Acute SGLT inhibition normalizes oxygen tension in the renal cortex but causes hypoxia in the renal medulla in anaesthetized control and diabetic rats.

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Running Title: SGLT inhibition results in medullary hypoxia.

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ABSTRACT

Early stage diabetic nephropathy is characterized by glomerular hyperfiltration and reduced renal tissue oxygen tension (PO$_2$). Recent, observations indicate that increased tubular sodium glucose linked transport (SGLT) plays a role in the development of diabetes-induced hyperfiltration. The aim of the current study was to determine how inhibition of SGLT impacts upon PO$_2$ in the diabetic rat kidney.

Diabetes was induced by streptozotocin in Sprague Dawley rats 2 weeks prior to experimentation. Renal hemodynamics, excretory function and renal oxygen homeostasis were measured in anaesthetized control and diabetic rats during baseline and after acute SGLT inhibition using phlorizin (200 mg/kg IP).

Baseline arterial pressure was similar in both groups and unaffected by SGLT inhibition. The diabetic animals displayed reduced baseline PO$_2$ in both cortex and medulla. SGLT inhibition improved cortical PO$_2$ in the diabetic kidney, whereas it reduced medullary PO$_2$ in both groups. SGLT inhibition reduced sodium transport efficiency (TNa/QO$_2$) in the control kidney whereas the already reduced TNa/QO$_2$ in the diabetic kidney was unaffected by SGLT inhibition.

In conclusion, these data demonstrate that when SGLT is inhibited, renal cortex PO$_2$ in the diabetic rat kidney is normalized, which implies that increased proximal tubule transport contributes to the development of hypoxia in the diabetic kidney. The reduction in medullary PO$_2$ in both control and diabetic kidneys during the inhibition of proximal sodium reabsorption suggests redistribution of active sodium transport to less efficient nephron segments such as the medullary thick ascending limb which results in medullary hypoxia.
INTRODUCTION

Diabetes affects up to 220 million people worldwide (15). Diabetic Nephropathy is a renal complication of type 1 and type 2 diabetes and is a major cause of morbidity and mortality affecting up to 40% of diabetic patients (9). More recently, SGLT (sodium glucose linked transport) inhibition has become a frontline pharmacological target in the treatment of diabetes because of its ability to lower blood glucose levels by promoting the excretion of glucose by the kidney.

Indeed, in a healthy kidney 99% of filtered glucose is reabsorbed, mostly via the high capacity SGLT2 which is expressed in the brush border membrane of the proximal tubule in the S1 segment (39) and to a lesser extent via the low capacity isoform, SGLT1, which is expressed in the S3 segment of the proximal tubule (2). Glucose is transported out of proximal tubules and into the surrounding interstitium via the glucose uniporter, GLUT2. The reabsorption of glucose from the tubule into the cell is dependent upon the simultaneous reabsorption of sodium. The reabsorption of sodium is in turn driven by the steep concentration gradient between the tubule and the cell and this gradient is maintained by the Na/K-ATPase pump on the basolateral side of the cell. Thus, glucose reabsorption via SGLT is secondarily an energy dependent process and when energy is utilized, oxygen is consumed (16).

It has been widely reported that the early stages of diabetic nephropathy are characterized by glomerular hyperfiltration and proteinuria (9, 19, 31). Indeed, previous studies have shown that the genetic ablation of SGLT2 and the chronic non-selective pharmacological inhibition of SGLT in diabetic rats and mice not only reverse hyperglycemia but also attenuate hyperfiltration and proteinuria (16, 34, 37, 40). Furthermore, others have observed that renal tubular SGLT2 and
GLUT2 are upregulated in the proximal tubule of diabetic rats (6, 42, 21, 30, 22, 1). Together these data suggest that SGLT activity and expression is increased in the diabetic kidney and that is directly related to the development of glomerular hyperfiltration and proteinuria. Mechanisms underlying the relationship between diabetes induced glomerular hyperfiltration and increased proximal reabsorption via SGLTs have yet to be fully understood but impaired tubuloglomerular feedback (TGF) regulation (39) and changes in intratubular pressure have been proposed (34).

