are similar to the enterocyte. However, re-evaluating glucose transport at the proximal tubule has received relatively little attention. It has been demonstrated that GLUT2 is translocated to the proximal tubule BBM in STZ-induced diabetes. Overnight fasting, which reduces plasma glucose concentrations, abolishes this pattern of expression (Marks, Carvou et al. 2003).

The importance of understanding how diabetes affects renal glucose handling is evident from the observation that renal glucose uptake plays a key role in reducing plasma glucose during hyperglycaemia (Cersosimo, Ajmal et al. 1997). In addition, since plasma glucose concentration can influence glucose handling and utilization by the kidney, changes in glucose transport in diabetes may lead to tubule cell injury and associated renal interstitial changes (Larkins and Dunlop 1992). In this context, it has also been shown that hyperglycaemia increases GLUT1 expression in mesangial cells, which has been linked to increased polyol pathway activity and production of PKC-βI, and this interaction may contribute to the development of diabetic nephropathy (Brosius and Heilig, 2005).

Previous studies have concentrated on GLUT2 expression in STZ-induced diabetes, a model of type I diabetes. However, effects on glucose transport have been hypothesised to be linked to blood glucose levels *per se*. Thus, it was our aim to establish whether a wide range of glucose concentrations has an effect on GLUT2 expression at the BBM of the proximal tubule. In this chapter the effects of different plasma glucose levels on GLUT2 expression at the BBM of the proximal tubule was measured using nicotinamide and STZ to modulate glucose concentration.

2.2 Methods

2.2.1 Induction of hyperglycaemia

Different levels of hyperglycaemia were induced in 230-260g male Sprague-Dawley rats according to the protocol of Masiello *et al.* (Masiello, Broca et al. 1998). Various doses of nicotinamide were dissolved in 0.9% saline and injected intraperitoneally (i.p). After 15 min., STZ (60 mg/kg dissolved in 0.05 M citrate buffer, pH 4.5) was administered via a tail vein injection under light isoflurane anaesthesia. Animals were divided into three groups representing different levels of hyperglycaemia. The first group received a single dose of STZ (60 mg/kg) without any nicotinamide treatment (STZ group). The second group was given an i.p. injection of 50 mg/kg nicotinamide followed by administration of STZ (50 mg/kg group), whereas the third group was administered 200 mg/kg nicotinamide i.p. followed by STZ injection (200 mg/kg group). Weight-matched animals without having received any treatment were used as controls (control group). Animals were allowed ad libitum access to food (standard rat chow; Diet RM1, SDS Ltd., Essex, UK) and water up to the time of experimentation, with exception of those subjected to an overnight fast, where the food source was removed 16 hours prior to experimentation. Sacrifice of the animals was undertaken two weeks after the injection of nicotinamide and STZ. The animals were terminally anaesthetised with i.p. pentobarbitone sodium (60 mg/kg; Pentoject, Animalcare Ltd., York, UK). Blood for analysis of blood glucose was taken via cardiac puncture and kidneys were removed and placed in ice-cold 154 mM NaCl. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986.

2.2.2 Brush border membrane (BBM) vesicle preparation

Two weeks after drug administration the kidneys were excised and transferred into ice-cold 154 mM NaCl. Individually they were placed onto a cold glass surface, where the surrounding fat was removed and capsules were peeled off; kidney weights were then recorded. Each kidney was cut horizontally into 2 mm slices and the cortex was carefully

dissected away. The cortical fragments of both kidneys per animal were then used for the preparation of BBM vesicles.

BBM vesicles were prepared following the method of Biber *et al.* (Biber, Stieger et al. 1981), with all steps carried out at 4ºC. The cortical fragments were added to 30 ml of R1 (resuspension 1) buffer containing 300 mM Mannitol, 12 mM Tris-HCL and 5 mM ethylene glycol-bis(2-aminoethyl)-N,N,N´,N´-tetraacetic acid (EGTA) at the pH of 7.4 and homogenised for 2 minutes using an Ultra Turrax homogeniser (Janke & Kunkel, FRG) at half speed. Afterwards, 10 ml of ice cold deionised water was added, followed by $MgCl_2$ to a concentration of 12 mM. The solution was stirred on ice for 15 minutes and centrifuged at 4500 x g for 15 min. The supernatant was re-centrifuged at 16,000 x g for 30 min. The resulting pellet was re-suspended in 20 ml R2 (resuspension 2) buffer containing 150 mM Mannitol, 6 mM Tris-HCL and 2.5 mM EGTA (pH 7.4), using 12 cycles of a hand-operated glass-Teflon homogeniser. $MgCl_2$ was added to a concentration of 12 mM and the solution was stirred on ice for 15 min. Afterwards, it was centrifuged again at 4500 x g for 15 min and the supernatant re-centrifuged at 16,000 x g for 30 min. The pellet was re-suspended in 20 ml R3 (resuspension 3) buffer, containing 300 mM Mannitol, 12 mM Tris-HCL and 2.5 mM EGTA and centrifuged 16,000 x g for 30 min. The resulting purified BBM pellet was finally re-suspended in approximately 0.5 ml R3 buffer (resulting protein concentration: 3-6 mg/ml) using 6 passes through a syringe fitted with a 21-gauge-needle.

After determination of the protein concentration and alkaline phosphatase activity the BBM vesicles were frozen and stored at -80ºC.

2.2.3 Analysis of plasma glucose levels

Blood obtained by cardiac puncture was placed into heparinised tubes containing 7500 Kallikrein Inactivator units (KIU) of the broad range protease inhibitor Aprotinin. It was then centrifuged for 10 minutes at 13000 rpm. The resulting plasma was frozen and stored at -80ºC until use. Plasma glucose levels were measured using the glucose oxidase method (assay kit no. 510, Sigma, Dorset, UK).

The principle of this assay relies on the conversion of glucose to gluconic acid and hydrogen peroxide (H_2O_2) by the enzyme glucose oxidase. The generated H_2O_2 is used for the oxidation of o-dianisidine by the enzyme peroxidase. Oxidation of o-dianisidine to oxidised o-dianisidine is accompanied by a colour change (colourless to brown). The colour change, which is proportional to the glucose concentration of the examined sample, is measured at 450nm, using a Du 650 spectrophotometer (Beckman-Coulter Bioresearch, Buckinghamshire, UK) (Fig.2.1).

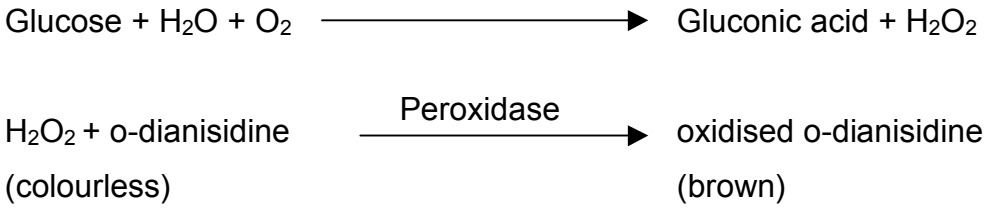

Fig. 2.1: Principle of plasma glucose assay

2.2.4 Validation of BBM vesicle purity

During the process of BBM preparation, it is inevitable that contamination with BLM and membranes of cellular organelles occurs (Murer and Gmaj 1986). A number of marker enzymes can be used to establish the degree of contamination of the BBM vesicles with membranes from other cellular locations. Amongst others, alkaline phosphatase is commonly used as a marker for the BBM (Biber, Stieger et al. 1981). In the experiments the fold-enrichment of the BBM marker enzyme alkaline phosphatase was derived from the activity of the enzyme in the initial homogenate and final BBM vesicle preparation using the method of Forstner *et al*. (Forstner, Sabesin et al. 1968).

The principle of this assay relies on the cleavage of phosphate (Pi) from the substrate, p-nitrophenol-phosphate, under alkaline conditions. This produces nitrophenol, which appears yellow and can be detected at 450 nm (Fig. 2.2)

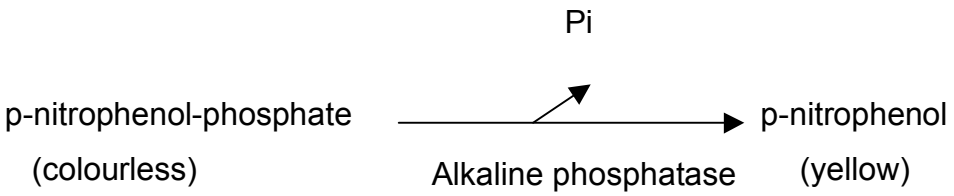

Fig. 2.2: Principle of alkaline phosphatase assay

2.2.5 Protein concentration

To measure the protein concentration of the initial homogenate and the BBM vesicle preparation, the Bradford method (Bradford, 1976) was used. The principle of this protein assay relies on binding of the dye, Coomassie Brilliant Blue G-250, to the proteins. This results in a shift in the absorption maximum of the dye (465 nm to 595 nm). Therefore, the absorption at 595 nm, measured with a Du 650 spectrophotometer (Beckman-Coulter Bioresearch, Buckinghamshire, UK), is relative to the protein concentration.

2.2.6 Western blotting

Polyclonal antibodies for GLUT2 and actin were purchased from Biogenesis and abcam, respectively. Mouse monoclonal antibodies raised against the first 14 amino acids of *Xenopus Laevus* β-actin protein (abcam, Cambridge, UK; product number: ab 6276) were used as a loading control. Affinity purified polyclonal GLUT2 antibodies were raised in rabbits against the C-terminal of the rat GLUT2 (Poole, UK).

For western blotting, BBM samples (15μg) were solubilised in Laemmli sample buffer containing 5% sodium dodecyl sulphate (SDS) and 5% mercaptoethanol and electrophoresed on a 10% SDS polyacrylamide gel using 0.1% (v/v) SDS running buffer (SIGMA Ltd, Poole, Dorset, UK) at 20 mAmps per gel. The proteins were transferred to nitrocellulose membranes by semi-dry electrophoretic blotting for 1 h at a constant current of 1mA/cm^2 (Trans-blot semi-dry transfer cell, Biorad Hertfordshire, UK), using transfer

buffer containing 10% (v/v) methanol, 25 mM tris, 192 mM glycine and 0.1% (w/v) SDS (pH 8.2-8.4). The non-specific protein–binding sites were blocked with 5% (w/v) fat-free milk powder in PBS-T (phosphate buffered saline (pH 7.4)) containing 0.1% Tween 20) for 1 hour at room temperature. The membranes were incubated with GLUT2 (1:1000) or actin (1:5000) antibodies diluted in PBS-T for 16 hours at 4C°. The membranes were washed (2 x 5 minutes, 1 X 15 minutes) with PBS-T and incubated with anti-rabbit IgG antibody conjugated to horseradish peroxidase (1:2000) (Amersham Pharmacia Biotech UK Limited, Bucks, UK) or anti-mouse IgG antibody conjugated to horseradish peroxidase (1:5000) (Sigma Ltd., Poole, Dorset, UK) for 2 hours at room temperature and finally washed again with PBS-T (2 x 5minutes, 1 x 15minutes). Bound antibodies were detected by an enhanced chemiluminescence system (Upstate Cell Signalling Solutions, Dundee, UK) and visualised and quantified using a Fluor-S Multilmager System (Biorad, Hertfordshire, UK). Band density was expressed in arbitrary units.

2.2.7 Statistics

Data are presented as mean ± standard error of the mean (SEM). Normal distribution was determined by Kolmogrov-Smirnov test of normality and statistical comparisons made using either an unpaired t-test or a one-way analysis of variance (ANOVA) with *post-hoc* comparisons performed using either the Bonferroni multiple comparisons test or the Kruskal-Wallis test. All analyses were performed using Graphpad Instat software with statistical significance taken as $P<0.05$.

2.3 Results

2.3.1 Plasma glucose levels

The original aim of this study was to use the nicotinamide model of type II diabetes following the protocol of (Masiello, Broca et al. 1998). Their protocol involves the injection of 230 mg nicotinamide (i.p.) 15 min. prior to STZ injection which produces a model of type II diabetes with blood glucose in the range of 155 mg/dl. This has been attributed to the protective effect of nicotinamide on the pancreatic β-cell cytotoxicity of STZ.

In the first set of experiments using this protocol, plasma glucose levels of the animals displayed values comparable to those of the untreated control animals (data not shown), suggesting that nicotinamide at this concentration conferred complete protection to the pancreatic β-cells. To avoid influences of different plasma glucose levels prior to the injections, the animals were fasted overnight before injection of nicotinamide and STZ. This protocol also failed to produce the model described by Masielle *et al.* (data not shown).

Therefore, various concentrations of nicotinamide (50 mg, 200 mg) were used to modulate plasma glucose concentration. Additionally, to assess the effect of short term reduction in blood glucose levels, subsets of each of the animal groups were fasted overnight before sacrifice (fasted group).

