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Title: Type II diabetes and related models of impaired glucose metabolism differentially regulate glucose transporters at the proximal tubule brush border

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Running Title: GLUT2, SGLT1 and SGLT2 in diabetes-related disease

Abstract: Objective: SGLT2 inhibitors are now in clinical use to reduce hyperglycemia in type II diabetes. However, renal glucose reabsorption across the brush border membrane (BBM) is not completely understood in diabetes. Increased consumption of a Western diet is strongly linked to type II diabetes. This study aimed to investigate the adaptations that occur in renal glucose transporters in response to experimental models of diet-induced insulin resistance. Research Design and Methods: The study used Goto-Kakizaki type II diabetic rats and normal rats rendered insulin resistant using junk-food or high-fat (HFD) diets. Levels of PKC-βI, GLUT2, SGLT1 and 2 were determined by western blotting of purified renal BBM. GLUT- and SGLT-mediated [3H]-glucose uptake by BBM vesicles was measured in the presence and absence of the SGLT inhibitor phlorizin. Results: GLUT- and SGLT-mediated glucose transport were elevated in type II diabetic rats, accompanied by increased expression of GLUT2, its upstream regulator PKC-βI, and SGLT1 protein.

Junk-food and HFD feeding also caused higher membrane expression of GLUT2 and its upstream regulator PKC-βI. However, the junk-food diet also increased SGLT1 and 2 levels at the proximal tubule BBM. Conclusions: Glucose reabsorption across the proximal tubule BBM, via GLUT2, SGLT1 and 2, is not solely dependent on glycemic status, but is also influenced by diet-induced changes in glucose metabolism. We conclude that different metabolic disturbances result in complex adaptation in renal glucose transporter protein levels and function.

New Findings: While SGLT2 inhibitors represent a promising treatment for patients suffering from diabetic nephropathy, the role of metabolic disruption on the expression and function of glucose transporters is largely unknown. In vivo models of metabolic disruption (type II rat and junk-food diet) demonstrate increased expression of SGLT1, SGLT2 and GLUT2 in the proximal tubule brush-border. In the type II model, this is accompanied by increased SGLT- and GLUT-mediated glucose uptake. A fasted model of metabolic disruption (high-fat diet) demonstrated increased GLUT2 expression only. The differential alterations in glucose transporters in response to varying metabolic stress offer insight into therapeutic values of inhibitors.

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Experimental type II diabetes and related models of impaired glucose metabolism differentially regulate glucose transporters at the proximal tubule brush border membrane

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In vivo models of metabolic disruption (Goto-kakizaki type II rat and junk-food diet) demonstrate increased expression of SGLT1, SGLT2 and GLUT2 in the proximal tubule brush-border. In the type II model, this is accompanied by increased SGLT- and GLUT-mediated glucose uptake. A fasted model of metabolic disruption (high-fat diet) demonstrated increased GLUT2 expression only. The differential alterations in glucose transporters in response to varying metabolic stress, offer insight into the therapeutic value of inhibitors.

Abstract

Objective: SGLT2 inhibitors are now in clinical use to reduce hyperglycemia in type II diabetes. However, renal glucose reabsorption across the brush border membrane (BBM) is not completely understood in diabetes. Increased consumption of a Western diet is strongly linked to type II diabetes. This study aimed to investigate the adaptations that occur in renal glucose transporters in response to experimental models of diet-induced insulin resistance.

Research Design and Methods: The study used Goto-Kakizaki type II diabetic rats and normal rats rendered insulin resistant using junk-food or high-fat (HFD) diets. Levels of PKC-βI, GLUT2, SGLT1 and 2 were determined by western blotting of purified renal BBM. GLUT- and SGLT-mediated [^{3}H]-glucose uptake by BBM vesicles was measured in the presence and absence of the SGLT inhibitor phlorizin.

Results: GLUT- and SGLT-mediated glucose transport were elevated in type II diabetic rats, accompanied by increased expression of GLUT2, its upstream regulator PKC-βI, and SGLT1 protein. Junk-food and HFD feeding also caused higher membrane expression of GLUT2 and its upstream regulator PKC-βI. However, the junk-food diet also increased SGLT1 and 2 levels at the proximal tubule BBM.

Conclusions: Glucose reabsorption across the proximal tubule BBM, via GLUT2, SGLT1 and 2, is not solely dependent on glycemic status, but is also influenced by diet-induced changes in glucose metabolism. We conclude that different metabolic disturbances result in complex adaptation in renal glucose transporter protein levels and function.

Introduction

Diabetic nephropathy is a major complication of diabetes and a leading cause of end-stage renal disease. Chronic hyperglycemia is associated with tubulo-interstitial changes that accompany progressive renal dysfunction . While it has been difficult to show a clear cardiovascular benefit from tighter glycemic control in diabetes, at least in the short-term (Boot-Handford & Heath, 1981; Phillips *et al.*, 1997), available evidence favors a long-term reduction in hyperglycemia as an important treatment goal in preventing nephropathy (Dluhy & McMahon, 2008; Duckworth *et al.*, 2009). Thus, renal glucose reabsorption has been considered to have a pathophysiological role in diabetes (Debnam & Unwin, 1996).