Importantly, Korner et al. in 1994 were among the first to demonstrate that glomerular hyperfiltration and proteinuria were also accompanied by elevated renal oxygen consumption in the proximal tubule of the diabetic rat kidney (16). This increase could be attributed to increases in intracellular sodium accumulation via SGLT, which in turn increases the activity of Na/K-ATPase pump and thereby proximal tubule oxygen consumption (16). More recently, Palm et al. 2003 reported that increased renal oxygen consumption was accompanied by a corresponding reduction in renal cortical and medullary oxygen availability (24). These data combined certainly raise the question as to whether the attenuation of glucose reabsorption via SGLT could correct renal hypoxia in the diabetic rat kidney?

Thus, the overall aim of the current study was to determine whether there is a relationship between SGLT-mediated sodium and glucose uptake in the proximal tubule, glomerular hyperfiltration and proteinuria and renal hypoxia in the diabetic rat kidney. We therefore hypothesize that acute non-selective inhibition of SGLT will reverse renal tissue hypoxia, in addition to correcting glomerular hyperfiltration, proteinuria and enhanced oxygen consumption, in a rat model of type 1 diabetes.
MATERIALS AND METHODS

All chemicals and assay kits were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise specified.

Animal model

Male Sprague Dawley rats (8 weeks old) were obtained from Charles River (Jackson, USA) and maintained under a 12h light-dark regime at 20±3°C in the Centre for Biomedical Resources, Linköping University. Animals received free access to standard chow and water. All experimental procedures were performed under the European Community Directive 86/609/EC and were approved by the local Animal Experimentation Ethical Committee. NIH guidelines for the care of experimental animals were adhered to throughout each protocol. Diabetes was induced in conscious rats (n=9) by the injection of streptozotocin (50 mg/kg) into the tail vein. Control animals (n=12) received vehicle. Animals were deemed to be diabetic if blood glucose levels (MediSense, Bedford MA) were >16mmol/L 24 hours post induction. Animals were submitted for acute experimentation 14-18 days post induction of diabetes. Control animals were 9 weeks old and diabetic animals were 10-11 weeks old when the acute experiments were carried out.

Surgical Protocol

Animals were anaesthetized via an IP injection of Inactin, (Diabetic animals: 80 mg/kg, Control animals: 120mg/Kg) and placed on a heated pad so that body temperature was maintained at 37°C. A tracheostomy was performed (PP240 tubing) to facilitate respiration. A cannula was inserted into the right carotid artery (PP50) to facilitate the measurement of mean arterial
pressure (MAP) and into right femoral artery to facilitate the infusion of $^{3}$H-inulin and $^{14}$C-para-aminiohippuric acid (PAH), (control animals: 5 ml/kg/h, diabetic animals: 10 ml/kg/h) (Fig. 1).

A cannula was inserted into the bladder to facilitate the excretion of urine produced by the right kidney. The left kidney was exposed by a left subcostal flank incision, stabilized in a cup and surrounded and covered by cotton wool soaked in paraffin oil (Apoteksbolaget, Gothenburg, Sweden). This was carried out to ensure that the kidney remained moist at body temperature. The left renal vein was dissected and prepared for the withdrawal of blood samples. A cannula was inserted into the left ureter to facilitate the measurement of left renal function. Animals were allowed to recover for 45 minutes prior to the experiment. At the end of the experiment, animals were terminated via an intra-arterial KCl bolus and the left kidney was removed and weighed.

Experimental Protocol (Fig. 1)

In vivo, IP administration of phlorizin

After the stabilization period, one forty minute clearance period was taken prior to and one approximately 30 minutes after the IP administration of phlorizin (200 mg/kg). Phlorizin was dissolved in propylene glycol (approximately 0.1 ml) and delivered directly into the peritoneum via the left subcostal flank incision site. As a control propylene glycol (0.1 ml vehicle) was administered to a separate group of normoglycemic animals (n=12). Vehicle per se did not affect any of the parameters involved in tubular sodium handling or tissue oxygen homeostasis (data not shown). Blood samples were collected at specific points during the experimental protocol for the measurement of blood gas parameters.

Measurement of hemodynamic parameters
MAP was measured via the right carotid artery cannula which was connected to a blood pressure transducer and a signal transduction amplifier. MAP was continuously monitored and recorded with a PowerLab instrument (AD Instruments, Hastings, UK). Renal blood flow (RBF) was determined by $^{14}$C-PAH extraction and glomerular filtration rate (GFR) was determined by the clearance of $^3$H-Inulin. $^3$H and $^{14}$C activities were measured in both urine and plasma by liquid scintillation. Blood gases and electrolytes were measured in blood withdrawn from the carotid artery and renal vein using the iSTAT system (Abbott Laboratories, Abbott Park, IL, USA).