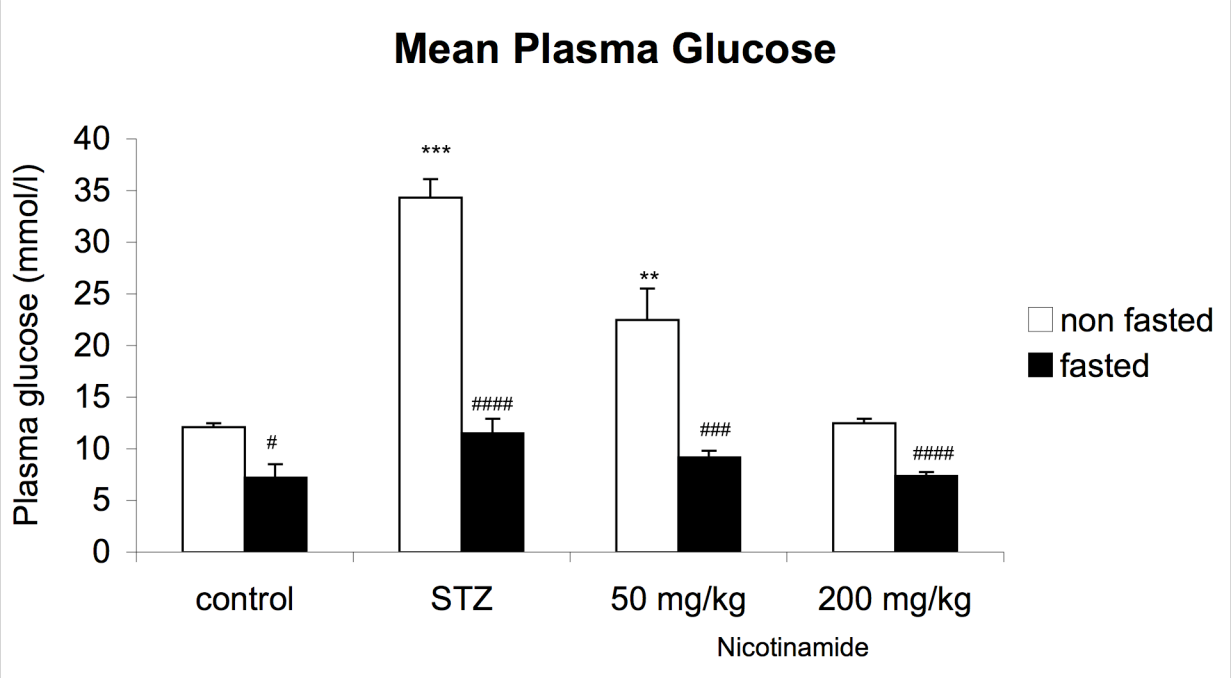

Figure 2.3: Effect of STZ and nicotinamide on plasma glucose levels in non fasted and overnight fasted animals. Results are expressed as mean ± SEM, n=6-12. **P<0.01, ***P<0.001 compared to non fasted control animals using ANOVA with Tukey-Kramer *post hoc* test; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001, $^{\#\#\#\#\#}$P<0.0001 compared with non fasted animals using unpaired *t* test.

Figure 2.3 shows a summary of the different plasma glucose levels of the non fasted and overnight fasted animals having received different doses of nicotinamide. In non fasted animals the mean plasma glucose level of the animals receiving only an STZ injection is significantly higher compared to the control values (2.8-fold, P<0.001 compared to control), whilst the mean plasma glucose level of the 50 mg nicotinamide animals is 1.8-fold higher (p<0.01 compared to control). The plasma glucose level of the 200 mg nicotinamide animals has returned to control levels (not significant).

Overnight fasting results in a dramatic reduction in blood glucose following an overnight fast. All plasma glucose levels of the overnight fasted animals are significantly lower compared to the corresponding non overnight fasted animals (Figure 2.3). Furthermore, the

STZ animals of both groups display higher glucose levels compared to the control values and nicotinamide injected animals of the same group.

2.3.2 Animal parameters

2.3.2.1 Weight gain

As changes in weight gain are a parameter used to describe changes in STZ-induced diabetic animals (Johns, Yoon et al. 1978) weights of the animals were continuously measured and compared after they were distributed according to the different groups of injection protocol (STZ, 50 mg and 200 mg nicotinamide).

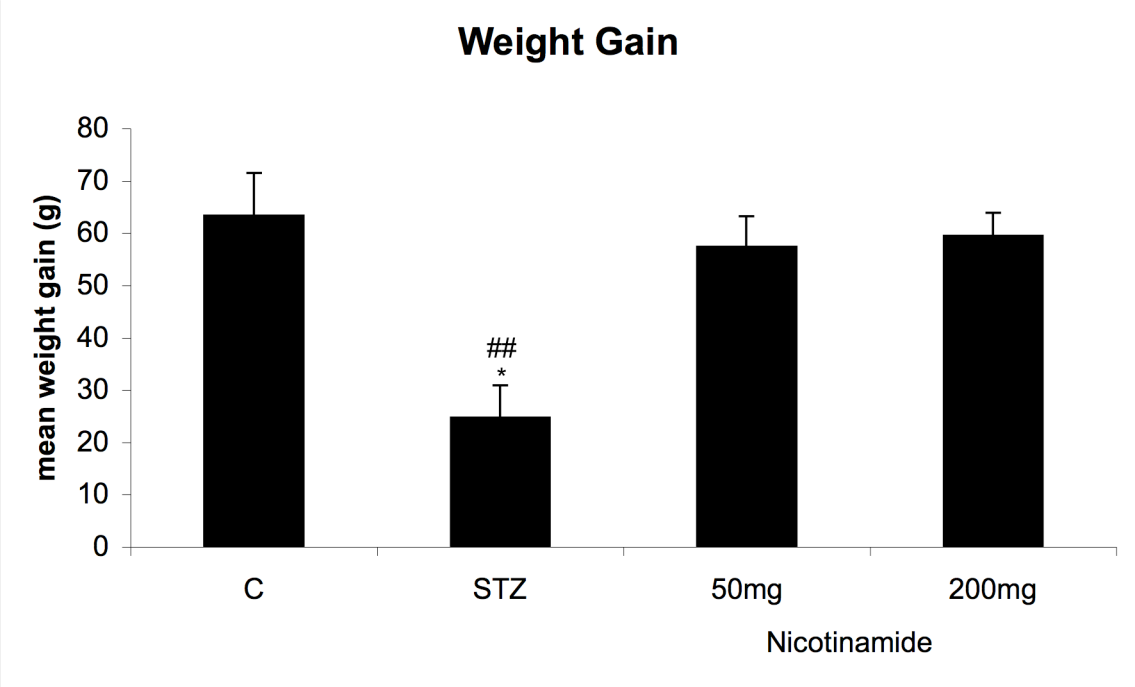

Figure 2.4: Weight gain of animal groups administered different nicotinamide doses. Results are expressed as mean values ±SEM,
n=18.*P<0.05 compared to control, $^{\#\#}$P<0.001 compared to 50 & 200mg using Anova with Tukey-Kramer post hoc test

Fig 2.4 shows the animals receiving a single dose of STZ have a 2.6-fold lower weight gain compared to the control (P<0.05). The mean weight gain of the animals receiving nicotinamide injections is similar to levels of the control animals.

2.3.2.2 Kidney weight

As mentioned in chapter 1, glomerular and tubuloepithelial hypertrophy are among the earliest changes in diabetic nephropathy (Adler 2004). Thus, kidney weight has also been described as a parameter in diabetes to evaluate the level of hypertrophy.

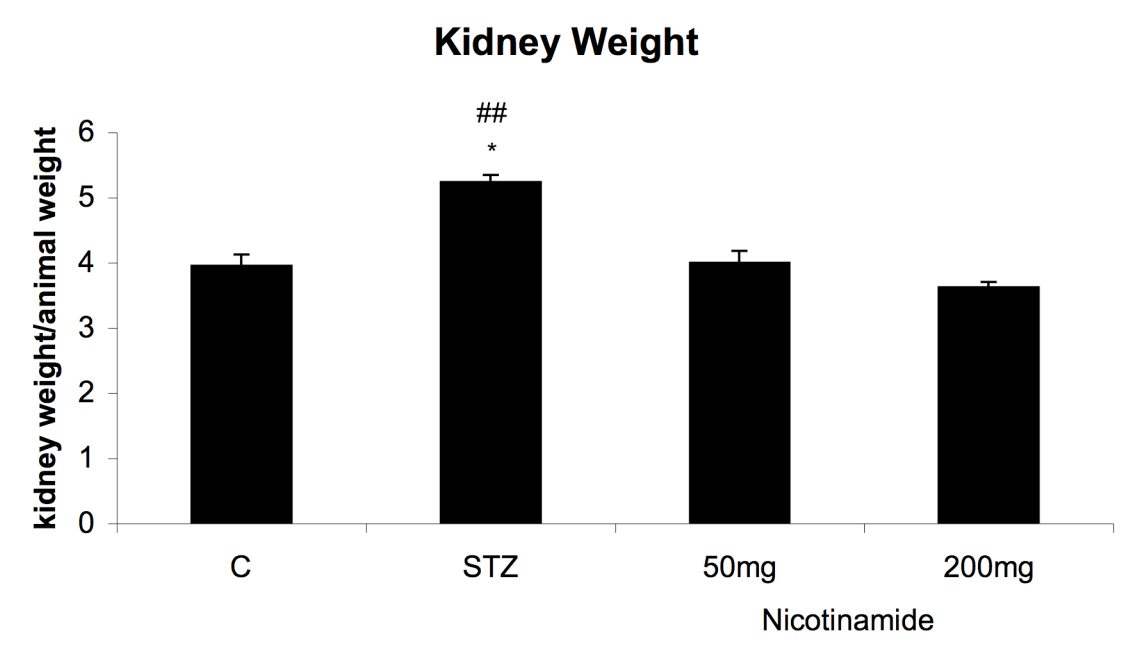

Figure 2.5: Mean kidney weights of relative kidney weights of animal groups given different nicotinamide doses. Results are expressed as mean values ±SEM, n=18.*P<0.001 compared to control; ##P<0.001 compared to 50 & 200 mg using Anova with Tukey-Kramer post hoc test.

The mean kidney weight of the STZ animals is 1.32-fold higher compared to control values (P<0.001). In 50 mg and 200 mg animals the kidney weight is similar to values of the control animals (Fig. 2.5).

A comparison of the kidney weight of the overnight fasted and non fasted animals has shown no effect of overnight fasting on kidney weight using a non parametric *t* test (data not shown).

2.3.3 Enrichment value

The enrichment level of alkaline phosphatase is a parameter used to validate the purity of BBM vesicle preparation (see chapter 2.2.4). Enrichment values of about 7-15 represent a valid proportion of BBM in the prepared vesicles (Biber et al., 1981). As shown in table 2.1 the enrichment values of different animal groups are between 9.5 and 13.48, validating the purity of the BBM vesicles used in this study. Various concentrations of nicotinamide injection do not cause any significant difference in enrichment values compared to the control and STZ animals. The difference between the enrichment value of non fasted and overnight fasted was also not significant.

	Non fasted	Overnight fasted
Control	12.05	10.59
STZ	12.985	13.31
50mg	12.105	12.7
200mg	11.49	12.05

Table 2.1: Enrichment values of different animal groups of the final BBM vesicle preparation. Results are expressed in average, n=6-12 each group. Since the data was not normally distributed, significance was tested using Kruskal-Wallis test non-parametric Anova with Dunn´s multiple comparisons.

2.3.4 GLUT2 expression

As described in chapter 1 it has been shown that changes in plasma glucose levels have an effect on GLUT2 expression at the BBM (Marks, 2003). To investigate the influence of moderate changes in plasma glucose levels *per se*, GLUT2 expression at the BBM was

measured via western blotting in animals treated with different concentrations of nicotinamide. Furthermore, GLUT2 expression levels were then correlated with the plasma glucose concentration of each animal.

2.3.4.1 Western Blot

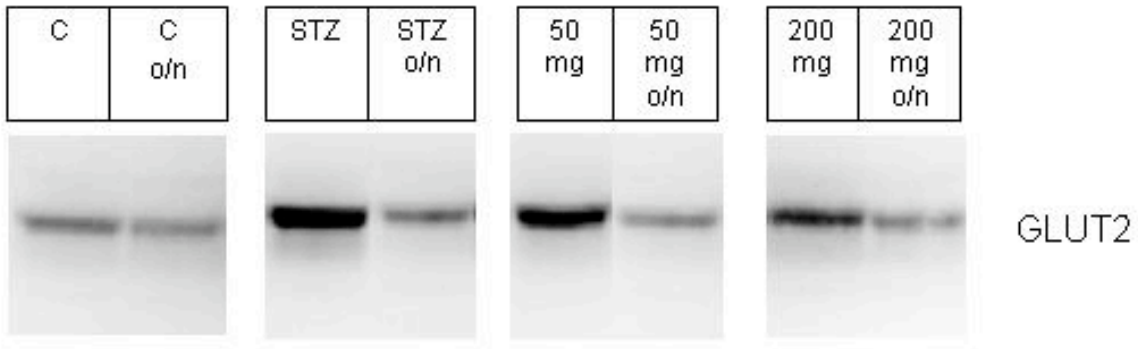

Figure 2.6: Representative Western Blot of GLUT2 in BBM vesicles of the different experimental groups

Figure 2.6 shows a representative Western Blot of GLUT2 expression in BBM vesicles of the different animal groups. GLUT2 protein levels were highest in STZ treated non fasted animals followed by the 50 mg group and visibly reduced in the overnight fasted group. GLUT2 protein levels of the 200 mg group were similar to the control.