In euglycemic conditions, the majority of filtered glucose is reabsorbed by the *high-capacity* transporter SGLT2 at the brush border membrane (BBM) of the early proximal convoluted tubule, while the remainder is scavenged by the *high-affinity* SGLT1 in the late proximal straight tubule (Turner & Silverman, 1977; Barfuss & Schafer, 1981; Cramer *et al.*, 1992; Brown, 2000). Adaptation of glucose transport at the BBM has been documented in diabetes; in particular, the facilitated transporter, GLUT2, is readily detectable at the BBM in animals 2-4 weeks after induction of type I diabetes (Marks *et al.*, 2003). Indeed, expression of GLUT2 shows a positive correlation with blood glucose levels, and it has been proposed that increased renal GLUT2 expression at the BBM is mediated by PKC-βI, since levels of this signalling molecule also show a positive correlation with both expression of

GLUT2 and blood glucose levels (Goestemeyer *et al.*, 2007). While there are conflicting reports of the impact of diabetes on SGLT-mediated transport (Marks *et al.*, 2003; Albertoni Borghese *et al.*, 2009), inhibitors of SGLT2 have been demonstrated to increase glycosuria and reduce hyperglycemia in type II diabetes (Han *et al.*, 2008). Indeed, Dapagliflozin, Canaglifozin and Empaglifozin, chemical inhibitors of this renal-specific glucose transporter, have all been approved for use in Europe as insulin-independent treatments for type II diabetes. However, the glucose transporters SGLT1 and GLUT2 also play a role in glucose reabsorption across the proximal tubule brush-border (Marks *et al.*, 2003; Hummel *et al.*, 2011), and may represent additional targets for the control of hyperglycemia in diabetes. Consistent with this, studies under euglycemic conditions indicate that SGLT1-mediated renal glucose transport increases to compensate for SGLT2 knockdown (Rieg *et al.*, 2014). Thus, the role of glucose transporters at the proximal tubule BBM in diabetes requires further study.

Western diets have changed dramatically over the last 30 years with a significant increase in consumption of calorie-dense processed foods. There is a general consensus that the rising prevalence of obesity, type II diabetes, and its essential prerequisite, insulin resistance, is related to consumption of these processed and calorie-dense foods, rich in saturated fat and carbohydrates with a high glycemic index (Bayol *et al.*, 2005; Fulgoni, 2008). Diet-induced obesity is a major risk factor for development of the metabolic syndrome, a disorder characterized by impaired glucose tolerance, hyperuricemia, hypertriglyceridemia, and hypertension, and which is considered to be pre-diabetic (Aguilar-Salinas *et al.*, 2005; Junien & Nathanielsz, 2007; AlSaraj *et al.*, 2009). Increased consumption of saturated and trans-saturated

fats and carbohydrates has also been shown to adversely affect glucose metabolism and induce insulin resistance (Moeller *et al.*, 2009). Consumption of artificial sweeteners such as sucralose and saccharin, has also increased significantly in recent years (Mattes & Popkin, 2009; Yang, 2010). Interestingly, both experimental studies and meta-analyses have linked the consumption of artificial sweeteners with the development of glucose intolerance (Pepino *et al.*, 2013; Suez *et al.*, 2014). Therefore, the Western diet likely contributes to the development of insulin resistance and type II diabetes through disturbances in glucose homeostasis that are determined more by its actual composition.

Since the kidneys play a major role in glucose homeostasis, the aim of the present study was to characterize glucose transport at the proximal tubule BBM in experimental models associated with metabolic disturbance. Models of early type I diabetes, type II diabetes, diet-induced obesity and insulin resistance and exposure to artificial sweetener were utilized. Studies on type II diabetes used Goto-Kakizaki (GK) rats, an established non-obese model of the condition characterized by glucose intolerance and impaired insulin secretion (Goto *et al.*, 1976). Since type II diabetes in man has been attributed to the high consumption of “junk-foods” in the Western diet (Hu *et al.*, 2001), an earlier stage in type II diabetes pathogenesis was studied using rats maintained either on a cafeteria diet composed of processed foods or a defined high-fat diet. Finally, animals acutely exposed to the artificial sweetener saccharinwere compared. Our studies demonstrate a differential expression profile of glucose transporters at the BBM in response to these various forms/stages of metabolic disease. We propose that specific glucose transporters could be targeted at the proximal tubule BBM to attenuate glucose reabsorption and potentially lessen

hyperglycemia in diabetes or pre-diabetes, and may even provide eventual renoprotection.

Methods

Ethical approval

All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 Amendment Regulations 2012. The protocol was approved by the University College London (Royal Free Campus) Comparative Biology Unit Animal Welfare and Ethical Review Body (AWERB) committee.

Animal models

Type I diabetes was induced by a single tail vein injection of streptozotocin (STZ) (55 $mg.kg^{-1}$) administered to male Sprague-Dawley rats (200-230 g) 7 days prior to experimentation. This protocol was chosen because hyperglycemia of at least 7 day duration is required for detection of increased GLUT2 expression at the BBM (data not shown). STZ was freshly prepared in 0.05 M citrate buffer and administered under light inhaled isofluorane anesthesia (2% isofluorane in 100% oxygen, non-terminal). Control animals received an injection of citrate buffer. Type II diabetes was studied in 8-9 week old non-obese Goto-Kakizaki (GK) rats (Goto *et al.*, 1976) purchased from Charles River, USA, and compared to Wistar controls.

Type I and Type II diabetic animals were allowed *ad libitum* access to a standard rat chow (diet RM1, SDS Ltd, Witham, Essex, UK) and water. For 'junk-food' ('cafeteria diet') feeding studies, male Wistar rats were allowed *ad libitium* access to water and either a standard rat maintenance diet (Diet RM3, SDS Ltd) or chow supplemented with junk-food over an 8-week period (Rothwell & Stock, 1979; Bayol *et al.*, 2005). The junk-food diet consisted of a choice of palatable, processed foods with high fat

and/or high sugar content, comprised of potato crisps (5.7% of consumed food), flapjacks (12.3% of consumed food), cheese (4.9% of consumed food), marshmallows (6.9% of consumed food), muffins (12.1% of consumed food), doughnut (19.8% of consumed food), biscuits (31.1% of consumed food) and chocolate bars (7.2% of consumed food). Daily energy consumption for chow fed and junk-food fed animals was 17.5 kcal (73.2 kJ) and 198 kcal (830.6 kJ), respectively. For the high-fat diet model, male Wistar rats (125-150 g) were purchased from Charles River (UK) and had *ad libitum* access to a 60% fat-as-calories chow (R12492, Research Diets, New Brunswick, USA) over a 5-week period or maintenance diet. Rats maintained on a high-fat diet were fasted overnight prior to being euthanised by exposure to gradually increasing concentration of carbon dioxide. At the end of the diabetic or junk feeding protocols, unfasted animals were terminally anesthetized with an intraperitoneal (i.p.) injection of pentobarbitone sodium (60 $mg.kg^{-1}$) (Pentoject, Animalcare Ltd, York, UK). Monitoring of the pedal and corneal reflex was undertaken to ensure deep anaesthesia was achieved before blood was taken by cardiac puncture, and the kidneys removed. Death after exsanguination was ensured by incising the heart. Cortical fragments were dissected at $4^{o}C$ and either snap-frozen for BBM preparation for Western blotting studies or BBM vesicles were freshly prepared for glucose uptake studies.