**Measurement of excretory parameters**

Urine flow was determined gravimetrically at baseline, during the IP administration of phlorizin. Urinary sodium and potassium concentrations were determined using flame spectrophotometry (model IL543, Instrumentation Lab, Milan, Italy). Urinary protein excretion was measured using a BCA protein assay (Bio-Rad Laboratories, Sundbyberg, Sweden) and urinary albumin excretion was determined using a rat albumin ELISA (Bethyl Laboratories, Montgomery, TX, USA). Urinary glucose was measured using a glucose hexokinase assay kit (Roche, Stockholm, Sweden).

**In vivo measurement of oxygen tension in the renal cortex and medulla**

At the end of each 40-minute clearance period, a Clark-type microelectrode (Unisense A/s, Aarhus, Denmark) was used to measure both cortical and medullary PO$_2$ as previously described (24). In brief, a small portion of the renal capsule is removed and the electrode inserted 0.5-1.0 and 3.5-4.0 mm into the kidney to measure cortical and medullary PO$_2$, respectively. This procedure was repeated 3-4 times before and after SGLT inhibition and the average calculated for each region and time point and used as N=1. Importantly after each set of cortical and medullary recordings the electrode was completely removed.
from the kidney and reinserted in an area immediately adjacent (“undamaged area”) to the previous point of insertion. Generally if the electrode is calibrated correctly and if the kidney is sufficiently stabilized in the cup, the readings stabilize quite quickly and the whole process, on average takes approximately 15 minutes.

Calculations

GFR was calculated using, \( GFR = \frac{U \times UV}{P} \), where \( U \) = urinary \(^3\)H activity, \( UV \) = urine volume, \( P \) = plasma \(^3\)H activity. Renal plasma flow (RPF) was calculated using, \( RPF = \frac{U \times UV}{A / ^{14}C} \) extraction, where \( U \) = urinary \(^{14}\)C activity, \( A \) = arterial \(^{14}\)C activity and \(^{14}\)C extraction = arterio-venous difference in \(^{14}\)C activity/arterial \(^{14}\)C activity. Renal blood flow (RBF) was then calculated using, \( RBF = \frac{RPF}{1 - \text{Hematocrit}/100} \). Renal vascular resistance (RVR) was determined by, \( RVR = \frac{\text{MAP}}{\text{RBF}} \). In vivo renal oxygen consumption (QO\(_2\)) was estimated from the arteriovenous difference in oxygen content and was given by, \( QO_2 = \) arterio-venous difference in oxygen content \( \times RBF \), where blood oxygen content = \( 1.34 \times \text{hemoglobin oxygen saturation} \times \text{Haemoglobin concentration} + \text{blood PO}_2 \times 0.003 \). Tubular sodium transport (TNa) was calculated by, \( TNa = [\text{PNa}] \times GFR - [\text{UNa}] \times UV \), where \([\text{PNa}] = \) plasma sodium concentration and \([\text{UNa}] = \) urinary sodium concentration. Fractional sodium excretion = sodium clearance/GFR, where sodium clearance = \([\text{UNa}] \times UV/[\text{PNa}] \).

Statistical analysis

Data are presented as mean±SEM. All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). All data were analyzed using 2x2 repeated measures ANOVA followed by Bonferroni post hoc test. Unpaired t-test was used where appropriate to compare baseline parameters between the control group and the diabetic group.
Differences were deemed to be statistically significant when $P < 0.05$ and all data presented as mean±SEM.
RESULTS

General Animal Characteristics

All animals administered streptozotocin developed hyperglycemia. Control and diabetic animals had similar body weight but both left kidney weight and kidney to body weight ratio were approximately 2-fold higher in the diabetic animals relative to the controls (Table 1).

MAP and RBF were largely unaffected by acute inhibition of SGLT by phlorizin. Although, phlorizin had no overall effect on RPF in either group, there was a significant interaction between groups because phlorizin had a tendency to increase this parameter in the control animals and reduce it in the diabetic group (Table 2). Phlorizin had no significant effect on GFR in the control group but significantly reduced the glomerular hyperfiltration in the diabetic group (Table 2).