2.3.4.2 Nicotinamide concentration and GLUT2 expression

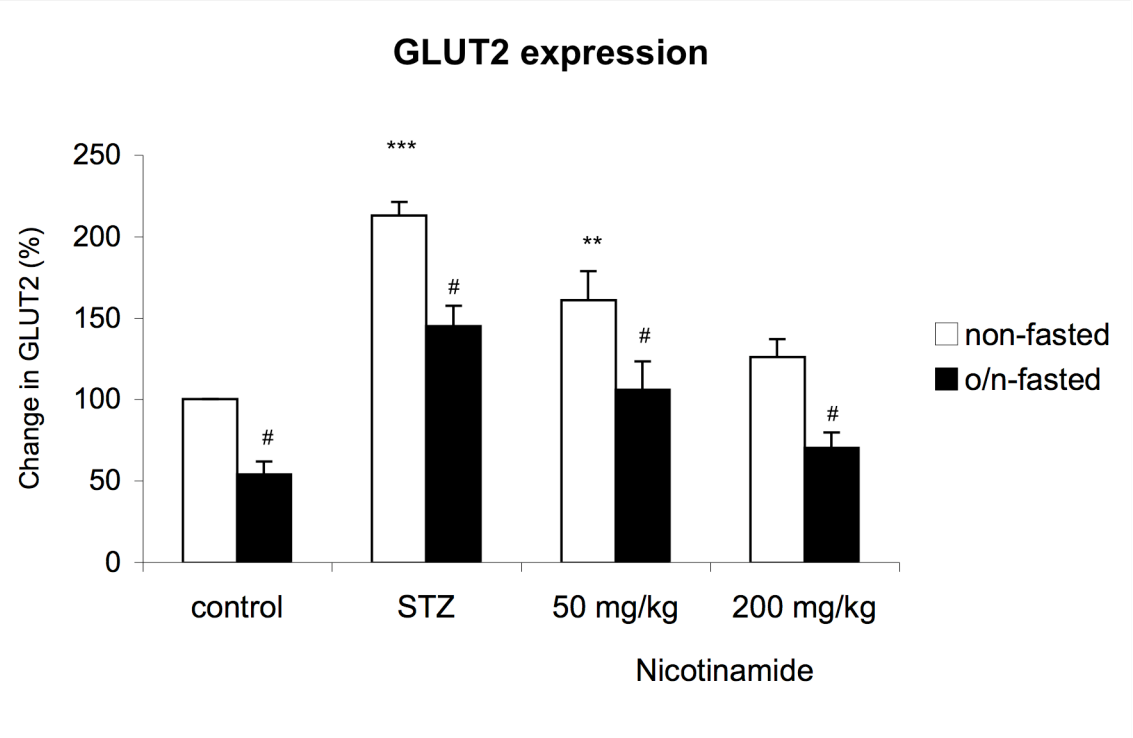

Figure 2.7**:** Quantification of GLUT2 in BBM vesicles of non fasted and overnight fasted animals in the different experimental groups. Results are expressed as a percentage of GLUT2 compared with the control, n=6-12. **P<0.001, ***P<0.001 compared with non-fasted control group using ANOVA with Dunn´s Multiple Comparison *post hoc* test; $^{\#}$P<0.05 compared with non fasted animals using unpaired *t* test.

Figure 2.7 shows the comparison of GLUT2 expression in overnight fasted and non fasted animals treated with nicotinamide. GLUT2 expression of the non fasted STZ animals is 2.13-fold higher compared to control animals (P<0.001). In the 50 mg group the GLUT2 expression is still 1.61-fold higher (P<0.05), whereas in 200 mg animals it is approaching values similar to the control (non significant).

Regarding the overnight fasted animals, GLUT2 expression of the STZ animals is just 1.45-fold higher (P<0.05) compared to the non fasted control. In 50 mg and in 200 mg overnight fasted animals there is no significant change in GLUT2 expression compared to the control.

2.3.4.3 Plasma glucose level and GLUT2 expression

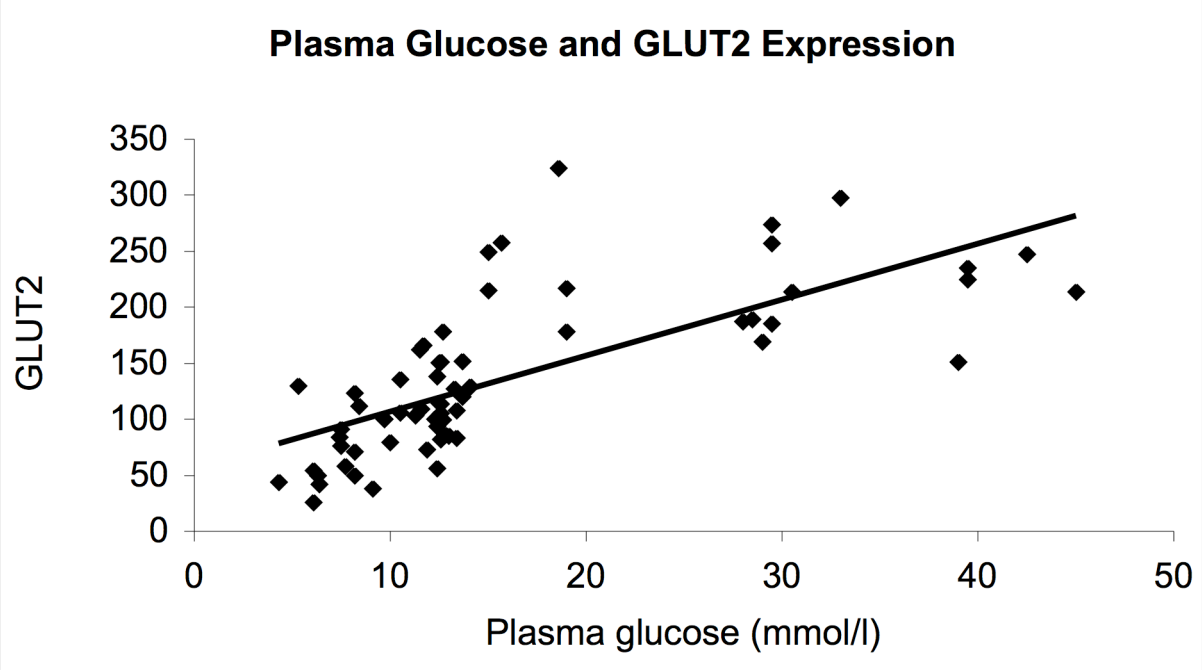

Figure 2.8: Pearson´s correlation between GLUT2 protein levels and plasma glucose concentration; r=0.7183, r^2=0.516, P<0.0001

The results of the GLUT2 expression (Fig. 2.7) and plasma glucose levels (Fig. 2.3) of the different experimental groups suggest a similar pattern of glucose and GLUT2 in each group. Therefore, a Pearson´s correlation was established. Figure 2.8 reveals a significant positive correlation between the plasma glucose level and GLUT2 protein concentration with an r value of 0.7183 (P<0.001).

2.4 Discussion

2.4.1 Nicotinamide model of type II diabetes

The original aim of this study was to use the nicotinamide model of type II diabetes following the protocol of Masiello *et al.* (Masiello, Broca et al. 1998). Their protocol involves the injection of 230 mg nicotinamide (i.p.) 15 min. prior to STZ injection (65 mg/kg), which was described to produce a model of type II diabetes with blood glucose in the range of 155 mg/dl. This has been attributed to the protective effect of nicotinamide on the pancreatic β-cell cytotoxicity of STZ.

Nicotinamide, also known as niacinamide, is the amide of nicotinic acid (vitamin B_3). Its biochemical importance is based on the fact that it is a component of the coenzymes NAD^+ and $NADP^+$, which are responsible for the hydrogen transfer in many reactions *in vivo*. Thus, the protective effect of nicotinamide against the STZ-induced β-cell cytotoxicity is thought to be dependant on the preservation of the intracellular NAD pool (Masiello, Broca et al. 1998). Furthermore, nicotinamide is an inhibitor of poly (ADP-ribose) synthetase, which has also been reported to be activated by STZ-induced DNA injury (Yamamoto, Uchigata et al. 1981). Masiello *et al.* demonstrated that nicotinamide injection prior to STZ administration produced stable levels of hyperglycaemia. Insulin response of the pancreatic β-cells to glucose elevation was still present, although significantly reduced with respect to controls. In further experiments this effect could be attributed to a quantitative reduction in β-cells, with maintenance of most of the metabolic function of the residual β-cells. However, decreased FAD-glycerophosphate dehydrogenase (mGDH) activity was present in the residual β-cells (Novelli, Fabregat et al. 2001), an effect which has been shown to be partly responsible for the impaired insulin secretion in GK rats, which is another model of type II diabetes (Ostenson, Abdel-Halim et al. 1993), and in human diabetic islet cells (Fernandez-Alvarez, Conget et al. 1994).

In summary, the STZ-nicotinamide model of type II diabetes shares a number of features with human type II diabetes. The insulin responsiveness to glucose and sulfonylureas, a feature that is not present in other established models of type II diabetes, makes this model

suitable for both biochemical and pharmacological studies. Indeed, there have been various studies using the STZ-nicotinamide model for induction of type II diabetes. Most follow the protocol of Masiello *et al.*, where 200-270 mg/kg and 60-65 mg/kg of nicotinamide and STZ respectively are administered (Kuntz, Pinget et al. 2002; Novelli, Pocai et al. 2004). The measured levels of blood glucose in these studies were reported to have the expected range of approximately 150 mg/dl. There is one study where the reported doses of STZ and nicotinamide were reduced (STZ: 45 mg/kg, nicotinamide: 110 mg/kg) and blood glucose levels were reported to be higher with a range of 262±15 mg/dl (Pari and Saravanan 2007).

Another approach which seems suitable for pharmacological research is the fat-fed, STZ-treated animal model of type II diabetes (Reed, Meszaros et al. 2000). In this model, the animals are treated with a high-fat diet (40% of calories as fat) and receive an STZ injection (50mg/kg), which induces hyperglycaemia and impaired glucose tolerance. A recent study compared the STZ-nicotinamide model and the fat-fed STZ-treated rat. It was reported that there was no significant change of blood glucose levels of the STZ-nicotinamide animals that were injected following the protocol of Masiello *et al*., whereas the fat-fed STZ-treated rats developed hyperglycaemia and a decreased insulin secretion (Islam and Choi 2007). In keeping with the findings of Islam *et al.* in the first set of our experiments using the protocol of Masiello *et al.,* plasma glucose levels were not elevated, suggesting nicotinamide at this concentration conferred complete protection to the pancreatic β-cells (data not shown).

Another well characterized animal model of type II diabetes that has been described in the literature is the db/db mouse. This mouse model has proven to have many similar features of human diabetic nephropathy and has therefore been used extensively to investigate the mechanisms that lead to diabetic renal disease (Sharma, McCue et al. 2003). Renal abnormalities have also been identified in Goto-Kakizaki (GK) rats, Obese Zucker rats (Janssen, Phillips et al. 1999) and Otsuka Long-Evans Tokushima Fatty (OLETF) rats, all of which are models of type II diabetes. Since these animal models were not available for

us, we were using nicotinamide at different concentrations (50 mg, 200 mg) to modulate plasma glucose levels.

2.4.2 Diabetic nephropathy and GLUT2

It has been demonstrated that hyperglycaemia is a key factor in the pathogenesis of diabetic nephropathy (Larkins and Dunlop 1992). The earliest renal pathological alterations associated with diabetes mellitus are glomerular and tubuloepithelial hypertrophy, which precede and may contribute to the renal abnormalities that occur at the later stages of the disease, such as glomerulosclerosis and tubulointerstitial fibrosis (Ziyadeh 1993). These abnormalities result from increased accumulation of extracellular matrix component, which has been demonstrated to be a consequence of hyperglycaemia (Larkins and Dunlop 1992; Wolf and Thaiss 1995). Hyperglycaemia-induced mesangial cell damage has been linked to increased expression of GLUT1 protein, leading to elevated intracellular glucose accumulation and ECM formation (Heilig, Concepcion et al. 1995). It has been shown that transforming growth factor β (TGF-β), a cytokine that is elevated in the glomeruli of diabetic animals and diabetic patients with nephropathy, can induce GLUT1 mRNA and protein expression (Inoki, Haneda et al. 1999). Unlike the glomerulus, exposure of human proximal tubular cells to elevated glucose levels does not effect collagen or fibronectin gene expression, but results in a change in their degradation and rate of turnover (Phillips, Steadman et al. 1997; Phillips, Morrisey et al. 1999).

Renal enlargement is one of the main pathological changes of diabetic nephropathy. Since the tubulointerstitium comprises much of the kidney substance, tubular hypertrophy might be responsible for most of this renal enlargement (Nath 1992). However, the causative factors involved in the hyperglycaemia-induced damage of proximal tubule cells have received relatively little attention. Previous studies focusing on the effect of diabetes on renal SGLT-mediated glucose transport in the proximal tubule have yielded conflicting results, with reports of both increased (Blank, Bode et al. 1985) and decreased (Yasuda, Kurokawa et al. 1990) rates of transport. In contrast, one previous study demonstrated that in STZ-induced diabetes the sodium-dependant component of renal glucose transport

remained unchanged, whilst the facilitative component was enhanced (Marks, Carvou et al. 2003). In accordance with these findings the presented data shows clearly that changes in circulating glucose concentration can rapidly influence the levels of GLUT2 protein at the proximal tubule BBM.