Plasma glucose concentration was measured using the glucose oxidase assay (Huggett & Nixon, 1957) and plasma insulin was quantified using a sandwich ELISA kit (Millipore, UK).

Animal procedures - Infusion studies

For infusion experiments, 8-9 week old male Sprague-Dawley rats, allowed *ad libitum* access to RM1 diet containing 65% carbohydrate, were anesthetized with an i.p. injection of pentobarbitone sodium (60 $mg.kg^{-1}$) and the jugular vein and bladder were cannulated. Test animals were infused with 154 mM NaCl at 5.5 $ml.h^{-1}$ for 1 h, followed by 1 mM saccharin in 154 mM NaCl for a further 2 h, while control animals continued to receive 154 mM NaCl. At the end of the infusion protocol blood was collected by cardiac puncture and the kidneys removed. Cortical fragments were dissected at 4^{o}C and BBM vesicles immediately prepared for studies of glucose uptake.

Preparation of renal BBM and glucose uptake studies

BBM vesicles were prepared from kidney cortex, using a double Mg^{2+} chelation protocol, as described previously (Marks *et al.*, 2003). Purity of the BBM preparation was confirmed by 6-8-fold enrichment of alkaline phosphatase. Protein concentration was determined using a Bradford assay (Bradford, 1976). Uptake studies were carried out as described previously (Marks *et al.*, 2003). In brief, the transport process was initiated by mixing equal volumes of vesicle suspension and uptake buffer consisting of (mM) 200 NaSCN, 20 Hepes, 0.1 MgSO4 containing D-[^{3}H]glucose and such that the final concentration of glucose was 30-960 μM to determine the transport kinetics of SGLT-mediated glucose transport. Uptake was terminated after 4 s by the addition of 3 ml of 154 mM NaCl containing 0.5 mM phlorizin, followed by vacuum filtration through 0.45 μm nitrocellulose filters (Sartorius, Germany). Three further washes were carried out. In order to assess GLUT-mediated transport, a higher glucose concentration (20 mM) was used in the presence of 1 mM phlorizin - this value is consistent with the low affinity of GLUT

transporters for glucose binding (Debnam & Unwin, 1996). The kinetic parameters of V_{max} (maximum transport capacity) and K_t (glucose concentration at half V_{max}) for phlorizin-sensitive uptake were derived using Lineweaver-Burk plot analysis of uptake date obtained using a glucose concentration in the μM range..

Western blotting

Western blotting of BBM was carried out as previously described (Marks J, 2003, 553, 137) using rabbit polyclonal antibodies raised against SGLT1 (a gift from Prof. G. Kellett, University of York, UK), SGLT2 (Santa Cruz Biotechnology, Santa Cruz, USA), GLUT2 (AbD Serotec, Kidlington, UK) and PKC-βI (Santa Cruz). Mouse monoclonal antibody for β-actin (Abcam, Cambridge, UK) was used as a loading control. Blots were visualized with enhanced chemiluminescence on a Fluor-S MultiImager system (BioRad, Hertfordshire, UK), and the abundance of each protein of interest was calculated relative to actin and expressed as a percentage of the control average.

Statistics

Values are expressed as mean ± S.E.M.; *n* values represent numbers per treatment group (i.e. 1 animal is equivalent to an n value). For each experiment, a comparison between metabolic-treatment group was made relative to control group. Studies were performed to ensure that both control and metabolic-treatment group were studied in at the same time point. Differences between groups were tested by Student's unpaired *t* test, with $p<0.05$ considered significant.

Results

Type II diabetes increases SGLT- and GLUT-mediated transport of glucose across the BBM

Long-term type I diabetes increases glucose transport across the proximal tubule BBM through enhanced levels of GLUT2 (Marks *et al.*, 2003). Our first aim was to assess the effect of experimental type II diabetes on renal glucose transport. In keeping with previously published observations (Noll *et al*., 2011), at 8-9 weeks of age GK rats weighed significantly less than control Wistar rats and had an increased plasma glucose concentration (Table 1) and glycosuria (data not shown), however, the increase in plasma insulin levels in GK rats did not reach significance (Table 1).

BBM vesicles isolated from the kidneys of Wistar control rats were enriched in alkaline phosphatase (6.26 ± 1.04-fold, n = 6) and type II diabetes had no significant effect on these values (6.26 ± 0.68-fold, n = 6). As previously reported, glucose uptake studies revealed the expected time dependent overshoot, which was blocked by phlorizin (data not shown) (Marks *et al.*, 2003). Vesicle-trapped space, as determined by incubation of BBM vesicles with 100 mM ^{3}H glucose for 15 min, was unaffected by type II diabetes (control: 2.84 ± 0.66 vs. GK: 3.60 ± 0.64 µl/mg protein, n = 6). At increasing concentrations of glucose, uptake across the BBM was significantly higher in GK rats compared with control animals (Figure 1a). The V_{max} for SGLT-mediated glucose uptake was 66% greater in BBM prepared from kidneys from type II diabetic rats (control: 1426.1 ± 66.3 vs. GK: 2364.6 ± 433.2 pmol.mg protein^{-1}, n = 6-8, $P<0.05$) and K_t increased by 80% (control: 147.1 ± 21.6 vs. GK: 264.3 ± 46.1 µM, n = 6-8, $P<0.05$). Phlorizin-insensitive, GLUT-mediated glucose

transport, measured at 20 mM, was 130% greater in BBM prepared from Type II diabetic animals (Figure 1b).

