Prompt apoptotic response to high glucose in SGLT expressing renal cells

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L.N. performed experiments, analyzed data, wrote manuscript. L.Z. performed experiments, analyzed data. A.B. designed PCR experiments. D.S. performed experiments, analyzed data. A.W. analyzed clinical data. H.B. designed study, reviewed and edited manuscript. L.S. designed study, analyzed data, reviewed and edited manuscript. A.A. designed study, wrote manuscript.

ABSTRACT

It is generally believed that cells that are unable to downregulate glucose transport are particularly vulnerable to hyperglycemia. Yet little is known about the relation between expression of glucose transporters and acute toxic effects of high glucose exposure.

Here we have, in an ex vivo study on rat renal cells, compared the apoptotic response to a moderate increase in glucose concentration. We have studied the cell types that commonly are targeted in diabetic kidney disease (DKD): proximal tubule cells (PTC) that express SGLT2, mesangial cells (MC) that express SGLT1, and podocytes that lack SGLT and take up glucose via the insulin dependent GLUT4.

PTC and MC responded within 4-8 h exposure to 15 mM glucose with translocation of the apoptotic protein Bax to mitochondria and increased apoptotic index. SGLT down-regulation and exposure to SGLT inhibitors abolished the apoptotic response. Onset of overt DKD generally coincides with onset of albuminuria. Albumin had an additive effect on the apoptotic response. Ouabain, which interferes with apoptotic onset, rescued from the apoptotic response. Insulin supplemented podocytes remained resistant to 15 and 30 mM glucose for at least 24 h.

Our study points to a previously unappreciated role of SGLT dependent glucose uptake as a risk-factor for diabetic complications and highlights the importance of therapeutic approaches that specifically target the different cell types in DKD.

INTRODUCTION

Diabetic kidney disease (DKD) is the most common cause of chronic kidney disease (CKD) and end-stage renal failure. It is associated with a large social and economic burden, and there is an unmet need for therapy to halt the progressive course of the disease (6, 37). DKD has been extensively studied during the last decade. Yet there is no uniform concept regarding the cellular mechanisms behind the disease and its progressive course. The majority of studies have focused on the role of one cell type, omitting comparisons. However, given the complexity of the kidney, it is likely that there are several ongoing disease processes, and the development of a therapeutic program that prevents or halts the progressive course of DKD will need to be based on an insight into the ongoing disease processes in each of the target cells in the kidney.

The podocytes, proximal tubule cells (PTC) and mesangial cells (MC) are the most commonly studied cells in DKD. Damage and loss of podocytes cause proteinuria and contribute to glomerulosclerosis (23, 47). Damage of tubular cells causes interstitial fibrosis and glomerular tubular dissociation (7, 34). Damage of MC leads to mesangial expansion and contributes to glomerulosclerosis (1, 31). Hyperglycemia and insulin resistance are main causes of diabetic complications (8, 41, 43). Tight glucose control reduces the overall incidence of micro- or macro-albuminuria and halts the progression to end-stage disease (38). Several factors mediate glycemic toxicity, including metabolic dysregulation and generation of advanced glycosylation end products (9). The question whether the adverse effects of glucose concentrations, exceeding the levels in non-diabetic individuals, will also depend on the cellular mechanisms for glucose uptake has often been discussed, but has rarely been addressed experimentally. PTC, which have a high level of aerobic metabolism

due to high reabsorption workload (19, 27), takes up glucose via sodium-dependent glucose transporters (SGLT) (24). MC are also reported to express SGLT (20). Podocyte glucose uptake occurs via the insulin sensitive glucose transporter type 4 (GLUT4) (14).

Glucose related apoptosis was first reported in 1997 by Ortiz and Neilson (36), who showed that immortalized murine renal epithelial cells exposed to 25 mM glucose for at least 24 h caused an upregulation of the apoptotic protein Bax, a downregulation of the anti-apoptotic protein Bcl-xl and triggered apoptosis. Subsequently, most studies of renal apoptosis in DKD have been performed on immortalized renal cells exposed to glucose concentrations that generally by far exceed those commonly observed in the clinical setting. Here we describe the early response of PTC, MC and podocytes when exposed to moderately high (10 and 15 mM) glucose concentrations. All studies were carried out on primary cells, since cell lines undergo mutations, progressively lose their phenotype and have a shift to more anaerobic metabolism. The onset of the mitochondrial apoptotic pathway was used to validate the response to high glucose, since apoptosis marks the transition from reversible to irreversible cell damage and is a common finding in studies of rodent models of DKD (7, 21).