SGLT inhibition increased urinary glucose excretion in both control and diabetic rats (Fig. 2A), with concomitantly increased urine flow in both groups (Fig. 2B). The reduced glucose transport by SGLT in the proximal tubules did not affect total tubular sodium transport in either group (Fig. 3A). However, both absolute (Fig. 3B) and fractional (Fig. 3C) urinary sodium excretions increased after SGLT inhibition. Interestingly, SGLT inhibition did not reduce total kidney QO₂ in either group (Fig. 4A) even though it reduced the diabetes-induced hyperfiltration. TNa/QO₂ was significantly reduced by SGLT inhibition in controls, whereas the already reduced TNa/QO₂ observed in diabetes was not significantly affected by SGLT inhibition (Fig. 4B). The reduced glucose and sodium transport in the proximal tubules was manifested as increased PO₂ in the diabetic kidney cortex (Fig. 5A). However, the increased sodium load to the more distal parts of the nephron after SGLT inhibition reduced medullary PO₂ in both control and diabetic kidneys.
Furthermore, the magnitude of the reduction in medullary PO$_2$ during SGLT inhibition was significantly greater in the control group (Fig. 5B). SGLT inhibition increased urinary protein excretion in controls whereas the already increased baseline urinary protein excretion in diabetic animals was not significantly affected by SGLT inhibition (Fig. 6A). However, SGLT inhibition had no significant effect on urinary albumin excretion in controls, but significantly increased this parameter in diabetics (Fig. 6B).
DISCUSSION

The current study sought to determine whether diabetes-induced renal hypoxia could be attenuated by inhibiting SGLT in the proximal tubule. Overall the main findings of this study are that acute SGLT inhibition by phlorizin reduces sodium and glucose reabsorption and attenuates glomerular hyperfiltration which reverses the renal cortical hypoxia in diabetic rats. On the other hand, SGLT inhibition promotes renal medullary hypoxia in both control and diabetic animals by promoting increased sodium load to the more distal parts of the nephron. Taken together these data clearly show that there is a link between the uptake of glucose and sodium via SGLT in the proximal tubule, glomerular hyperfiltration and tissue hypoxia in the renal cortex of diabetic rats. Importantly, these data also reveal that the improvement of cortical tissue PO$_2$ observed during SGLT inhibition may have deleterious consequences for oxygen metabolism in the medullary region.

The present study provides evidence for a causative link between proximal tubule cell glucose accumulation via SGLT and hypoxia in the renal cortex of the diabetic rat kidney. In agreement with reports by Palm et al 2003 (24), the elevated renal QO$_2$ and TNa and reduced TNa/QO$_2$ in the diabetic animals in the current study are consistent with the observed hypoxia in the renal cortex. Indeed, previous studies by others provide evidence for a direct and positive correlation between diabetes induced increases in QO$_2$, SGLT activity and Na/K-ATPase activity in the renal cortex of the diabetic kidney (16). More recently, it was demonstrated that the enhanced uptake of glucose via SGLT also promotes the production of reactive oxygen species (ROS), such as peroxynitrite in the renal cortex and medulla in a rat model of type 1 diabetes (23). In the same set of experiments, phlorizin additionally had an inhibitory effect on antioxidant enzymes
such as catalase and a stimulatory effect on pro oxidant enzymes such as glutathione peroxidase (23).

Importantly, previous research has also demonstrated that diabetes induced oxidative stress augments renal $Q_O_2$ by stimulating mitochondrial uncoupling in proximal tubule cells (7, 8, 24). In this regard mitochondria are consuming more oxygen in the absence of generating ATP (10, 11), which may explain the reduced $T_{Na}/Q_O_2$ observed in the diabetic animals at baseline in the current study. Overall the existing body of evidence provided by the literature, coupled with the observations of the present study suggests that SGLT mediated intracellular accumulation of sodium and glucose during chronic hyperglycemia increases $Na^+/K^+\text{ATPase}$ activity and the generation of ROS. The resultant increase in $Q_O_2$ results in a reduction in the availability of oxygen in the renal cortex and thereby hypoxia.