Western blotting revealed higher levels of GLUT2, SGLT1, and PKC-βI in BBM vesicles prepared from GK rats (Figure 2a). As previously demonstrated (Marks *et al.*, 2003; Goestemeyer *et al.*, 2007), exposure to streptozotocin induced hyperglycaemia (Table 1). Interestingly, while GLUT2 and PKC-βI expression at the BBM was significantly increased, SGLT1 and SGLT2 levels were unaffected one week after STZ injection (Figure 2b). Therefore our data demonstrate that renal glucose transport by SGLT1 and GLUT2 is upregulated at the BBM in a non-obese, hyperglycemic model of type II diabetes, but that there are differences in the adaptation of renal glucose transport induced in models of type I and type II diabetes.

Differential expression profile of glucose transporters at the renal BBM in rodent models of diet-induced obesity and insulin resistance

We next sought to examine whether diet-induced models of obesity and insulin resistance affect glucose transporter expression at the renal BBM using two well-characterized models of diet-induced obesity (Bayol *et al.*, 2005; Anderson *et al.*, 2009). Both cafeteria diet and high-fat diet fed rats gained significantly more body weight and displayed elevated plasma glucose and insulin levels versus their chow-fed control groups (Table 1). Western blotting of BBM prepared from rats maintained on both the junk-food and high-fat diet revealed significantly higher GLUT2 levels compared with chow fed animals, which was accompanied by increased levels of PKC-βI (Figure 2c and d, respectively). Interestingly, BBM protein levels of SGLT1

and 2 was significantly increased in animals fed the junk-food, but not high-fat, diet (Figure 1c and d, respectively). These data demonstrate that expression of glucose transporters at the proximal tubule BBM is dependent on the model of diet-induced obesity used and is not solely regulated by glycemia or insulinemia.

The artificial sweetener saccharin regulates SGLT-mediated glucose transport at the renal BBM

Activation of sweet taste receptors, localized to the small intestine, have been proposed to regulate intestinal glucose transporter expression via activation of sweet taste receptors (Mace *et al.*, 2007; Mace *et al.*, 2009). Given the similarity in renal glucose handling in the intestine and kidney we wanted to establish whether short-term i.v. infusion of the artificial sweetener, saccharin, influenced renal glucose transport. A 2 hour infusion of 1 mM saccharin had no effect on plasma glucose concentration (Table 1). As expected, BBM vesicles isolated from the kidneys of saline-infused rats and saccharin-infused rats were enriched in alkaline phosphatase (6.81 ± 0.61-fold, n = 6 and 6.64 ± 0.57-fold, n = 6 respectively) and vesicle-trapped space was also unaffected by saccharin (in µl/mg protein), saline: 1.49 ± 0.24 *vs.* saccharin: 1.44 ± 0.21, n = 6). The V_{max} for SGLT-mediated glucose uptake was 143% greater in the renal BBM of saccharin infused rats (control: 959.4 ± 90.5 vs. saccharin: 2333 ± 447 pmole.mg protein^{-1}, n = 5, $P<0.05$), whilst the K_t was unaffected (control: 947.4 ± 120.9 vs. saccharin: 1282.4 ± 329 µM, n = 5) (Figure 3a). Western blotting revealed that SGLT1, but not SGLT2, expression was significantly increased at the proximal tubule BBM (Figure 4). Renal GLUT-mediated glucose transport, measured using 20 mM glucose, was unaffected by

saccharin infusion (Figure 3b) and in keeping with this finding, protein levels of GLUT2 and its upstream regulator PKC-βI were also unchanged (Figure 4).

To establish whether sweet taste receptors might mediate the effect of saccharin on SGLT1-dependent glucose transport, we next studied the mRNA expression of sweet taste receptors in the rat kidney. Using RNA from rat small intestine as a positive control, qPCR revealed that T1R2, T1R3 and their downstream G-protein, alpha-gustducin, were not expressed in the rat kidney (data not shown). This finding is in keeping with RNA-seq data published by Lee *et al,* who, using microdissected tubular segments, were unable to demonstrate expression of these receptors in any part of the mouse kidney (Lee *et al.*, 2015b). In conflict with this RNA data, the antibodies used by Mace *et al* (Mace *et al.*, 2007; Mace *et al.*, 2009) to investigate the effect of saccharin on intestinal taste receptor expression (purchased from Santa Cruz Biotechnology) were able to detect a strong signal in renal BBM vesicles. However the use of these antibodies in immunohistochemistry demonstrated that there was a high degree of non-specific binding, with no particular region of the kidney showing positive staining. Taken together our findings suggest a role for saccharin in enhancing renal BBM glucose transport through SGLT1, which unlike in the small intestine, appears to be independent of the sweet taste receptor T1R2/3.

Discussion

Diabetic nephropathy is an important late complication of diabetes, especially when glycaemic status has been poorly controlled. The use of SGLT2 inhibitors is a new treatment modality for maintaining glycemic control in patients with type II diabetes, and although it has been suggested that this approach may also limit the

long-term renal consequences of diabetic nephropathy, to date it has not been shown to provide unequivocal 'renoprotection' other than by improving blood sugar control. Lack of more direct renoprotection might be a consequence of adaptation in glucose transport mechanisms in response to SGLT2 inhibition. For example it is known that in healthy volunteers inhibiting SGLT2 reduces reabsorption of filtered glucose by only ~ 30–50% (Liu *et al.*, 2012), which may be due to scavenging of the abnormally high glucose levels reaching the late proximal tubule by SGLT1 (Brown, 2000; Wright, 2001). However, this explanation is largely based on studies in non-diabetic mice and does not take account of the potential for additional adaptive changes in renal glucose transport that may occur in diabetes. In this context, it has also been shown by Vallon *et al* that although SGLT2 gene knockout reduces glycemia in experimental type I diabetes, lack of SGLT2 did not abrogate the effects of diabetes on renal growth or markers of renal injury, inflammation and fibrosis (Vallon *et al.*, 2013); others have made similar findings for SGLT2 inhibition (O'Neill *et al.*, 2015). Thus, understanding how glucose is transported across the renal BBM and how the process is upregulated in diabetes and pre-diabetes may offer new insights for reducing potential damaging effects of increased glucose load and transport on tubular function.