It has previously been reported that the substrates for GLUT2 and GLUT5 can positively regulate the expression and activity of these transporter proteins. GLUT2 is regulated by both glucose and fructose, whilst GLUT5 is regulated by fructose only (Burant and Saxena 1994). This data is consistent with the proposal that GLUT2 can transport both fructose and glucose (Colville, Seatter et al. 1993), whereas *in vivo* GLUT5 only transports fructose (Inukai, Katagiri et al. 1995). Previous studies carried out in the laboratory of Dr Debnam revealed an increase in both GLUT2 and GLUT5 protein at the proximal tubule BBM of STZ-induced diabetic animals, with no change in GLUT1 levels (Marks, Carvou et al. 2003). Furthermore, the upregulation of GLUT2 could be abolished by overnight fasting. This finding has also been reported by Freitas *et al.* who conclude that plasma glucose concentration is the key regulator of the GLUT2 gene (Freitas, D'Agord Schaan et al. 2007). However, the modulation of GLUT5 protein levels appears to be distinct from that of GLUT2. During diabetes the expression of GLUT5 is increased but overnight fasting failed to completely normalise its expression. This increase in GLUT5 levels may therefore be a consequence of increased intracellular fructose concentration produced as a consequence of increased polyol pathway activity (chapter 1, Fig.1.5).

Our present results demonstrate that a reduction in plasma glucose concentration of STZ-diabetic rats treated with nicotinamide reduces STZ-induced renal hypertrophy, as well as GLUT2 protein levels. Therefore, since GLUT5 is considered to be a fructose transporter *in vivo* with a low affinity for glucose (Miyamoto, Tatsumi et al. 1994) and GLUT1 protein levels in the proximal tubule cells is generally considered to be reduced (Dominguez, Camp et al. 1994; Kamran, Peterson et al. 1997) or unaffected by diabetes (Marks, Carvou et al. 2003) it is plausible to hypothesise that GLUT2 is responsible for glucose-induced tubular hypertrophy in this condition. Additionally, the importance of GLUT2 in the development of renal hypertrophy also comes from the evidence that this transporter is

responsible for the development of tubular hypertrophy in patients with Fanconi-Bickel Syndrome (Santer, Schneppenheim et al. 1998). In this rare autosomal disorder, mutations in the gene for the GLUT2 transporter are responsible for the typical clinical and laboratory findings; amongst these, proximal tubular nephropathy is one of the pathological changes.

2.4.3 Clinical implications

It has been well established in controlled clinical trials that diabetic nephropathy is more likely to develop in patients with poor glycaemic control and high HbA1c levels (DCCT, 1993; UKPDS, 1998). In one previous study 11,140 patients were randomly assigned to undergo either standard glucose control or intensive glucose control. After a median of 5 years follow-up there was a 21% relative reduction in cases of diabetic nephropathy in the intensive glucose control group (ADVANCE, Patel, MacMahon et al. 2008). As shown in our results, even modest changes in plasma glucose levels have an effect on glucose transport at the proximal tubule BBM, which is suggested to be a causative factor of diabetic nephropathy. This finding is consistent with reports from recent clinical trials (ADVANCE, Patel, MacMahon et al. 2008) and highlights the importance of tight control of blood glucose levels for the prevention of diabetic nephropathy.

It should be mentioned that to date no randomized clinical trial has demonstrated a beneficial effect of intensive therapy on macrovascular outcomes in type II diabetes. Previous studies have shown that there was no significant change in cardiovascular events and mortality in the intensive glucose control group of patients with type II diabetes (UKPDS, 1998). Furthermore, one recent study reported even an increased mortality and no significant reduction in major cardiovascular events in a group of patients receiving intensive glucose lowering therapy to achieve normal HbA1c levels (Gerstein, Miller et al. 2008). A specific cause for the increased mortality could not be identified. However, since the duration of this study was 3.5 years, it could be considered that the long-term risks of diabetes, e.g. diabetic nephropathy, which have an influence on long-term mortality, have not been fully investigated in this study. Thus, further investigations are necessary to

assess the approaches from clinically relevant outcomes in the treatment of type II diabetes.

2.4.4 Role of renal SGLT on GLUT2 expression

There are two sodium-dependent transporters expressed in the kidney; the bulk of filtered glucose is reabsorbed in the S1 segment by the low affinity, high capacity transporter, SGLT2; whereas the high affinity, low capacity transporter, SGLT1, scavenges the remaining glucose that is presented to the distal regions of the proximal tubule (Debnam and Unwin 1996) (chapter 1, Fig. 1.2). However, it has been shown that the predicted Km values for these two transporters are 0.5 mM and 2 mM for SGLT1 and SGLT2, respectively. Therefore, in situations of high concentrations of filtered glucose, such as those presented to the proximal tubule during diabetes, these transporters would be saturated. It has been shown that in the small intestine high glucose concentrations are "sensed" by SGLT proteins, which results in the insertion of GLUT2 into the BBM via the activation of Ca^{2+} channels and PKC-βII (Mace, Morgan et al 2007) (chapter 1 fig. 1.3). It could be envisaged that in the kidney a situation occurs that is similar to the small intestine. Indeed, previous studies in the laboratory of Dr Debnam have shown rapid insertion of GLUT2 into the BBM under conditions of STZ-induced hyperglycaemia. Furthermore, with a predicted Km of 20-40 mM (Debnam and Unwin 1996), this protein would provide a component for absorption that is considerably higher than that provided by the SGLT proteins. It could therefore be hypothesised that the effectiveness of SGLT-inhibitors in the treatment of diabetes occurs through the inability of this protein to signal for the insertion of GLUT2 into the BBM. It has been shown that the use of T-1095, a prodrug and selective inhibitor of renal SGLTs, suppresses the elevated renal GLUT2 mRNA expression in STZ rats. Expression of SGLT2 remained unchanged, suggesting that inhibition of renal glucose reabsorption could modulate GLUT2 expression in the kidney of diabetic rats (Adachi, Yasuda et al. 2000). In contrast, Freitas *et al*. have shown no effect of phlorizin, an inhibitor of SGLT, on GLUT2 mRNA in the proximal tubule of diabetic rats (Freitas, D'Agord Schaan et al. 2007).

Further studies have shown that phlorizin prevents proteinuria, hyperfiltration and kidney hypertrophy, but not glomerular hypertrophy, in an animal model of STZ-induced diabetes (Malatiali, Francis et al. 2008). This approach is currently being tested for the treatment of diabetes. In human studies, oral application of the SGLT2 inhibitor sergliflozin resulted in dose-dependant urinary glucose excretion (Hussey 2007). Another SGLT2 inhibitor, dapagliflozin, has shown to induce renal glucose excretion and reduce hyperglycaemia in diabetic rats (Han, Hagan et al. 2008). One study, where type II diabetic patients were randomly assigned to dapagliflozin treatment, metformin or placebo for 12 weeks, demonstrated improved hyperglycaemia by inducing controlled glucosuria (List, Woo et al 2009). However, further clinical studies are needed to assess the role of SGLT2 inhibitors in the treatment of type II diabetes.

In conclusion, this study demonstrates that raised circulating glucose levels promote GLUT2 protein to the proximal tubule BBM. Furthermore, there is a direct correlation between even modest changes in blood glucose and GLUT2 expression. These findings support previous studies that have shown a link between hyperglycaemia, GLUT2 expression and changes in diabetic nephropathy.

Chapter 3:
Involvement of protein kinase C (PKC) signalling pathway in the regulation of GLUT2-mediated glucose transport

3.1 Introduction

It is now well established that hyperglycaemia is a key factor involved in the pathogenesis of diabetic nephropathy. In addition to the typical histological changes of the mesangium, tubulointerstitial changes are responsible for most of the renal enlargement seen in diabetic nephropathy. However, the processes leading to these histological changes are still not known in detail. There have been four major hypotheses proposed as to how this cellular damage occurs (chapter 1.4). One of them is based on the observation that activation of PKC under conditions of hyperglycaemia results in increased production of cytokines and extracellular matrix proteins (Meier, Menne et al 2009). These studies have mainly focused on changes of the glomerulus; however, PKC activation in the proximal tubule is assumed to play a role in tubulointerstitial changes of diabetic nephropathy.

Previous studies have shown that many PKC isoforms are expressed in rat and mouse kidney. In the proximal tubule of the rat kidney the PKC isoforms alpha (α), beta I (βI), beta II (βII), delta (δ) and epsilon (ε) have been detected (Karim, Defontaine et al. 1995; Kang, Alexander et al. 1999), with prominent staining for PKC-α (Dong, Stevens et al. 1991; Pfaff, Wagner et al. 1999) and -βI (Pfaff, Wagner et al. 1999) at the BBM. Studies have also detected altered levels of PKC isoforms in proximal tubule cells following exposure to high glucose concentrations: specifically, PKC-α, -ε (Kang, Alexander et al. 1999) and -βI (Hsieh, Chen et al. 2006) are increased. Furthermore, Kellet and co-workers have shown that in the enterocyte, where transport mechanisms are similar to the proximal tubule, a high level of luminal glucose leads to rapid insertion of GLUT2 into the BBM via a calcium and PKC-βII -dependent mechanism (Mace, Morgan et al. 2007).

These findings suggest that diabetes-induced alterations in the levels and activity of PKC isoforms may affect proximal tubular function, as well as having their already established effects on glomerular cells.

Therefore, in this chapter, it was the aim to establish the relationship between changes in circulating glucose concentration, protein levels of PKC-βI and other PKC isoforms, together with GLUT2 at the proximal tubule BBM.

3.2 Methods

3.2.1 BBM vesicle preparation

Preparation of the BBM vesicles was carried out following the same protocol as described in chapter 2.2.2.

3.2.2 Western Blotting

Western Blotting for the various PKC isoforms was performed as described in chapter 2.2.6 using PKC antibodies (Table 3.1) purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, United States).

antibody	Product number	Raised against	dilution
PKC-α	sc-8393	C terminus of human PKC-α	1:400
PKC-βI	sc-209	C terminus of human PKC-βI	1:200
PKC-βII	sc-210	C terminus of human PKC-βII	1:200
PKC-δ	sc-937	C terminus of human PKC-δ	1:500
PKC-ε	sc-214	C terminus of human PKC-ε	1:500

Table 3.1: Details of the PKC antibodies used for western blotting of BBM vesicles

3.3 Results

3.3.1 Expression of different PKC isoforms

Since PKC signalling has been shown to regulate intestinal and glomerular glucose transport, the expression of different PKC isoforms in BBM samples of STZ and control animals was measured.

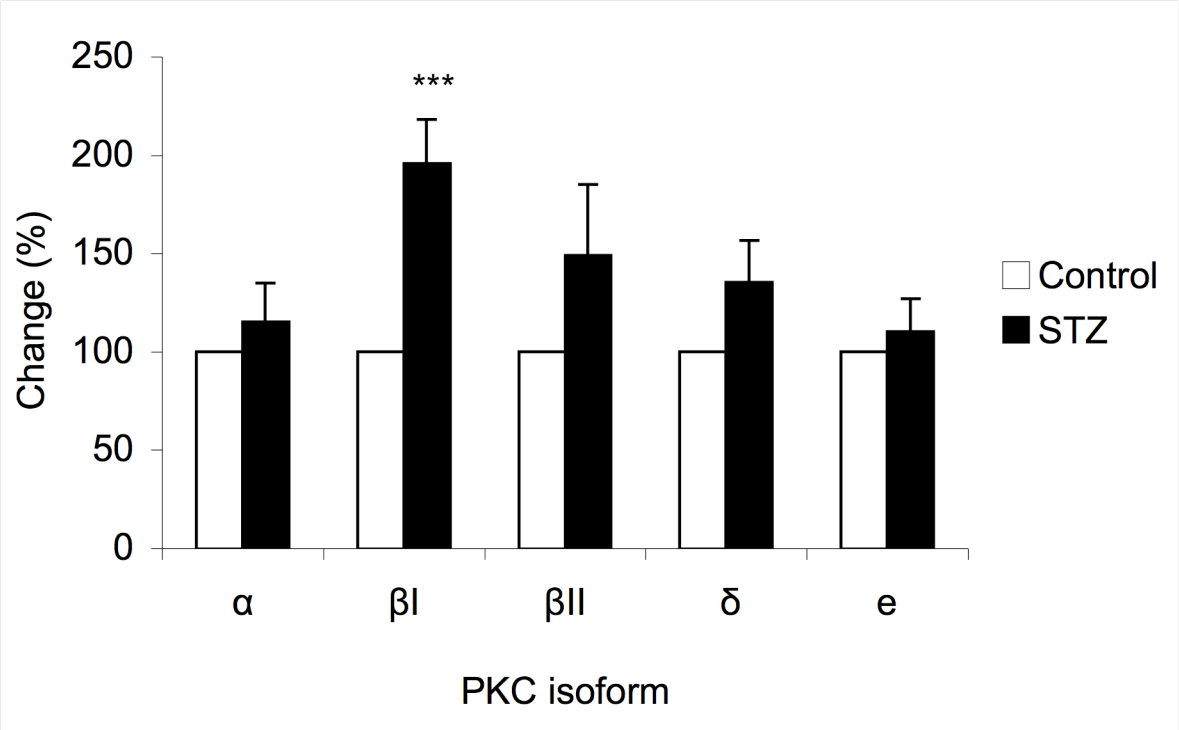

Figure 3.1: Quantification of PKC isoforms in BBM vesicles of control and STZ-treated animals. Results are expressed as a percentage compared with control, n=6-12; ***P<0.001 compared with control using unpaired *t* test.

Figure 3.1 demonstrates that the only significant change in PKC expression compared to the control animal can be demonstrated in the isoform PKC-βI ($P<0.01$). All changes in the expression of the other isoforms are not significant compared to the control.