Interestingly, phlorizin induced normalization of renal cortex $PO_2$ was not accompanied by a corresponding decrease in renal $Q_O_2$ or $T_{Na}$ in either the control or diabetic rat kidney in the present study. Furthermore, SGLT inhibition had no significant effect on $T_{Na}/Q_O_2$ in the diabetic animals but actually reduced this parameter in the control group. These observations may be explained by the fact that acute SGLT inhibition actually promoted hypoxia in the renal medulla in both groups but particularly in the control group. One might be tempted to assume that the reduction in medullary $PO_2$ in the diabetic animals following phlorizin administration was due to a reduction in oxygen supply. However, this seems unlikely since phlorizin induced medullary hypoxia was not accompanied by a corresponding decrease in RBF.

Alternatively, the data described above might indeed indicate that when SGLT is inhibited $T_{Na}$ is diverted to other nephron segments such as the medullary thick ascending limb, distal tubule
or collecting duct where TNa increasingly requires more energy and thereby oxygen (13, 32). This proposal is somewhat supported by the observations of others in rats in vivo. Indeed, it was demonstrated previously that nitric oxide synthase (NOS) inhibitors also block the proximal TNa (5) and reduce medullary PO2 and RBF without altering total renal TNa (4). However, other vasoconstrictors such as endothelin and norepinephrine also reduced RBF but were without effect on medullary PO2 (4), indicating that factors other than a reduction in oxygen delivery interfere with PO2 in the medulla during NOS inhibition. Furthermore, others later demonstrated that NOS inhibition does in fact increase QO2 in the dog kidney (17). These observations taken together with the findings of the present study show that a reduction in PO2 in the renal medulla during the inhibition of proximal tubule TNa is possibly due to a compensatory shift in renal TNa to the less efficient medullary regions, thereby resulting in an increase in QO2 (4).

The present data demonstrate that SGLT inhibition has a positive effect on oxygen availability in the cortex of the diabetic kidney. However, the same treatment induces medullary hypoxia in both control and diabetic rat kidneys. Indeed, these data do not give any indication as to what may happen to renal oxygen availability during the exclusive administration of SGLT2 inhibitors, which are used clinically to lower plasma glucose levels in diabetic patients. Whether SGLT1 activity and expression might become upregulated in this scenario in an effort to compensate is presently unknown. On the one hand, in phase II clinical trials it was recently demonstrated that the dual SGLT1/SGLT2 inhibitor, LX 4211, did not increase urinary glucose excretion any more than what was observed when patients were just administered a specific SGLT2 antagonist (29). On the other hand, it has been widely reported that even the most potent SGLT2 inhibitors can only inhibit 30-50% of total tubular glucose reabsorption. Indeed a recent mathematical model has predicted that SGLT1 compensation may be an underlying factor (18).
The ability of SGLT inhibition to significantly reduce diabetes-induced glomerular hyperfiltration in the current study was not surprising as this has been well established in numerous studies in diabetic rats and mice (20, 27, 39, 37, 34). These observations clearly suggest that the uptake of glucose and thereby sodium via SGLT plays a role in promoting increased GFR in the diabetic rat kidney. The proposed mechanisms underlying this relationship are controversial. Indeed Vallon and co-workers would argue that increased proximal sodium uptake via SGLT reduces the distal delivery of sodium to the macula densa (39). This results in the inhibition of TGF, dilation of the afferent arteriole and thereby glomerular hyperfiltration (39). This viewpoint is based on data collected from in vivo micropuncture studies carried out in a rat model of type 1 diabetes whereby intratubular administration of phlorizin reduced proximal TNa, increased distal sodium concentration and attenuated single nephron glomerular hyperfiltration (39). These findings were later substantiated when it was shown that diabetes induced glomerular hyperfiltration was virtually absent in type 1 diabetic mice who were deficient in the adenosine A1-receptor and therefore lacking a functional TGF response (41).

In contrast Sällström et al. reported that genetic ablation of the adenosine A1-receptor had no effect on glomerular hyperfiltration in a mouse model of type 1 diabetes (33). Furthermore, it was later demonstrated that SGLT inhibition by phlorizin could still attenuate glomerular hyperfiltration in the above mentioned mouse model (34). These data certainly imply that mechanisms other than a defective TGF response cause diabetes induced glomerular hyperfiltration. Indeed it has been proposed that diabetes induced increases in sodium and glucose uptake in the proximal tubule and resultant reductions in the distal delivery of sodium (35), might cause a reduction in proximal tubule hydrostatic pressure (12), a corresponding increase in net filtration pressure and thereby GFR (26, 34). Either way, it is clear that increased
sodium and glucose uptake via SGLT in the diabetic rat kidney plays a role in promoting glomerular hyperfiltration. Whether this is due to a defective TGF response or a reduction in proximal tubule hydrostatic pressure or indeed a combination of both is currently unclear.