The present study used animal models of diabetes and models of diet-induced obesity and insulin resistance of differing aetiology to assess the effects of insulin resistance and hyperglycaemia on expression of glucose transporters at the renal BBM. Our previous findings have shown that expression of GLUT2 and its regulator, PKC-βI, are increased in a model of established type I diabetes (Marks *et al.*, 2003), and that their expression levels correlate with changes in glucose concentration

within the pathophysiological range (Goestemeyer *et al.*, 2007). In keeping with these findings, we have demonstrated that expression of GLUT2 and PKC-βI at the renal BBM is higher in type II diabetes and after one week induction of type I diabetes. However, we also show increased levels of both GLUT2 and PKC-βI at the BBM in animals maintained on junk-food and high-fat diets, even though these animals demonstrated only mild elevations in plasma glucose. The high renal GLUT2 expression in the absence of a pronounced glycemic stimulus suggests that increased glucose uptake across the BBM can occur in models of insulin resistance and impaired glucose tolerance (pre-diabetes), a state known to precede the onset of type II diabetes.

Also in agreement with our previous studies using long-term type 1 diabetic rats (Marks *et al.*, 2003), we observed no alteration in SGLT1 or SGLT2 expression at the renal BBM in response to recent induction of type I diabetes. However, in the GK model of type II diabetes, the capacity for SGLT-mediated glucose transport was augmented and this was accompanied by a rise in the level of SGLT1, but not SGLT2, protein. Studies using exfoliated proximal tubular epithelial cells obtained from the urine of type 2 diabetic patients also show increased SGLT-mediated glucose transport, but this was attributed to an increase in SGLT2 activity as a result of enhanced mRNA and protein levels (Rahmoune *et al.*, 2005). In addition, some studies in genetic mouse models of both type I (Akita mice) and type II (db/db mice) diabetes also report increased renal SGLT2 expression and SGLT activity respectively (Arakawa *et al.*, 2001; Vallon *et al.*, 2013). However, it is important to note that numerous studies over the past 30 years have provided conflicting results on diabetes-induced changes in sodium-dependent glucose transport, and SGLT

transporter expression (Debnam & Unwin, 1996; Poulsen *et al.*, 2015), with discrepancies being attributed to the dose of STZ used to induce type I diabetes, the use of chemical *vs* genetic models of diabetes, and the severity of the model being studied. The complexity of these changes in renal glucose transporter expression is also highlighted by the differing transporter expression profiles observed in our junk-food and high-fat feed models. As with GLUT2, renal SGLT1 and SGLT2 expression does not follow a clear pattern with respect to plasma glucose concentrations or levels of insulin. Furthermore, the differential effects of a junk-food or high-fat diet on expression of SGLTs and GLUT2 suggest that these transporters are not regulated by the same factor(s) in these different dietary models.

At present the identity of this factor(s) is unknown; however, given the similarity between the process of glucose transport across enterocytes and renal tubular cells, parallels can be drawn over regulation of glucose transport and the possibility of glucose 'sensing' by the kidney. Sweet taste receptors are transmembrane G-protein-coupled receptors formed by heterodimerisation of T1R2 and T1R3; this heterodimer is sensitive to a variety of different sweet taste molecules including sugars, artificial sweeteners and sweet proteins (Li *et al.*, 2002). Recent studies suggest that activation of sweet taste receptors in intestinal epithelium enhances both SGLT1 expression and GLUT2 insertion into the BBM during carbohydrate digestion (Mace *et al.*, 2007; Mace *et al.*, 2009). Intestinal sweet taste receptor activation by artificial sweeteners regulates glucose-dependent GLP1 secretion from L cells, but not GIP secretion from K cells (Jang *et al.*, 2007; Parker *et al.*, 2009). Interestingly, although our studies demonstrate that the artificial sweetener saccharin enhances renal SGLT-mediated glucose transport and SGLT1 protein expression, we were unable to detect

T1R2, T1R3 and the downstream signalling protein alpha-gustducin in the kidney by qPCR. While numerous sensory receptors have been reported to be expressed in the kidney (including taste receptors) (Rajkumar *et al.*, 2014), recent RNAseq data using microdissected tubular segments was unable to demonstrate expression of these sweet taste receptors in any part of the mouse kidney (Lee *et al.*, 2015b). Thus it is unlikely that the proximal tubule response to saccharin results from 'classical' sweet taste sensing.

An alternative glucose sensor, SGLT3, is expressed in the submucosal and myenteric plexuses of the small intestine (Diez-Sampedro *et al.*, 2003) and duodenal enterochromaffin cells (Lee *et al.*, 2015a). Recent studies have shown that SGLT3 is involved in the coordinated modulation of glucose absorption (via an increase in distal jejunal SGLT1 expression) and GLP-1 secretion (Lee *et al.*, 2015a; Pal *et al.*, 2015), with these effects occurring via a vagally-mediated pathway (Pal *et al.*, 2015). However, although SGLT3 may play a significant role in glucose sensing in the intestine, it is unlikely to be responsible for the increased renal SGLT1 activity in response to artificial sweeteners, since, compared with intestinal SGLT3 expression, renal levels of SGLT3 mRNA are extremely low (Barcelona *et al.*, 2012) or undetectable (Matus Sotak, personal communication).

A potential mechanism through which glucose transporter expression is regulated at the proximal tubule BBM is through the local action of insulin. The insulin receptor is present throughout the nephron, including in the proximal tubule, with higher expression at the basolateral membrane than at the BBM (Feraille *et al.*, 1995). The action of SGLT2 inhibitors on renal glucose handling is notably independent on insulin

action, but this is not the case for GLUT2 activity. Lowered circulating level of insulin in type I diabetes might provide a stimulus for recruitment of GLUT2 to the BBM, an action of the hormone that has been noted in jejunal enterocytes (Tobin *et al.*, 2008), but would not explain the raised GLUT2 levels at the BBM in the prediabetic state seen following feeding of junk-food or high-fat diet. Therefore, GLUT2 expression at the renal BBM, like SGLT expression, is likely to be mediated through an insulin–independent pathway in metabolic conditions.