3.3.2 PKC-βI expression

3.3.2.1 Nicotinamide concentration and PKC-βI expression

As already shown in figure 3.1, STZ-induced hyperglycaemia results in significant changes in the level of PKC-βI expression. To evaluate the effect of different blood glucose levels, changes in the PKC-βI expression of BBM vesicles prepared from the different animal groups treated with nicotinamide were examined (fig. 3.2).

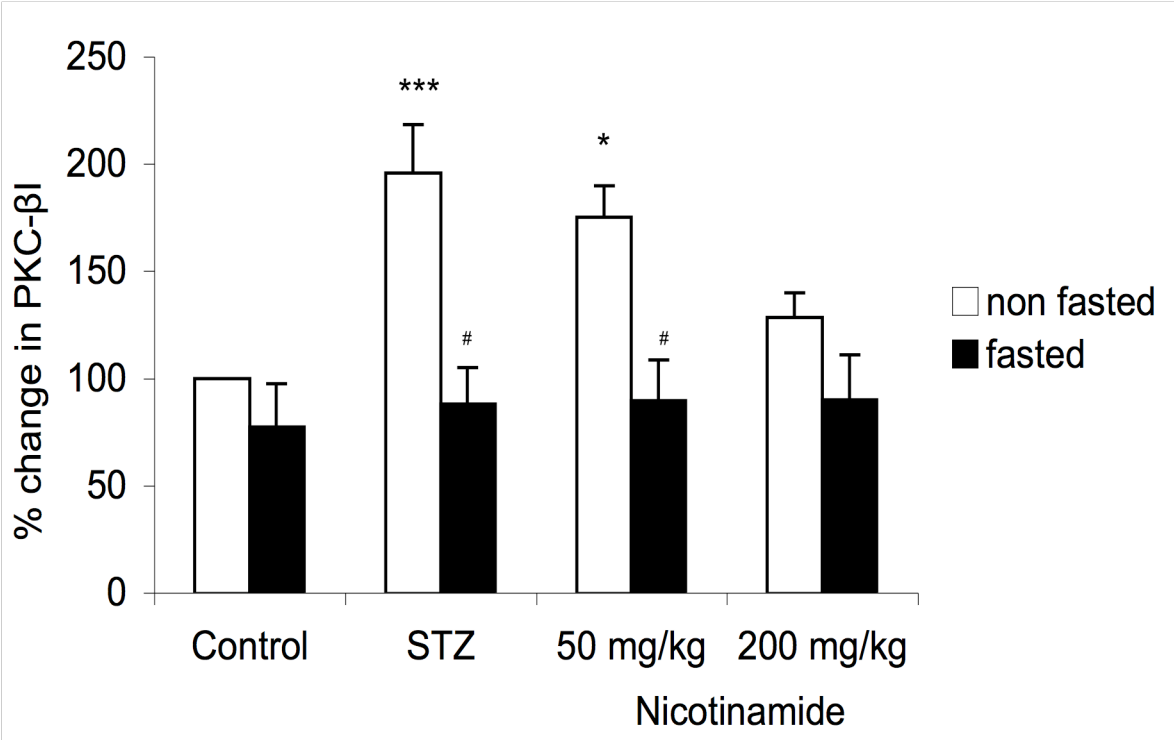

Figure 3.2: Quantification of PKC-βI expression in BBM vesicles of control and STZ-injected animals, with or without nicotinamide treatment or overnight fasting. Results are expressed as percentage change in PKC-βI expression compared with control, n=6-12;

*P<0.05, ***P<0.001 compared with non fasted control animals using Kruskal-Wallis ANOVA; #P<0.05 compared with non fasted animals using unpaired *t* test.

Figure 3.2 shows that PKC-βI levels are elevated in STZ treated animals and that treatment with 50 mg/kg nicotinamide reduces the expression level of PKC-βI. Treatment with 200 mg/kg nicotinamide or overnight fasting results in PKC-βI levels that are similar to the control.

3.3.2.2 Plasma glucose concentration and PKC-βI expression

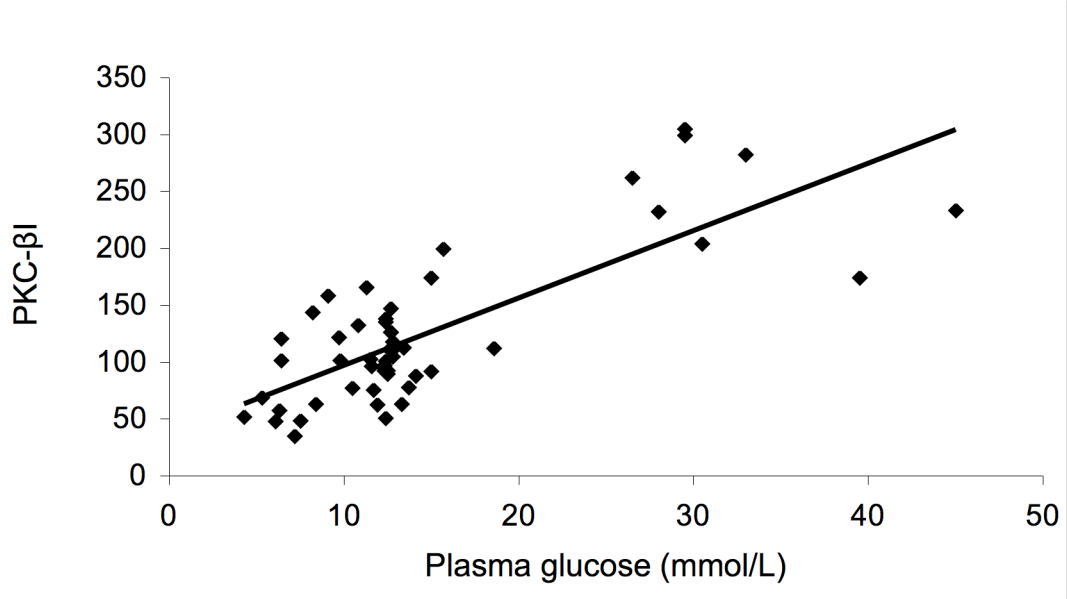

Figure 3.3: Pearson`s correlation between PKC-βI protein levels and plasma glucose concentration. r=0.779, P<0.0001

The results of the PKC-βI expression and nicotinamide concentration (fig. 3.2) together with nicotinamide and glucose concentration (fig. 2.3) suggest a similar pattern of plasma glucose and PKC-βI. Therefore, a Pearson´s correlation was established. Figure 3.3

reveals a significant positive correlation between plasma glucose and PKC-βI protein concentration with an r value of 0.779 (P<0.0001).

3.3.2.3 GLUT2 expression and PKC-βI expression

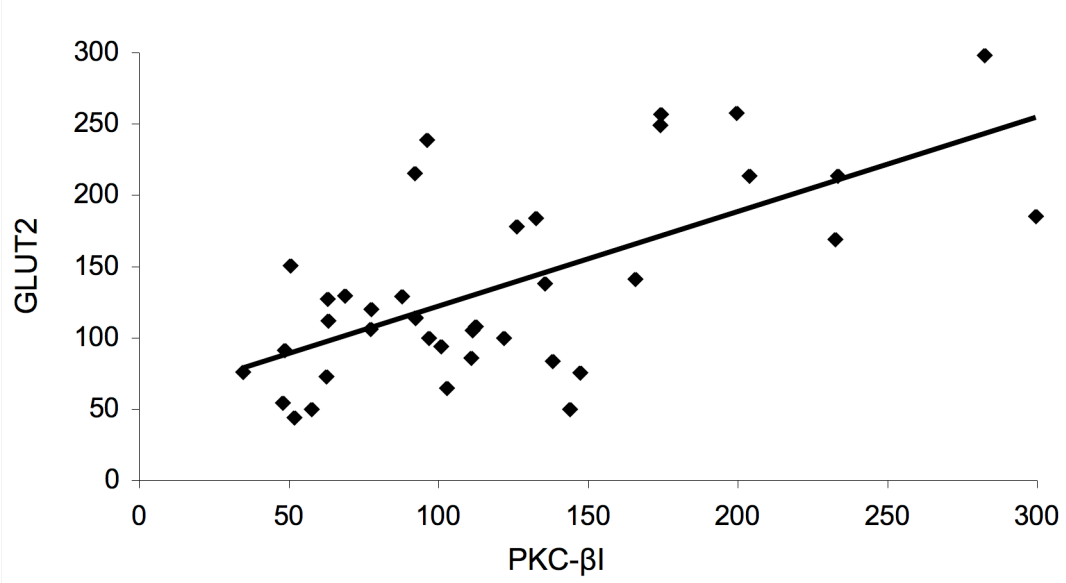

Figure 3.4: Pearson`s correlation between GLUT2 and PKC-βI protein levels. r=0.648, P<0.0001

The pattern of PKC-βI expression as shown in fig. 3.2 is similar to the GLUT2 expression pattern described in fig. 2.7 (chapter 2.3.4.2). Therefore, a Pearson`s correlation between the levels of PKC-βI and GLUT2 expression was established. There is a positive correlation (r=0.648) between PKC-βI and GLUT2 protein levels (fig. 3.4).

3.4 Discussion

3.4.1 PKC and diabetic nephropathy

Diabetic nephropathy has been linked to chronically elevated plasma glucose levels. Clinical studies have shown that intensive blood glucose control in diabetic patients prevents the onset and progression of diabetic complications like retinopathy, neuropathy and nephropathy (DCCT, 1993). The overall pathways linking high glucose concentration and end-organ damage in diabetes mellitus are currently under intensive investigation (Schena and Gesualdo 2005). There have been four major hypotheses proposed as to how this cellular damage occurs:

-Formation of reactive oxygen species (ROS)
-Activation of the aldose reductase pathway
-accelerated formation of advanced glycation end products (AGEs)
-Activation of protein kinase C (PKC) isoforms
(chapter 1.4)

In this study the main focus was set on the role of PKC isoforms as the potential link between hyperglycaemia and diabetic nephropathy.

It was recently shown that the process of GLUT2 recruitment to the apical membrane in the enterocytes is mediated by activation of PKC-βII under conditions of high luminal glucose (Mace, Morgan et al. 2007). Furthermore, a similar mechanism has been described for the uptake of amino acids in the small intestine. This suggests the existence of a wider transport network which acts via stimulation of a common enterocytic pool of PKC-βII for nutrient absorption (Mace, Lister et al. 2009). In the mesangial cells of the kidney, hyperglycaemia-induced cell damage has been linked to increased expression of GLUT1 protein *in vitro* (Heilig, Concepcion et al. 1995). Furthermore, it has been shown that mesangial cells cultured under conditions of high glucose concentrations display increased PKC and mitogen-activated protein kinase activity in their membrane fraction (Haneda,

Kikkawa et al. 1995). In elucidating the cellular mechanisms involved in PKC activation, Haneda *et al* have demonstrated *in vivo* and *in vitro* that the DAG-PKC-ERK (extracellular regulatory protein kinase) pathway is involved in the cellular damage that occurs during hyperglycaemia (Haneda, Koya et al. 2001). Incubation of mesangial cells under conditions of high glucose in the presence of PD98059, a specific inhibitor of MAP/ERK kinase (MEK), which is an upstream kinase activator of ERK, has been shown to inhibit the glucose induced production of TGF-βI and ECM proteins, such as collagen and fibronectin (Isono, Cruz et al. 2000). In turn, PKC activation in glomerular endothelial cells results in increased production of cytokines like TGF-β and stimulates the production of ECM proteins by glomerular endothelial cells (Ziyadeh, Fumo et al. 1995; Chen, Cohen et al. 2001). This effect can be mimicked by phorbol esters (activators of PKC) and reversed by PKC inhibitors, such as staurosporine, calphostin C and ruboxistaurin (Fumo, Kuncio et al. 1994).

In keeping with the findings *in vitro* it was shown that GLUT1 and PKC expression is increased in the glomerulus of STZ-induced diabetic rats (Brosius and Heilig 2005). There are reports of increased PKC-α (Koya, Jirousek et al. 1997; Kang, Alexander et al. 1999; Ohshiro, Ma et al. 2006), -βI (Koya, Jirousek et al. 1997; Kelly, Zhang et al. 2003; Ohshiro, Ma et al. 2006), -δ (Ha, Yu et al. 2001) and -ε (Kang, Alexander et al. 1999; Ha, Yu et al. 2001). However, it appears that PKC-βI is the main pathophysiological isoform (Koya, Haneda et al. 2000; Kelly, Zhang et al. 2003). In summary, it has been hypothesised that increased GLUT1 expression leads to enhanced polyol pathway activity and production of PKC-βI, which in turn enhances the synthesis of fibronectin and other ECM proteins (Phillips, Morrisey et al. 1999; Brosius and Heilig 2005) (fig. 3.5).

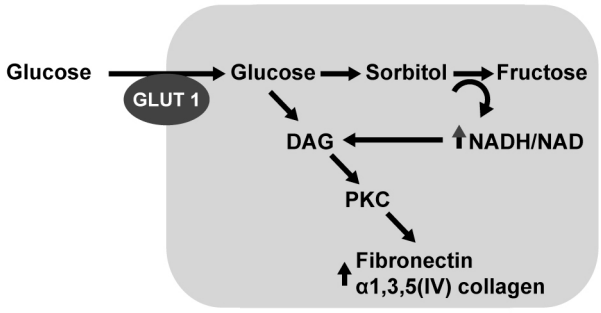

Fig 3.5: GLUT1 signalling in mesangial cells

Findings in the enterocyte and the glomerulus, and the findings of the present study, draw attention to the link between PKC activation and increased expression of GLUT transporters in diabetes-induced renal damage.