The phlorizin induced attenuation of glomerular hyperfiltration observed in diabetic animals in the current study was accompanied by a significant increase in the urinary excretion of glucose and sodium. This was anticipated and provides evidence that SGLTs were inhibited by phlorizin as has been demonstrated previously by others in diabetic rats and mice (20, 34, 39). Importantly, additional studies have shown that SGLT2 activity and expression is up-regulated in the diabetic rat kidney (22, 30, 42). However, this is not reflected by the current observations, whereby phlorizin produced increases in sodium and glucose excretion of a similar magnitude in control and diabetic animals. Indeed, SGLT activity may well have been elevated at baseline in the diabetic group but this was possibly masked by the parallel reductions in GFR and thereby the filtered load of glucose following the acute inhibition of SGLT (37, 40).

A modest total proteinuria was observed in the diabetic group at baseline. Interestingly the acute administration of phlorizin augmented urinary protein excretion particularly in the control animals. The observed phlorizin induced increases in urinary protein excretion in the current study are supported by the findings of others in a rat model of type 1 diabetes (20). This group additionally demonstrated that this increase in urinary protein excretion was related to an independent inhibitory effect of phlorizin on the tubular reabsorption of beta 2 microglobulin (20). Indeed earlier studies in type 1 diabetic humans revealed a positive correlation between tubular flow rate and the urinary excretion of beta 2 microglobulin (43). Phlorizin caused a robust diuresis in the present study in both control and diabetic animals. This observation combined with the previous findings of others (20, 43) suggests that the increases in urinary
protein excretion following the acute administration of phlorizin are possibly due to flow
induced decreases in beta 2 microglobulin reabsorption. It is unclear from the present data why
phlorizin-increased urinary protein excretion is much greater in the control animals and there is
currently no data in the literature to explain such observations. However, it might be tempting to
speculate that the diabetic nephrons are more adapted to high urine flow. It may well be that
acute increases in urine flow following SGLT inhibition in the diabetic group only result in mild
increases in urinary protein excretion because the tubule has adapted mechanisms at this early
phase in the disease process in an effort to counteract the urinary loss of protein.

Surprisingly, proteinuria was not accompanied by albuminuria in the diabetic animals at
baseline. Indeed, diabetes induced proteinuria and specifically albuminuria have been
demonstrated clinically and in animal models of type 1 and type 2 diabetes (1, 20, 25, 37, 43).
On the other hand, it has been reported that when glomerular injury is mild, as may be the case in
the present study, the tubules still have a large capacity for albumin reabsorption but a
comparably lower capacity to reabsorb larger molecular weight proteins such as IgG (3). This
may indeed account for the occurrence proteinuria in the absence of albuminuria in the present
study. Importantly, Blantz and co-workers, demonstrated hyperfiltration in diabetic rats in the
absence of albuminuria, 10 days post streptozotocin administration (36). Interestingly, lysine was
also acutely administered to inhibit tubular albumin reabsorption. Lysine had no effect on urinary
albumin excretion in the control rats but caused a significant increase in this parameter in the
diabetic group (36). In the context of the current study, these data clearly suggest that the filtered
load of albumin may well be increased at this early stage in the disease but albumin is not
detected in the urine because it is being reabsorbed in the proximal tubule (36).
Finally, the phlorizin induced increase in urinary albumin excretion in the diabetic group in this study was not anticipated given that others have shown the opposite effect in mouse models of type 1 and 2 diabetes (1, 37). This discrepancy may be related to the fact that SGLTs were chronically inhibited (12 weeks) in these studies (1, 37). Furthermore, the chronic SGLT inhibition blunted diabetes induced diuresis over time (1) in a mouse model of type 2 diabetes. Although Vallon et al. appear not to have measured urine volume in their experiments in Akita mice, it may be inferred that SGLT inhibition had a similar effect on urine flow in these experiments since diabetes induced polydipsia was also blunted after 3 months of treatment (37). Taking all previous studies into account, it may well be that the acute increase in urine flow following SGLT inhibition by phlorizin in the current study was somehow interfering with the tubular reabsorption of albumin in the diabetic animals. The mechanism underlying this is currently unclear but may be due to a non-specific interaction between the drug and the luminal proteins (megalin and cubulin) (35) involved in the reabsorption of albumin. Interestingly, phlorizin had no effect on albumin excretion in the control animals. This may be related to the fact that the filtration of albumin was not increased at baseline in the first place. On the other hand, the filtered load of albumin may well have been increased in the diabetic animals but as Tucker et al. suggest this may have been masked by an initial reciprocal increase in the tubular reabsorption of albumin (36).