The composition of diets has been demonstrated to play a role in glucose transporter expression in the small intestine. Consumption of a high-starch/low-fat pellet diet, by rats, increases jejunal gene expression of SGLT1 and GLUT2 compared with a low-starch/high-fat diet, and is likely to increase glucose transport across the intestinal brush-border (Inoue *et al.,* 2015). Interestingly, obese subjects who consume a high-fat/low-carbohydrate diet display increased brush-border GLUT2 expression under fasting conditions (Ait-Omar *et al.,* 2011). Studies by Gai *et al* demonstrate that 2441 genes are differentially expressed in renal tissue following chronic high-fat diet (Gai *et al.,* 2014), however no studies focused on renal glucose transporter expression. Our findings show that consumption of a high-fat diet chow elevates GLUT2 expression, whereas a diet high in fat and sugars (junk-food diet) increases SGLT1, SGLT2 and GLUT2. It is possible that specific components of the diet, and the ratio of carbohydrate to fat, can play a role in renal glucose transporter expression to regulate glucose reabsorption.

In conclusion, metabolic dysregulation associated with diabetes evokes changes in sodium-dependent and sodium–independent glucose transport across the renal BBM

that have the potential to worsen hyperglycaemia or cause diabetic renal injury. Although clearly SGLT2 plays a key role in glucose transport across the proximal tubule BBM, our data show that the kidney displays differential responses in glucose transporter expression depending on the severity and type of metabolic dysfunction present. These findings demonstrate the complexity of the adaptive response in renal tubular glucose transport in pre-diabetic syndromes and type II diabetes. Furthermore, the efficacy of the anti-hyperglycemic effect of SGLT2 inhibitors may vary with the progression of disease. Further characterisation of the signalling pathways involved in renal glucose handling may allow a deeper understanding of the effect of SGLT2 inhibitors in patients with type II diabetes and promote the development of further new therapies for the treatment of diabetes and associated kidney disease.

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Table 1. Body weight, plasma glucose and insulin levels in models of insulin resistance and diabetes. Results are expressed as mean ± SEM, n=6-12. *P<0.05, **P<0.01, ***P<0.005, ****P<0.001 compared with the corresponding control rats. nd = not determined. #animals were fasted overnight before samples taken. n=6-8 for each disease model.

	Treatment group	Body weight (g)	Plasma glucose (mM)	Plasma insulin ($ng.ml^{-1}$)
Artificial sweetener study	**Saline**	397.7 ± 11.8	5.39 ± 0.2	nd
	Saccharin	409.7 ± 15.3	6.27 ± 0.8	nd
Type I diabetes study	**Vehicle**	268.5 ± 11.3	10.3 ± 0.9	nd
	Streptozotocin	244.4 ± 7.1	35.7 ± 3.4 ****	nd
Type II diabetes study	**Wistar rat**	397 ± 7.4	13.3 ± 0.4	4.83 ± 1.12
	Goto-kakizaki rat	316.6 ± 5.2****	20.3 ± 1.3 ****	6.35 ± 1.38
Diet-induced obesity study	**Control diet**	367.7 ± 15.3	8.5 ± 0.30	2.15 ± 1.09
	Junk-food diet	429 ± 12.4***	10.7 ± 0.4 ***	9.18 ± 1.51 **
	Control diet#	392.8 ± 4.2	4.2 ± 0.1	4.91 ± 0.87
	High-fat diet#	417.6 ± 9.5*	4.7 ± 0.2 *	10.07 ± 1.76 *

Figure legends

Figure 1 – Effect of type II diabetes on sodium-dependent and independent glucose transport across the proximal tubule BBM. (a) Sodium-dependent glucose uptake across the BBM from control Wistar (solid line) and Goto-Kakizaki (dashed line) rats at different concentrations of glucose from 29.7 to 957 μM. (b) Sodium-independent glucose transport was measured with or without phlorizin at 20 mM glucose concentration. Values are given as mean ± SEM. n=6, *p<0.05, ***P<0.005 compared to saline infused controls.

Figure 2 – Effects of diabetes and diet-induced metabolic disruption on expression of SGLT1, SGLT2, GLUT2 and PKC-βI at the proximal tubule BBM. Western blotting was performed on proximal tubule BBM from rats under control conditions or differing models of metabolic disruption; (a) Type II diabetes, (b) Type I diabetes, (c) junk-food diet and (d) high-fat diet. Representative blots (top panel) and protein levels of transporters (lower panel) are shown. Band intensities for each transporter are normalised to those of β-actin, expressed as a percentage of the control average and are given as mean ± SEM, n=6-8. *P<0.05, **P<0.01, ***P<0.005, ****P<0.001 compared to control animals.

Figure 3 – Effect of saccharin infusion on sodium-dependent and independent glucose transport across the proximal tubule BBM. (a) Sodium-dependent glucose uptake across the BBM from rats exposed to i.v. infusion of saline (solid line) and saccharin (dashed line) using concentrations of glucose from 29.7 to 957 μM. (b) Sodium-independent glucose transport was measured in the presence and

absence of phlorizin at 20 mM glucose concentration. Values are given as mean ± SEM. n=5, *p<0.05, ***P<0.005 compared to saline infused controls.

Figure 4 – Effect of saccharin infusion on expression of SGLT1, SGLT2, GLUT2 and PKC-βI at the proximal tubule BBM. Western blotting was performed on proximal tubule BBM from rats exposed to i.v. infusion of saline (open bar) or saccharin (closed bar). Representative blots are shown (top panel) and protein levels of transporters (lower panel) are shown. Band densities for each transporter are normalised relative to β-actin, expressed as a percentage of the control average and given as mean ± SEM, n=6-8. *P<0.05, ***P<0.005 compared to control animals.