3.4.2 Hyperglycaemia, GLUT2 and specific PKC isoforms

The mechanisms controlling glucose uptake by proximal tubule cells are similar to those in enterocytes. In both cell types, activation of the protein kinase A (PKA) signalling pathway increases intracellular cAMP levels and promotes SGLT1-mediated glucose transport (Sharp and Debnam 1994; Marks, Debnam et al. 2003). Short-term modulation of SGLT1-mediated transport occurs via apical membrane insertion of additional SGLT1 protein (Williams and Sharp 2002) and through changes in the membrane electrochemical gradient (Sharp and Debnam 1994). In enterocytes, activation of the PKC signalling pathway by phorbol 12-myristate (PMA) and the influx of Ca^{2+} through L-type channels has been shown to induce cytoskeletal re-arrangement, leading to insertion of GLUT2 protein at the BBM (Kellett and Helliwell 2000; Mace, Morgan et al. 2007). Less information is available concerning the regulation of GLUT2 in the kidney; however, studies in the laboratory of Dr Debnam have shown that incubation of isolated proximal tubules with PMA and thapsigargin, which causes release of Ca^{2+} from intracellular stores, increases GLUT-

mediated, but not sodium-dependent glucose transport (Goestemeyer, Marks et al. 2007). This indicates that, similar to the small intestine, PKC and calcium are potential signalling pathways regulating renal BBM GLUT2 protein levels. It is also known that basal levels of cytosolic calcium in proximal tubule cells are elevated in rats with STZ-induced diabetes (Marcinkowski, Zhang et al. 1997). Furthermore, it has been shown that high extracellular glucose increases cytosolic calcium in cultured proximal tubule cells by activation of L-type Ca^{2+} channels (Park, Choi et al. 2001). The present study shows that in the proximal tubule, although numerous PKC isoforms are detectable, increased GLUT2 expression at the BBM is correlated only with an increase in one PKC isoform: PKC-βI. In contrast, in the small intestine, there is a strong correlation between the levels of GLUT2 and PKC-βII (Helliwell, Richardson et al 2000; Kellett and Helliwell 2000; Morgan, Mace et al. 2007). These differences of the PKC-β isoforms that are responsible for the regulation of GLUT2 in the kidney and small intestine may be due to differential tissue expression of these splice variants. In keeping with our findings, Hsieh *et al.* have reported that glucose-induced damage to proximal tubule cells is dependant on PKC-βI (Hsieh, Chen et al. 2006). Additional evidence that PKC-βI is the predominant pathophysiological isoform in the kidney comes from studies using PKC-β deficient (PKC-$\beta^{-/-}$) mice. Levels of the extracellular matrix proteins collagen and fibronectin, and the profibrotic cytokine TGF-β, are decreased in PK-C$\beta^{-/-}$ mice. Furthermore, there is a significant reduction in renal hypertrophy and glomerular enlargement when PKC-$\beta^{-/-}$ mice are made diabetic and compared with wild type (Ohshiro, Ma et al. 2006; Meier, Park et al. 2007).

3.4.3 PKC in extrarenal tissue in diabetes mellitus

Since long-term diabetic complications do not only affect the kidney, many studies have investigated the effect of PKC-induced diabetic changes in other tissues that are normally involved in diabetic complications, such as the retina (retinopathy), nerves (neuropathy) and the aorta (macroangiopathy). Studies have shown an increase in PKC activity and its stimulated product diacylglycerol (DAG) in the retina of STZ-induced diabetic rats. Furthermore, exposure of retinal cells to elevated glucose levels has shown an increase in DAG. This effect could be prevented by tocopherol, an inhibitor of PKC (Kunisaki, Bursell et al. 1995). The role of PKC in the pathogenesis of diabetic neuropathy is not clear yet.

There have been studies reporting reduced PKC activity in the peripheral nerves of diabetic mice (Uehara, Yamagishi et al. 2004). Another study has shown that treatment of diabetic animals with WAY 151003, a PKC inhibitor, improves motor nerve conduction velocity (Cameron, Cotter et al. 1999). In aortic rings of STZ-induced diabetic rats, it has been shown that the administration of phorbol esters, which are activators of PKC, is associated with delayed contractions. This effect has been attributed to the interference of PKC with the opening of calcium channels (Hattori, Kawasaki et al. 1995). Inoguchi *et al.* reported that during hyperglycaemia there is an activation of PKC-β in the heart and aorta of diabetic rats (Inoguchi, Battan et al. 1992). These studies support the role of PKC activation in diabetic complications.

3.4.4 Role of PKC inhibitors

To further specify the role of PKC activation in diabetic complications, many studies have used PKC inhibitors, such as d-alpha tocopherol and ruboxistaurin (RBX). Tocopherol (vitamin E) is a potent antioxidant which has an inhibitory effect on the DAG-PKC pathway but does not inhibit PKC directly. Studies have shown that treatment of diabetic rats with tocopherol prevents glomerular hyperfiltration and minimizes the development of proteinuria (Koya, Lee et al. 1997). RBX, also known as LY333531, is a competitive inhibitor with selectivity for PKC-βI and PKC-βII. Feeding of RBX to animals with STZ-induced diabetes prevents the increase of extracellular matrix components like fibronectin and collagen in the glomeruli of diabetic rats (Koya, Jirousek et al. 1997) and reduces albuminuria and structural injury to the glomerulus (Haneda, Koya et al. 2001). Interestingly, inhibition of PKC-β by RBX also attenuated the diabetes-induced changes in the tubulointerstitium (Kelly, Zhang et al. 2003; Wu, Wu et al. 2006). Also in other models of diabetes, such as the db/db mouse and the STZ-Ren 2 rat model, RBX prevented mesangial expansion (Koya, Haneda et al. 2000) normalized glomerular hyperfiltration, decreased urinary albumin excretion and reduced glomerular TGF-β and production of ECM (Kelly, Zhang et al. 2003; Tuttle and Anderson 2003).

In summary, experimental data on the PKC-β selective inhibitor, RBX, provides supportive evidence that PKC-β activation is involved in the development of diabetic nephropathy and other forms of diabetic complications. These findings also support the potential role of RBX as a therapeutic agent for the treatment and prevention of diabetic complications.

3.4.5 Clinical trials

There have been human clinical trials of orally administered RBX to evaluate its role in the prevention of diabetic complications, which have provided conflicting results. In one study there was no effect of RBX on prevention of the progression of diabetic retinopathy (PKC-DRS, 2005). A meta-analysis of three diabetic retinopathy trials, where kidney outcomes were also documented, showed no difference between active therapy and placebo (Tuttle, McGill et al. 2007). Another study carried out on 123 patients with early diabetic nephropathy demonstrated a stabilization of diabetic nephropathy (reduction in albuminuria, stabilization in GFR) after one year of treatment with orally administered RBX (Tuttle, Bakris et al. 2005). However, the clinical implications of PKC inhibitors still need to be evaluated in larger clinical trials.

In conclusion, this study demonstrates that high plasma glucose levels correlate with GLUT2 and PKC-βI expression at the BBM of the proximal tubule. Furthermore, renal hypertrophy was linked to elevated circulating glucose levels. These findings highlight the potential significance of altered PKC-βI in diabetic tubulopathy.

Chapter 4:
Summary of Discussion

Summary of Discussion

The mechanisms of epithelial glucose transport are currently under intensive investigation. Studies in the small intestine, where transport mechanisms are similar to the proximal tubule, have demonstrated GLUT2 insertion into the BBM of enterocytes under conditions of high luminal glucose. GLUT2 recruitment is mediated via PKC-βII activation through L-type Ca^{2+} channels and sugar sensing receptors which are located at the apical membrane (Mace, Lister et al 2009). Studies elucidating glucose transport in the mesangial cells have shown elevated GLUT1 expression under conditions of hyperglycaemia *in vitro* (Heilig, Liu et al. 1997). GLUT1 overexpression leads to an increased influx of glucose which in turn triggers the activation of PKC (Brosius and Heilig 2005). Studies focusing on the glomerulus of STZ-induced diabetic rats have shown that PKC-βI seems to be the main pathophysiological isoform that is activated in hyperglycaemia (Kelly, Zhang et al. 2003). Our results demonstrate that changes in plasma glucose concentration promote GLUT2 expression at the renal proximal tubule BBM. Furthermore, GLUT2-mediated transport correlates strongly with PKC-βI protein expression at the proximal tubule BBM.

Hyperglycaemia is a key factor in the pathogenesis of diabetic nephropathy (Larkins and Dunlop 1992). Clinical studies have shown that intensive blood glucose control in diabetic patients prevents the onset and progression of diabetic nephropathy (ADVANCE: Patel, MacMahon et al. 2008). From studies focusing on mesangial cells, where typical histological changes of diabetic nephropathy are present, it has been hypothesised that increased GLUT1 expression leads to enhanced polyol pathway activity and production of PKC-βI; this, in turn, enhances the synthesis of fibronectin and other ECM proteins and TGF-β (Brosius and Heilig 2005). However, renal enlargement is one of the main pathological changes in diabetic nephropathy. Since the tubulointerstitium comprises much of the kidney substance, tubular hypertrophy might be responsible for most of this renal enlargement. Our results demonstrate that high plasma glucose leads to an increase in kidney weight. Furthermore, glucose levels correlate with expression of GLUT2 and PKC-βI at the proximal tubule BBM. These findings indicate that GLUT2 and PKC are possible factors involved in the pathogenesis of diabetic nephropathy (Fig. 4.1)

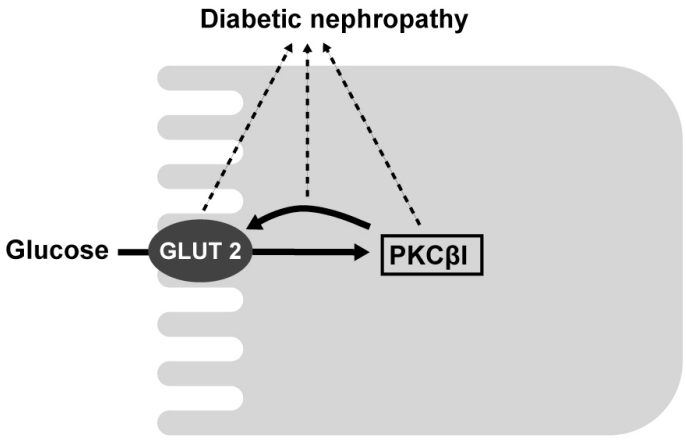

Figure 4.1: Possible involvement of GLUT2 and PKC-βI in the pathogenesis of diabetic nephropathy

List of References

(DCCT,1993). "The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group." N Engl J Med **329**(14): 977-86.

(UKPDS, 1998). "Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group." Lancet **352**(9131): 837-53.

(PKC-DRS, 2005). "The effect of ruboxistaurin on visual loss in patients with moderately severe to very severe nonproliferative diabetic retinopathy: initial results of the Protein Kinase C beta Inhibitor Diabetic Retinopathy Study (PKC-DRS) multicenter randomized clinical trial." Diabetes **54**(7): 2188-97.

Adachi, T., K. Yasuda, et al. (2000). "T-1095, a renal Na+-glucose transporter inhibitor, improves hyperglycemia in streptozotocin-induced diabetic rats." Metabolism **49**(8): 990-5.

Adler, S. (2004). "Diabetic nephropathy: Linking histology, cell biology, and genetics." Kidney Int **66**(5): 2095-106.

Asada, T., T. Ogawa, et al. (1997). "Recombinant insulin-like growth factor I normalizes expression of renal glucose transporters in diabetic rats." Am J Physiol **273**(1 Pt 2): F27-37.

Baud, L., B. Fouqueray, et al. (1992). "Reactive oxygen species as glomerular autacoids." J Am Soc Nephrol **2**(10 Suppl): S132-8.

Biber, J., B. Stieger, et al. (1981). "A high yield preparation for rat kidney brush border membranes. Different behaviour of lysosomal markers." Biochim Biophys Acta **647**(2): 169-76.

Blank, M. E., F. Bode, et al. (1985). "Kinetic studies of D-glucose transport in renal brush-border membrane vesicles of streptozotocin-induced diabetic rats." Biochim Biophys Acta **844**(3): 314-9.

Bojestig, M., H. J. Arnqvist, et al. (1994). "Declining incidence of nephropathy in insulin-dependent diabetes mellitus." N Engl J Med **330**(1): 15-8.

Bradford M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal. Biochem. 72 : 248-254

Brosius, F. C. and C. W. Heilig (2005). "Glucose transporters in diabetic nephropathy." Pediatr Nephrol **20**(4): 447-51.

Burant, C. F. and M. Saxena (1994). "Rapid reversible substrate regulation of fructose transporter expression in rat small intestine and kidney." Am J Physiol **267**(1 Pt 1): G71-9.

Cameron, N. E., M. A. Cotter, et al. (1999). "Protein kinase C effects on nerve function, perfusion, Na(+), K(+)-ATPase activity and glutathione content in diabetic rats." Diabetologia **42**(9): 1120-30.

Cersosimo, E., M. Ajmal, et al. (1997). "Role of the kidney in plasma glucose regulation during hyperglycemia." Am J Physiol **272**(5 Pt 1): E756-61.

Chen, S., M. P. Cohen, et al. (2001). "Glycated albumin stimulates TGF-beta 1 production and protein kinase C activity in glomerular endothelial cells." Kidney Int **59**(2): 673-81.