Indeed, the phlorizin-induced increase in albumin excretion observed exclusively in the diabetic animals may also be explained by the fact that non-selective SGLT inhibitors have been shown to increase plasma levels of glucagon like protein 1 (GLP-1) (28). GLP-1 stimulates release of ANP (14). In turn, ANP has been reported to selectively increase the urinary excretion of albumin in type 1 diabetic patients (44). It has been proposed that the glomerular permeability of
albumin was not increased by non-selective SGLT inhibition in control subjects because the presumably intact glomerular filtration barrier in this group was not sensitive to ANP (44). These observations may explain why urinary albumin excretion was not altered by SGLT inhibition in the present study.

Summary and significance

Overall the data presented in this study shows that the SGLT mediated accumulation of sodium and glucose in the proximal tubules of the diabetic rat kidney promote renal cortex hypoxia as well as glomerular hyperfiltration. On the other hand, the phlorizin-induced normalization of renal cortex PO$_2$ was paralleled by a reduction in this parameter in the renal medulla. This coupled with the fact that phlorizin did not alter either renal oxygen consumption, TNa or TNa/QO$_2$ in the diabetic animals indicates that TNa may have been diverted to other nephron segments such as mTAL, where sodium transport is less efficient and therefore requires more oxygen. Whether this is indeed the case or not, warrants further investigation. The association between SGLT activity and diabetes-induced proteinuria and particularly albuminuria is less clear and cannot be sufficiently explained by the present data. However, one might speculate that the robust diuresis induced by SGLT inhibition may have inadvertently interfered with the tubular reabsorption of certain proteins including albumin.

These studies are clinically relevant because SGLT2 inhibitors are now used in the treatment of diabetes and are for the most part reno-protective. However, the present data also shows that PO$_2$ in the medulla becomes compromised by the acute administration of these drugs. If medullary hypoxia persists during the long-term use of these drugs diabetic related complications such as hypertension, diabetic nephropathy and chronic kidney disease may in fact be exacerbated.
However, it must be noted that data presented in the current study are based on observations in an acute setting and are thus limited. Indeed, future studies should extend the present findings and examine the impact of chronic SGLT inhibition on intrarenal oxygen homeostasis in the diabetic kidney.
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J.O.N. and F.P. designed the study, J.O.N., A.F., L.P., D.P., and S.F. performed experiments, J.O.N. and F.P analyzed data, J.O.N. drafted the manuscript, J.O.N. and F.P. edited and revised manuscript, and all authors approved final version of manuscript.
REFERENCES


Table 1. General characteristics of control and diabetic rats at baseline.

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<th>Blood Glucose (mmol/L)</th>
<th>Body Weight (g)</th>
<th>Left Kidney Weight (g)</th>
<th>Left Kidney Weight/Body Weight (/1000)</th>
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<td>Control (n=12)</td>
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<td>339±5</td>
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<td>Diabetes (n=9)</td>
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<td>357±12</td>
<td>2.2±0.1*</td>
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Results are expressed as means ± SEM. * denotes P<0.05 versus Control.
Table 2. Hemodynamic and filtration effects of SGLT inhibition by Phlorizin in control and diabetic rats.