Additional Information

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Author Contributions: All experiments were performed at the London Epithelial Group (Department of Neuroscience, Physiology & Pharmacology, University College London) and Department of Veterinary Basic Sciences (Royal Veterinary College). All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

1. Conception or design of the work; HC, JM, ED, RU, KS
2. Acquisition, analysis, or interpretation of data for the work; HC, JM, MC
3. Drafting the work or revising it critically for important intellectual content; HC, JM, ED, RU, KS, MC
4. Approved the final version of the manuscript: HC, JM, ED, RU, KS, MC
5. Agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: HC, JM, ED, RU, KS, MC

Supporting Information

Supplementary Figure 1 - Representative Western blots performed on proximal tubule BBM from rats under control conditions or differing models of metabolic disruption.

Supplementary Figure 2 - Representative Western blots performed on proximal tubule BBM from rats exposed to i.v. infusion of saline or saccharin.

Figure 1

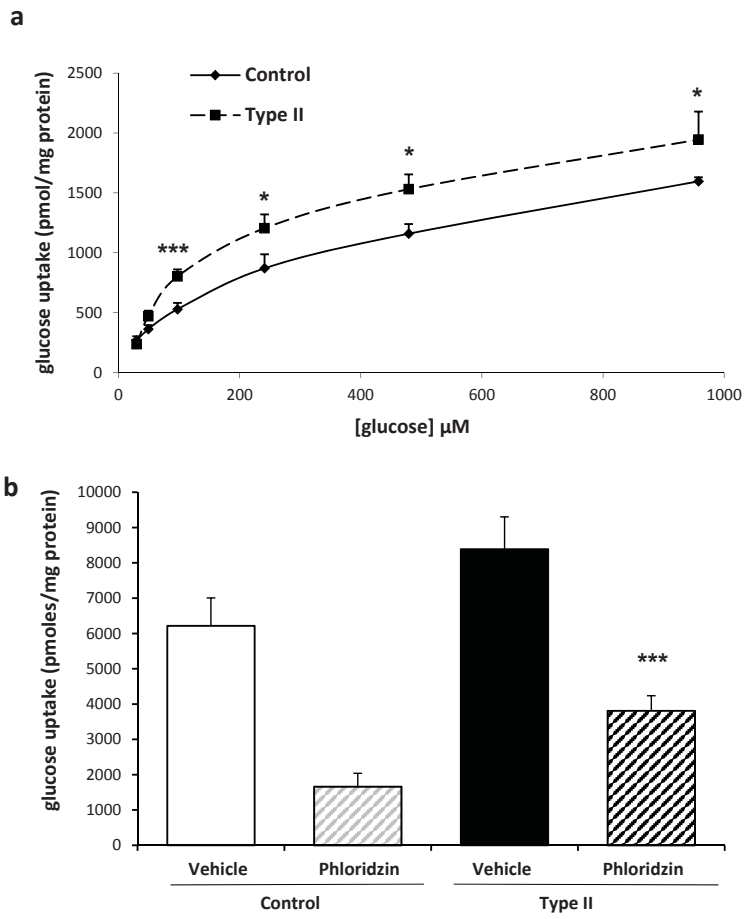
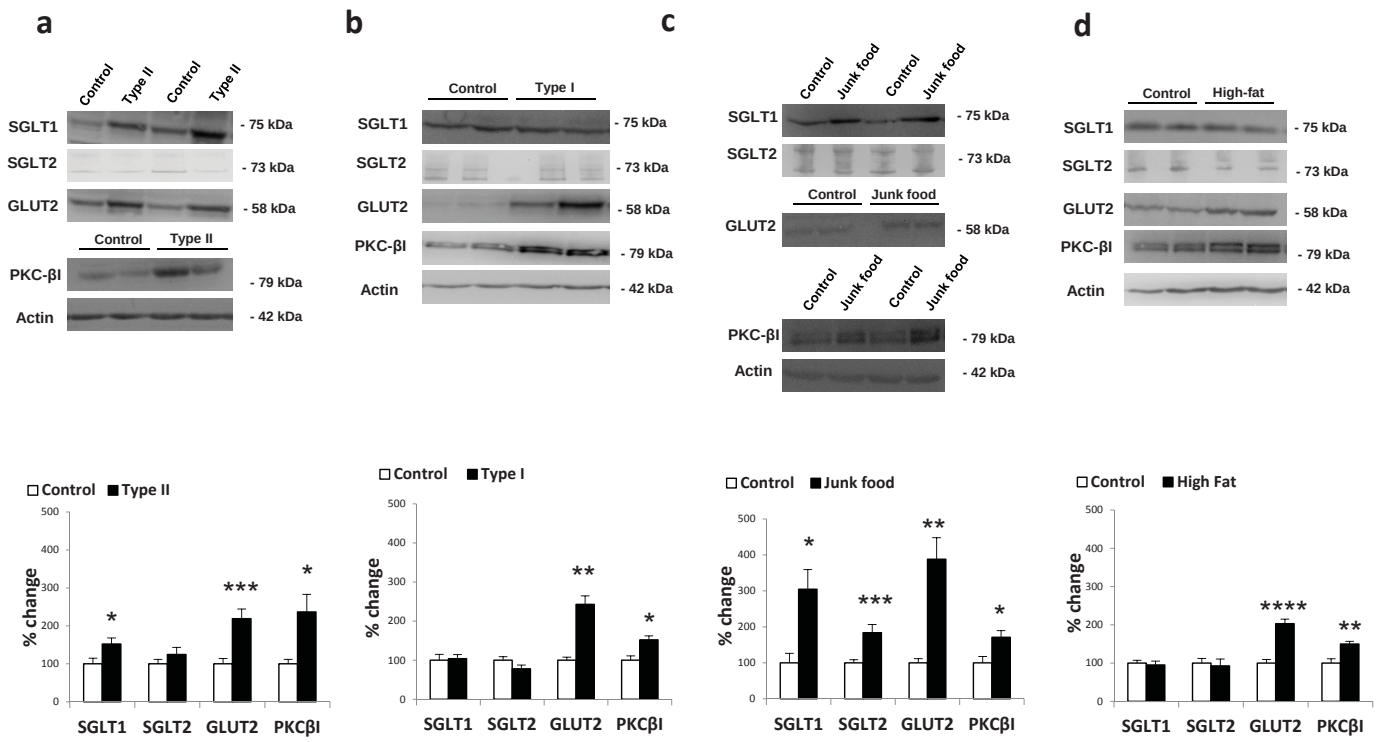

a
Control
Type II
glucose uptake (pmol/mg protein)
[glucose] µM
***
*
*
*
b
glucose uptake (pmoles/mg protein)
***
Vehicle
Phloridzin
Vehicle
Phloridzin
Control
Type II