Chin, E., A. M. Zamah, et al. (1997). "Changes in facilitative glucose transporter messenger ribonucleic acid levels in the diabetic rat kidney." Endocrinology **138**(3): 1267-75.

Chin, E., J. Zhou, et al. (1993). "Anatomical and developmental patterns of facilitative glucose transporter gene expression in the rat kidney." J Clin Invest **91**(4): 1810-5.

Cochrane, S. M. and G. B. Robinson (1995). "In vitro glycation of glomerular basement membrane alters its permeability: a possible mechanism in diabetic complications." FEBS Lett **375**(1-2): 41-4.

Cohen, M. P., F. N. Ziyadeh, et al. (1999). "Glycated albumin stimulation of PKC-beta activity is linked to increased collagen IV in mesangial cells." Am J Physiol **276**(5 Pt 2): F684-90.

Colville, C. A., M. J. Seatter, et al. (1993). "Kinetic analysis of the liver-type (GLUT2) and brain-type (GLUT3) glucose transporters in Xenopus oocytes: substrate specificities and effects of transport inhibitors." Biochem J **290 (Pt 3)**: 701-6.

Corpe, C. P., M. M. Basaleh, et al. (1996). "The regulation of GLUT5 and GLUT2 activity in the adaptation of intestinal brush-border fructose transport in diabetes." Pflugers Arch **432**(2): 192-201.

Craven, P. A. and F. R. DeRubertis (1989). "Protein kinase C is activated in glomeruli from streptozotocin diabetic rats. Possible mediation by glucose." J Clin Invest **83**(5): 1667-75.

Das Evcimen, N. and G. L. King (2007). "The role of protein kinase C activation and the vascular complications of diabetes." Pharmacol Res **55**(6): 498-510.

Debnam, E. S., M. W. Smith, et al. (1995). "The effects of streptozotocin diabetes on sodium-glucose transporter (SGLT1) expression and function in rat jejunal and ileal villus-attached enterocytes." Pflugers Arch **430**(2): 151-9.

Debnam, E. S. and R. J. Unwin (1996). "Hyperglycemia and intestinal and renal glucose transport: implications for diabetic renal injury." Kidney Int **50**(4): 1101-9.

Dominguez, J. H., K. Camp, et al. (1994). "Molecular adaptations of GLUT1 and GLUT2 in renal proximal tubules of diabetic rats." Am J Physiol **266**(2 Pt 2): F283-90.

Dominguez, J. H., K. Camp, et al. (1992). "Glucose transporters of rat proximal tubule: differential expression and subcellular distribution." Am J Physiol **262**(5 Pt 2): F807-12.

Dominguez, J. H., B. Song, et al. (1994). "Gene expression of epithelial glucose transporters: the role of diabetes mellitus." J Am Soc Nephrol **5**(5 Suppl 1): S29-36.

Dong, L. Q., J. L. Stevens, et al. (1991). "Biochemical and immunological characterization of renal protein kinase C." Am J Physiol **261**(4 Pt 2): F679-87.

Dunlop, M. (2000). "Aldose reductase and the role of the polyol pathway in diabetic nephropathy." Kidney Int Suppl **77**: S3-12.

Fernandez-Alvarez, J., I. Conget, et al. (1994). "Enzymatic, metabolic and secretory patterns in human islets of type 2 (non-insulin-dependent) diabetic patients." Diabetologia **37**(2): 177-81.

Forstner, G. G., S. M. Sabesin, et al. (1968). "Rat intestinal microvillus membranes. Purification and biochemical characterization." Biochem J **106**(2): 381-90.

Freitas, H. S., B. D'Agord Schaan, et al. (2007). "Insulin but not phlorizin treatment induces a transient increase in GLUT2 gene expression in the kidney of diabetic rats." Nephron Physiol **105**(3): p42-51.

Fumo, P., G. S. Kuncio, et al. (1994). "PKC and high glucose stimulate collagen alpha 1 (IV) transcriptional activity in a reporter mesangial cell line." Am J Physiol **267**(4 Pt 2): F632-8.

Gerstein, H. C., M. E. Miller, et al. (2008). "Effects of intensive glucose lowering in type 2 diabetes." N Engl J Med **358**(24): 2545-59.

Goestemeyer, A. K., J. Marks, et al. (2007). "GLUT2 protein at the rat proximal tubule brush border membrane correlates with protein kinase C (PKC)-betal and plasma glucose concentration." Diabetologia **50**(10): 2209-17.

Gwinner, W., U. Deters-Evers, et al. (1998). "Antioxidant-oxidant balance in the glomerulus and proximal tubule of the rat kidney." J Physiol **509 (Pt 2)**: 599-606.

Ha, H. and K. H. Kim (1999). "Pathogenesis of diabetic nephropathy: the role of oxidative stress and protein kinase C." Diabetes Res Clin Pract **45**(2-3): 147-51.

Ha, H. and H. B. Lee (2000). "Reactive oxygen species as glucose signaling molecules in mesangial cells cultured under high glucose." Kidney Int Suppl **77**: S19-25.

Ha, H., M. R. Yu, et al. (2001). "Activation of protein kinase c-delta and c-epsilon by oxidative stress in early diabetic rat kidney." Am J Kidney Dis **38**(4 Suppl 1): S204-7.

Han, S., D. L. Hagan, et al. (2008). "Dapagliflozin, a selective SGLT2 inhibitor, improves glucose homeostasis in normal and diabetic rats." Diabetes **57**(6): 1723-9.

Haneda, M., R. Kikkawa, et al. (1995). "Abnormalities in protein kinase C and MAP kinase cascade in mesangial cells cultured under high glucose conditions." J Diabetes Complications **9**(4): 246-8.

Haneda, M., D. Koya, et al. (2001). "Cellular mechanisms in the development and progression of diabetic nephropathy: activation of the DAG-PKC-ERK pathway." Am J Kidney Dis **38**(4 Suppl 1): S178-81.

Harris, R. C., B. M. Brenner, et al. (1986). "Sodium-hydrogen exchange and glucose transport in renal microvillus membrane vesicles from rats with diabetes mellitus." J Clin Invest **77**(3): 724-33.

Hattori, Y., H. Kawasaki, et al. (1995). "Phorbol esters elicit Ca(2+)-dependent delayed contractions in diabetic rat aorta." Eur J Pharmacol **279**(1): 51-8.

Heilig, C. W., L. A. Concepcion, et al. (1995). "Overexpression of glucose transporters in rat mesangial cells cultured in a normal glucose milieu mimics the diabetic phenotype." J Clin Invest **96**(4): 1802-14.

Heilig, C. W., Y. Liu, et al. (1997). "D-glucose stimulates mesangial cell GLUT1 expression and basal and IGF-I-sensitive glucose uptake in rat mesangial cells: implications for diabetic nephropathy." Diabetes **46**(6): 1030-9.

Helliwell, P. A., M. Richardson, et al. (2000). "Stimulation of fructose transport across the intestinal brush-border membrane by PMA is mediated by GLUT2 and dynamically regulated by protein kinase C." Biochem J **350 Pt 1**: 149-54.

Henry, D. N., J. V. Busik, et al. (1999). "Glucose transporters control gene expression of aldose reductase, PKCalpha, and GLUT1 in mesangial cells in vitro." Am J Physiol **277**(1 Pt 2): F97-104.

Hsieh, T. J., R. Chen, et al. (2006). "Upregulation of osteopontin gene expression in diabetic rat proximal tubular cells revealed by microarray profiling." Kidney Int **69**(6): 1005-15.

Hussey, E., Dobbins R, Stolz RR et al. A Double-blind Randomized Repeat Dose Study to Assess the Safety, Tolerability, Pharmacokinetics and Pharmacodynamics of Three Times Daily Dosing of Sergliflozin, a Novel Inhibitor of Renal Glucose Reabsorption, in Healthy Overweight and Obese Subjects. Chicago, IL:American Diabetes Association, 57th Annual Scientific Sessions2007 0491-P (2007). "A Double-blind Randomized Repeat Dose Study to Assess the Safety, Tolerability, Pharmacokinetics and Pharmacodynamics of Three Times Daily Dosing of Sergliflozin, a Novel Inhibitor of Renal Glucose Reabsorption, in Healthy Overweight and Obese Subjects." IL:American Diabetes Association, 57th Annual Scientific Sessions: 0491-P.

Inoguchi, T., R. Battan, et al. (1992). "Preferential elevation of protein kinase C isoform beta II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycemic control by islet cell transplantation." Proc Natl Acad Sci U S A **89**(22): 11059-63.

Inoki, K., M. Haneda, et al. (1999). "TGF-beta 1 stimulates glucose uptake by enhancing GLUT1 expression in mesangial cells." Kidney Int **55**(5): 1704-12.

Inukai, K., H. Katagiri, et al. (1995). "Characterization of rat GLUT5 and functional analysis of chimeric proteins of GLUT1 glucose transporter and GLUT5 fructose transporter." Endocrinology **136**(11): 4850-7.

Islam, M. S. and H. Choi (2007). "Nongenetic model of type 2 diabetes: a comparative study." Pharmacology **79**(4): 243-9.

Isono, M., M. C. Cruz, et al. (2000). "Extracellular signal-regulated kinase mediates stimulation of TGF-beta1 and matrix by high glucose in mesangial cells." J Am Soc Nephrol **11**(12): 2222-30.

Isshiki, K., M. Haneda, et al. (2000). "Thiazolidinedione compounds ameliorate glomerular dysfunction independent of their insulin-sensitizing action in diabetic rats." Diabetes **49**(6): 1022-32.

Janssen, U., A. O. Phillips, et al. (1999). "Rodent models of nephropathy associated with type II diabetes." J Nephrol **12**(3): 159-72.

Johns, P. R., M. G. Yoon, et al. (1978). "Directed outgrowth of optic fibres regenerating in vitro." Nature **271**(5643): 360-2.

Kamran, M., R. G. Peterson, et al. (1997). "Overexpression of GLUT2 gene in renal proximal tubules of diabetic Zucker rats." J Am Soc Nephrol **8**(6): 943-8.

Kanai, Y., W. S. Lee, et al. (1994). "The human kidney low affinity Na+/glucose cotransporter SGLT2. Delineation of the major renal reabsorptive mechanism for D-glucose." J Clin Invest **93**(1): 397-404.

Kang, N., G. Alexander, et al. (1999). "Differential expression of protein kinase C isoforms in streptozotocin-induced diabetic rats." Kidney Int **56**(5): 1737-50.

Kapor-Drezgic, J., X. Zhou, et al. (1999). "Effect of high glucose on mesangial cell protein kinase C-delta and -epsilon is polyol pathway-dependent." J Am Soc Nephrol **10**(6): 1193-203.

Karim, Z., N. Defontaine, et al. (1995). "Protein kinase C isoforms in rat kidney proximal tubule: acute effect of angiotensin II." Am J Physiol **269**(1 Pt 1): C134-40.

Keembiyehetty, C., R. Augustin, et al. (2006). "Mouse glucose transporter 9 splice variants are expressed in adult liver and kidney and are up-regulated in diabetes." Mol Endocrinol **20**(3): 686-97.

Kellett, G. L. (2001). "The facilitated component of intestinal glucose absorption." J Physiol **531**(Pt 3): 585-95.

Kellett, G. L. and E. Brot-Laroche (2005). "Apical GLUT2: a major pathway of intestinal sugar absorption." Diabetes **54**(10): 3056-62.

Kellett, G. L., E. Brot-Laroche, et al. (2008). "Sugar absorption in the intestine: the role of GLUT2." Annu Rev Nutr **28**: 35-54.

Kellett, G. L. and P. A. Helliwell (2000). "The diffusive component of intestinal glucose absorption is mediated by the glucose-induced recruitment of GLUT2 to the brush-border membrane." Biochem J **350 Pt 1**: 155-62.

Kelly, D. J., Y. Zhang, et al. (2003). "Protein kinase C beta inhibition attenuates the progression of experimental diabetic nephropathy in the presence of continued hypertension." Diabetes **52**(2): 512-8.

Koya, D., M. Haneda, et al. (2000). "Amelioration of accelerated diabetic mesangial expansion by treatment with a PKC beta inhibitor in diabetic db/db mice, a rodent model for type 2 diabetes." Faseb J **14**(3): 439-47.

Koya, D., M. R. Jirousek, et al. (1997). "Characterization of protein kinase C beta isoform activation on the gene expression of transforming growth factor-beta, extracellular matrix components, and prostanoids in the glomeruli of diabetic rats." J Clin Invest **100**(1): 115-26.

Koya, D., I. K. Lee, et al. (1997). "Prevention of glomerular dysfunction in diabetic rats by treatment with d-alpha-tocopherol." J Am Soc Nephrol **8**(3): 426-35.

Krolewski, M., P. W. Eggers, et al. (1996). "Magnitude of end-stage renal disease in IDDM: a 35 year follow-up study." Kidney Int **50**(6): 2041-6.

Kunisaki, M., S. E. Bursell, et al. (1995). "Vitamin E prevents diabetes-induced abnormal retinal blood flow via the diacylglycerol-protein kinase C pathway." Am J Physiol **269**(2 Pt 1): E239-46.

Kuntz, E., M. Pinget, et al. (2002). "Effects of cholecystokinin octapeptide on the exocrine pancreas in a new rat model of type 2 diabetes." Eur J Pharmacol **448**(2-3): 253-61.

Larkins, R. G. and M. E. Dunlop (1992). "The link between hyperglycaemia and diabetic nephropathy." Diabetologia **35**(6): 499-504.