<table>
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<th>Mean Arterial Blood Pressure (mmHg)</th>
<th>Renal Plasma Flow (ml/min)</th>
<th>Renal Blood Flow (ml/min)</th>
<th>Renal Vascular Resistance (mmHg/ml/min)</th>
<th>Glomerular Filtration Rate (ml/min)</th>
<th>Filtration Fraction (%)</th>
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2x2 RM Interaction ns P<0.05 ns ns ns ns
AVOVA Group ns ns P<0.05 ns P<0.05 ns
Treatment ns ns ns ns P<0.05 ns

Data are expressed as means ± SEM. ns = non-significant * denotes P<0.05 versus corresponding baseline whereas # denotes P<0.05 versus corresponding control.
FIGURE LEGENDS

Figure 1. Outline of experimental protocol used to investigate the role played by proximal tubule sodium glucose linked transport (SGLT) in the pathogenesis of renal hypoxia in the diabetic rat kidney.

Figure 2. Urinary glucose excretion (A) and urine flow (B) in control (n=12) and diabetic rats (n=9) during baseline and after SGLT inhibition by phlorizin. ns = no significance. * denotes P<0.05 versus corresponding baseline.

Figure 3. Transported sodium (A), absolute urinary sodium excretion (B) and fractional urinary sodium excretion (C) in control (n=12) and diabetic rats (n=9) during baseline and after SGLT inhibition by phlorizin. ns = no significance. * denotes P<0.05 versus corresponding baseline whereas # denotes P<0.05 versus corresponding control.

Figure 4. Total kidney oxygen consumption (A) and tubular electrolyte transport efficiency (B) in control (n=12) and diabetic rats (n=9) during baseline and after SGLT inhibition by phlorizin. ns = no significance. * denotes P<0.05 versus corresponding baseline whereas # denotes P<0.05 versus corresponding control.
**Figure 5.** Renal cortical (A) and medullary tissue oxygen tension (B) in control (n=12) and diabetic rats (n=9) during baseline and after SGLT inhibition by phlorizin. ns = no significance. * denotes P<0.05 versus corresponding baseline whereas # denotes P<0.05 versus corresponding control.

**Figure 6.** Urinary excretion of protein (A) and albumin (B) in control (n=12) and diabetic rats (n=9) during baseline and after SGLT inhibition by phlorizin. ns = no significance. * denotes P<0.05 versus corresponding baseline whereas # denotes P<0.05 versus corresponding control.
Figure 1

Diabetes: 50mg/Kg STZ

14-18 days

Recovery

45 mins

Baseline: 40 mins

@30 mins: Arterial sample

Urine collection

30 mins

IP Phlorizin: 200mg/Kg (0.1ml bolus)

@30 mins: Arterial sample

Urine collection

Renal tissue PO2 measurement (Clarke electrodes)
Renal vein sample

3H inulin and 14C-PAH infusion

Surgery
Urinary Glucose Excretion (mg/min)

- Phlorizin
- Control
- Diabetic

2x2 RM ANOVA
Interaction ns
Treatment P<0.0001
Group P=0.0545
Urine Volume (µl/min)

Phlorizin

Control

Diabetic

2x2 RM ANOVA
Interaction ns
Treatment P<0.0001
Group P<0.001
Transported Sodium (mol/min)
Absolute Sodium Excretion (mol/min)

Phlorizin

Control  Diabetic

2x2 RM ANOVA
Interaction ns
Treatment P<0.0001
Group ns
Fractional Sodium Excretion (%)

**Phlorizin**

**Control Diabetic**

2x2 RM ANOVA

Interaction ns

Treatment P<0.0001

Group ns
Oxygen Consumption (μmol/min)

- Phlorizin
  - Control
  - Diabetic

2x2 RM ANOVA
Interaction ns
Treatment ns
Group P<0.01
2x2 RM ANOVA
Interaction ns
Treatment P<0.01
Group P<0.05
Renal Cortex Oxygen Tension (mmHg)

Control

Diabetic

Phlorizin

* Interaction P < 0.0001
* Treatment P < 0.0001
* Group P < 0.001
Renal Medulla Oxygen Tension (mmHg)

Phlorizin

Control Diabetic

2x2 RM ANOVA
Interaction P<0.05
Treatment P<0.0001
Group ns
Urinary Protein Excretion (mg/min)

Phlorizin

Control Diabetic

2x2 RM ANOVA
Interaction P<0.001
Treatment P<0.0001
Group P<0.05
Urinary Albumin Excretion (µg/min)

- Phlorizin
- Control
- Diabetic

2x2 RM ANOVA
Interaction P<0.01
Treatment P<0.01
Group ns