Figure 2


a
Control
Type II
Control
Type II
SGLT1
SGLT2
GLUT2
- 75 kDa
- 73 kDa
- 58 kDa
Control
Type II
PKC-βI
Actin
- 79 kDa
- 42 kDa
b
Control
Type I
SGLT1
SGLT2
GLUT2
PKC-βI
Actin
- 75 kDa
- 73 kDa
- 58 kDa
- 79 kDa
- 42 kDa
c
Control
Junk food
Control
Junk food
SGLT1
SGLT2
- 75 kDa
- 73 kDa
Control
Junk food
GLUT2
- 58 kDa
Control
Junk food
Control
Junk food
PKC-βI
Actin
- 79 kDa
- 42 kDa
d
Control
High-fat
SGLT1
SGLT2
GLUT2
PKC-βI
Actin
- 75 kDa
- 73 kDa
- 58 kDa
- 79 kDa
- 42 kDa
Control
Type II
% change
SGLT1
SGLT2
GLUT2
PKCβI
Control
Type I
% change
SGLT1
SGLT2
GLUT2
PKCβI
Control
Junk food
% change
SGLT1
SGLT2
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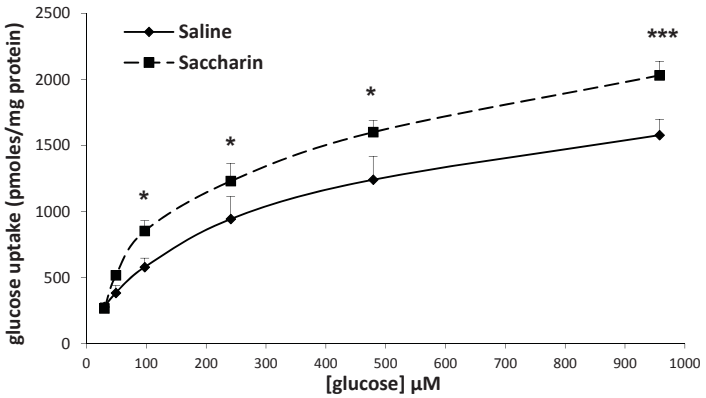
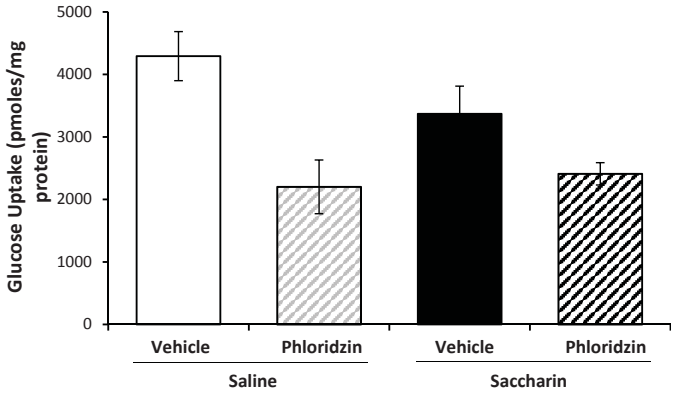
PKCβI
Control
High Fat
% change
SGLT1
SGLT2
GLUT2
PKCβI

Figure 3

a


Saline
Saccharin
glucose uptake (pmoles/mg protein)
[glucose] µM
0
500
1000
1500
2000
2500
0
100
200
300
400
500
600
700
800
900
1000
*
*
*
***

b


Glucose Uptake (pmoles/mg protein)
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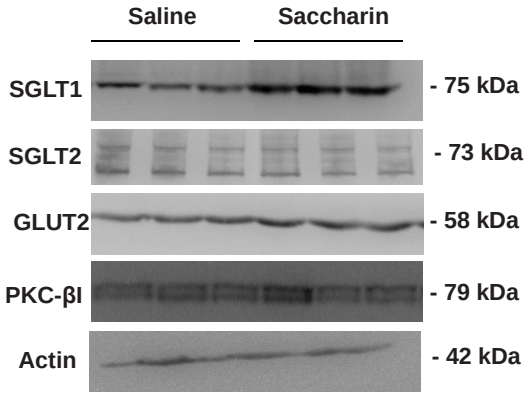
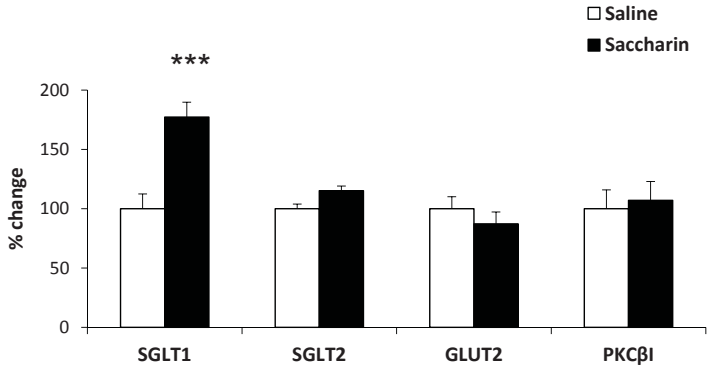
1000
2000
3000
4000
5000
Vehicle
Phloridzin
Vehicle
Phloridzin
Saline
Saccharin

Figure 4


Saline
Saccharin
SGLT1
- 75 kDa
SGLT2
- 73 kDa
GLUT2
- 58 kDa
PKC-βI
- 79 kDa
Actin
- 42 kDa


Saline
Saccharin
***
% change
200
150
100
50
0
SGLT1
SGLT2
GLUT2
PKCβI