Le Gall, M., V. Tobin, et al. (2007). "Sugar sensing by enterocytes combines polarity, membrane bound detectors and sugar metabolism." J Cell Physiol **213**(3): 834-43.

Lee, W. S., Y. Kanai, et al. (1994). "The high affinity Na+/glucose cotransporter. Re-evaluation of function and distribution of expression." J Biol Chem **269**(16): 12032-9.

Linden, K. C., C. L. DeHaan, et al. (2006). "Renal expression and localization of the facilitative glucose transporters GLUT1 and GLUT12 in animal models of hypertension and diabetic nephropathy." Am J Physiol Renal Physiol **290**(1): F205-13.

List, J. F., V. Woo et al. (2009). "Sodium-glucose cotransport inhibition with dapagliflozin in type 2 diabetes." Diabetes Care **32**(4): 650-7

Mace, O. J., N. Lister, et al. (2009). "An energy supply network of nutrient absorption coordinated by calcium and T1R taste receptors in rat small intestine." J Physiol **587**(Pt 1): 195-210.

Mace, O. J., E. L. Morgan, et al. (2007). "Calcium absorption by Cav1.3 induces terminal web myosin II phosphorylation and apical GLUT2 insertion in rat intestine." J Physiol **580**(Pt. 2): 605-16.

Malatiali, S., I. Francis, et al. (2008). "Phlorizin prevents glomerular hyperfiltration but not hypertrophy in diabetic rats." Exp Diabetes Res **2008**: 305403.

Manolescu, A. R., K. Witkowska, et al. (2007). "Facilitated hexose transporters: new perspectives on form and function." Physiology (Bethesda) **22**: 234-40.

Marcinkowski, W., G. Zhang, et al. (1997). "Elevation of [Ca2+]i of renal proximal tubular cells and down-regulation of mRNA of PTH-PTHrP, V1a and AT1 receptors in kidney of diabetic rats." Kidney Int **51**(6): 1950-5.

Marks, J., N. J. Carvou, et al. (2003). "Diabetes increases facilitative glucose uptake and GLUT2 expression at the rat proximal tubule brush border membrane." J Physiol **553**(Pt 1): 137-45.

Marks, J., E. S. Debnam, et al. (2003). "Detection of glucagon receptor mRNA in the rat proximal tubule: potential role for glucagon in the control of renal glucose transport." Clin Sci (Lond) **104**(3): 253-8.

Masiello, P., C. Broca, et al. (1998). "Experimental NIDDM: development of a new model in adult rats administered streptozotocin and nicotinamide." Diabetes **47**(2): 224-9.

McGill, J. B., G. L. King, et al. (2006). "Clinical safety of the selective PKC-beta inhibitor, ruboxistaurin." Expert Opin Drug Saf **5**(6): 835-45.

Meier , M., M. Menne, et al. (2009). "Targeting the protein kinase C family in the diabetic kidney: lessons from analysis of mutant mice." Diabetologia **52** (5): 765-75

Meier, M., J. K. Park, et al. (2007). "Deletion of protein kinase C-beta isoform in vivo reduces renal hypertrophy but not albuminuria in the streptozotocin-induced diabetic mouse model." Diabetes **56**(2): 346-54.

Miyamoto, K., S. Tatsumi, et al. (1994). "Characterization of the rabbit intestinal fructose transporter (GLUT5)." Biochem J **303 (Pt 3)**: 877-83.

Mogyorosi, A. and F. N. Ziyadeh (1999). "GLUT1 and TGF-beta: the link between hyperglycaemia and diabetic nephropathy." Nephrol Dial Transplant **14**(12): 2827-9.

Morgan, E. L., O. J. Mace, et al. (2007). "Apical GLUT2 and Cav1.3: regulation of rat intestinal glucose and calcium absorption." J Physiol **580**(Pt. 2): 593-604.

Morgan, E. L., O. J. Mace, et al. (2003). "A role for Ca(v)1.3 in rat intestinal calcium absorption." Biochem Biophys Res Commun **312**(2): 487-93.

Murer, H. and P. Gmaj (1986). "Transport studies in plasma membrane vesicles isolated from renal cortex." Kidney Int **30**(2): 171-86.

Nath, K. A. (1992). "Tubulointerstitial changes as a major determinant in the progression of renal damage." Am J Kidney Dis **20**(1): 1-17.

Nishikawa, T., D. Edelstein, et al. (2000). "The missing link: a single unifying mechanism for diabetic complications." Kidney Int Suppl **77**: S26-30.

Norman, J. T. and L. G. Fine (1999). "Progressive renal disease: fibroblasts, extracellular matrix, and integrins." Exp Nephrol **7**(2): 167-77.

Novelli, M., M. E. Fabregat, et al. (2001). "Metabolic and functional studies on isolated islets in a new rat model of type 2 diabetes." Mol Cell Endocrinol **175**(1-2): 57-66.

Novelli, M., A. Pocai, et al. (2004). "Alteration of beta-cell constitutive NO synthase activity is involved in the abnormal insulin response to arginine in a new rat model of type 2 diabetes." Mol Cell Endocrinol **219**(1-2): 77-82.

Ohshiro, Y., R. C. Ma, et al. (2006). "Reduction of diabetes-induced oxidative stress, fibrotic cytokine expression, and renal dysfunction in protein kinase Cbeta-null mice." Diabetes **55**(11): 3112-20.

Olefsky, J. M. (2001). "Prospects for research in diabetes mellitus." Jama **285**(5): 628-32.

Orchard, T. J., J. S. Dorman, et al. (1990). "Prevalence of complications in IDDM by sex and duration. Pittsburgh Epidemiology of Diabetes Complications Study II." Diabetes **39**(9): 1116-24.

Ostenson, C. G., S. M. Abdel-Halim, et al. (1993). "Deficient activity of FAD-linked glycerophosphate dehydrogenase in islets of GK rats." Diabetologia **36**(8): 722-6.

Pari, L. and R. Saravanan (2007). "Beneficial effect of succinic acid monoethyl ester on erythrocyte membrane bound enzymes and antioxidant status in streptozotocin-nicotinamide induced type 2 diabetes." Chem Biol Interact **169**(1): 15-24.

Park, S. H., H. J. Choi, et al. (2001). "High glucose stimulates Ca2+ uptake via cAMP and PLC/PKC pathways in primary cultured renal proximal tubule cells." Kidney Blood Press Res **24**(1): 10-7.

Parker, P. J. and J. Murray-Rust (2004). "PKC at a glance." J Cell Sci 117: 131-132

Patel, A., S. MacMahon, et al. (2008). "Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes." N Engl J Med **358**(24): 2560-72.

Pfaff, I. L., H. J. Wagner, et al. (1999). "Immunolocalization of protein kinase C isoenzymes alpha, beta1 and betaII in rat kidney." J Am Soc Nephrol **10**(9): 1861-73.

Phillips, A. O., K. Morrisey, et al. (1999). "Decreased degradation of collagen and fibronectin following exposure of proximal cells to glucose." Exp Nephrol **7**(5-6): 449-62.

Phillips, A. O., R. Steadman, et al. (1997). "Exposure of human renal proximal tubular cells to glucose leads to accumulation of type IV collagen and fibronectin by decreased degradation." Kidney Int **52**(4): 973-84.

Redling, S., I. L. Pfaff, et al. (2004). "Immunolocalization of protein kinase C isoenzymes alpha, beta I, beta II, delta, and epsilon in mouse kidney." Am J Physiol Renal Physiol **287**(2): F289-98.

Reed, M. J., K. Meszaros, et al. (2000). "A new rat model of type 2 diabetes: the fat-fed, streptozotocin-treated rat." Metabolism **49**(11): 1390-4.

Ritz, E. and S. R. Orth (1999). "Nephropathy in patients with type 2 diabetes mellitus." N Engl J Med **341**(15): 1127-33.

Ritz, E. and A. Stefanski (1996). "Diabetic nephropathy in type II diabetes." Am J Kidney Dis **27**(2): 167-94.

Santer, R., R. Schneppenheim, et al. (1998). "Fanconi-Bickel syndrome--the original patient and his natural history, historical steps leading to the primary defect, and a review of the literature." Eur J Pediatr **157**(10): 783-97.

Schena, F. P. and L. Gesualdo (2005). "Pathogenetic mechanisms of diabetic nephropathy." J Am Soc Nephrol **16 Suppl 1**: S30-3.

Schiffer, M., K. Susztak, et al. (2005). "Localization of the GLUT8 glucose transporter in murine kidney and regulation in vivo in nondiabetic and diabetic conditions." Am J Physiol Renal Physiol **289**(1): F186-93.

Scivittaro, V., M. B. Ganz, et al. (2000). "AGEs induce oxidative stress and activate protein kinase C-beta(II) in neonatal mesangial cells." Am J Physiol Renal Physiol **278**(4): F676-83.

Sharma, K., P. McCue, et al. (2003). "Diabetic kidney disease in the db/db mouse." Am J Physiol Renal Physiol **284**(6): F1138-44.

Sharp, P. A. and E. S. Debnam (1994). "The role of cyclic AMP in the control of sugar transport across the brush-border and basolateral membranes of rat jejunal enterocytes." Exp Physiol **79**(2): 203-14.

Shen, G. X. (2003). "Selective protein kinase C inhibitors and their applications." Curr Drug Targets Cardiovasc Haematol Disord **3**(4): 301-7.

Thorens, B. (1996). "Glucose transporters in the regulation of intestinal, renal, and liver glucose fluxes." Am J Physiol **270**(4 Pt 1): G541-53.

Tuttle, K. R. and P. W. Anderson (2003). "A novel potential therapy for diabetic nephropathy and vascular complications: protein kinase C beta inhibition." Am J Kidney Dis **42**(3): 456-65.

Tuttle, K. R., G. L. Bakris, et al. (2005). "The effect of ruboxistaurin on nephropathy in type 2 diabetes." Diabetes Care **28**(11): 2686-90.

Tuttle, K. R., J. B. McGill, et al. (2007). "Kidney outcomes in long-term studies of ruboxistaurin for diabetic eye disease." Clin J Am Soc Nephrol **2**(4): 631-6.

Uehara, K., S. Yamagishi, et al. (2004). "Effects of polyol pathway hyperactivity on protein kinase C activity, nociceptive peptide expression, and neuronal structure in dorsal root ganglia in diabetic mice." Diabetes **53**(12): 3239-47.

Vestri, S., M. M. Okamoto, et al. (2001). "Changes in sodium or glucose filtration rate modulate expression of glucose transporters in renal proximal tubular cells of rat." J Membr Biol **182**(2): 105-12.

Whiteside, C. I. and J. A. Dlugosz (2002). "Mesangial cell protein kinase C isozyme activation in the diabetic milieu." Am J Physiol Renal Physiol **282**(6): F975-80.

Williams, M. and P. Sharp (2002). "Regulation of jejunal glucose transporter expression by forskolin." Biochim Biophys Acta **1559**(2): 179-85.

Wolf, G. and F. Thaiss (1995). "Hyperglycaemia--pathophysiological aspects at the cellular level." Nephrol Dial Transplant **10**(7): 1109-12.

Wolf, G. and F. N. Ziyadeh (1997). "The role of angiotensin II in diabetic nephropathy: emphasis on nonhemodynamic mechanisms." Am J Kidney Dis **29**(1): 153-63.

Wu, Y., G. Wu, et al. (2006). "Protein kinase C beta inhibitor LY333531 attenuates intercellular adhesion molecule-1 and monocyte chemotactic protein-1 expression in the kidney in diabetic rats." J Pharmacol Sci **101**(4): 335-43.

Yamamoto, H., Y. Uchigata, et al. (1981). "Streptozotocin and alloxan induce DNA strand breaks and poly(ADP-ribose) synthetase in pancreatic islets." Nature **294**(5838): 284-6.

Yasuda, H., T. Kurokawa, et al. (1990). "Decreased D-glucose transport across renal brush-border membrane vesicles from streptozotocin-induced diabetic rats." Biochim Biophys Acta **1021**(2): 114-8.

Ziyadeh, F. N. (1993). "The extracellular matrix in diabetic nephropathy." Am J Kidney Dis **22**(5): 736-44.

Ziyadeh, F. N., P. Fumo, et al. (1995). "Role of protein kinase C and cyclic AMP/protein kinase A in high glucose-stimulated transcriptional activation of collagen alpha 1 (IV) in glomerular mesangial cells." J Diabetes Complications **9**(4): 255-61.

Anhang

Selbständigkeitserklärung

Erklärung

„Ich, Anne-Katrin Göstemeyer, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: „Glucose transport at the renal brush border membrane: GLUT2 and the involvement of PKC- βI “ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

CURRICULUM VITAE

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Publikationsliste

Goestemeyer, A. K., J. Marks, et al. (2007). "GLUT2 protein at the rat proximal tubule brush border membrane correlates with protein kinase C (PKC)-betal and plasma glucose concentration." Diabetologia **50**(10): 2209-17.

Hermle T, Goestemeyer AK, Sweny P, Burns A. (2007). “Succesful therapeutic use of rituximab in refractory Wegener´s granulomatosis after transplantation”
Clin Nephrol. **68**(5): 322-6

05/06
Physiology Society Meeting Manchester
Poster: “GLUT2 expression at the rat proximal tubule brush border membrane correlates with plasma glucose concentration”

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JASN Meeting Philadelphia
Poster: “GLUT2 transporter expression at the proximal tubule brush border membrane correlates with plasma glucose concentration”