MATERIAL AND METHODS

Antibodies and Chemicals

The following primary antibodies and dilutions were used: mouse monoclonal anti-Bax [6A7] 5 μg/ml, rabbit polyclonal anti-Bax 1:100, rabbit polyclonal anti-SGLT1 1:50, rabbit polyclonal anti-SGLT2 1:100, rabbit polyclonal anti-GLUT4 1:500 and mouse monoclonal anti-alpha smooth muscle actin 1:100 (all from Abcam,

Cambridge, UK), rabbit polyclonal anti-SGLT2 1:100 (Fitzgerald Industries International, Acton, MA, USA), rabbit monoclonal anti-Bcl-xl (54H6) 1:200 (Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit polyclonal anti-WT1 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), sheep polyclonal anti-nephrin 1:200 (R&D Systems, Inc., Minneapolis, MN, USA) and rabbit polyclonal anti-synaptopodin 1:500 (Sigma-Aldrich, St. Louis, MO, USA). The following fluorescence secondary antibodies were used: Alexa Flour 488 goat anti-rabbit IgG, Alexa Flour 546 goat anti-mouse IgG and Alexa Flour 546 goat anti-rabbit IgG (all from Life Technologies, Carlsbad, CA, USA), Star 635P goat anti-rabbit IgG (Abberior, Göttingen, Germany) and Star 635P conjugated to donkey anti-sheep IgG (ThermoFisher Scientific, Waltham, MA, USA) all used in a concentration of 1:500. All antibodies used are commercially available and validated by each manufacturer.

All chemicals and reagents were purchased from Sigma-Aldrich, Stockholm, Sweden, and all cell culture and molecular biology materials were purchased from ThermoFisher Scientific, Stockholm, Sweden, unless otherwise stated.

Microscopy

A Zeiss LSM 510 confocal microscope equipped with 25X/0.8NA oil, 40X/1.3NA oil, 63X/1.4NA oil and 40X/1.2NA water objectives was used for all imaging of proximal tubule cells (PTC), mesangial cells (MC), primary podocytes and patient material. A Zeiss LSM 780 confocal microscope equipped with 20X/0.8 air and 40X/1.2NA water objectives was used for all imaging of podocyte cell line. Immunofluorescence was detected as follows; CFP with excitation at 405 nm and detection 454-580 nm, DAPI and NucBlue with excitation at 405 nm and detection 420-480 nm, Alexa Fluor 488 with excitation at 488 nm and detection 510-550 nm, Alexa Fluor 546 and TUNEL labeling with excitation at 543 nm and 575 nm long pass detection, Star 635P and

DRAQ5 with excitation at 633 nm and 650 nm long pass detection. JC-1 fluorescence ratios were recorded with excitation at 488 nm and simultaneous 505-530 nm and 560 nm long pass detection. 2-NBDG fluorescence was detected with 488 nm excitation and 505 nm long pass detection and DCFDA fluorescence was recorded with 488 nm excitation and 505-550 nm detection.

Animals and Primary cultures

Twenty-day-old male Sprague Dawley rats were used for primary cell preparations. All animals were housed under controlled conditions of light and dark (12:12 h) and given a standard diet containing 20 % protein by weight and tap water were available *ad libitum*. All experiments were performed according to Karolinska Institutet regulations concerning care and use of laboratory animals and were approved by the Stockholm North ethical evaluation board for animal research.

Primary culture of rat PTC were prepared as previously described (11). PTC were characterized after 3 days in culture, 99 % of cells were SGLT2-positive (11).

Glomeruli isolation and podocyte culture were performed as follows. Rats were anesthetized by intraperitoneal injection of pentobarbital and perfused trough the left ventricle with HBSS to clear out blood followed by a solution of HBSS containing Dynabeads M-450. For each animal, $8x10^7$ dynabeads in 20 ml of solution was used. After perfusion, kidneys were removed and the medulla was discarded. The cortex was cut to small pieces and digested in 1 mg/ml collagenase I and 10 U/ml DNase in HBSS at 37°C for 30 min with gentle shaking. The digested tissue was gently pressed through a 100-µm cell strainer (BD Falcon, Bedford, MA, USA). Glomeruli containing dynabeads were collected using a magnetic particle concentrator, washed three times with cold HBSS and seeded on 12 or 18-mm glass coverslips in 12 or 24-well Petri dishes. Podocytes migrated out of glomeruli and were cultured for 3 days in

pH 7.4 MEM-NEEA medium supplemented with 3.6 g/l HEPES, 0.5 % insulin-transferrin-selenium-sodium pyruvate, 0.5 % sodium pyruvate, 5 % FBS, 10 μg/ml penicillin and 10 μg/ml streptomycin in 37°C at an approximate humidity of 95–98 % with 5 % CO_2.

Primary MC cultures were prepared from isolated glomeruli. The glomeruli were decapsulated by mixing the glomerular suspension with a 1 ml syringe and a 21-gauge needle a couple of times, resuspended in HBSS containing 1 mg/ml collagenase I and digested at 37°C for 15 min with gentle shaking. Cells were resuspended in DMEM supplemented with 2 mM L-glutamine, 20 % FBS, 10 μg/ml penicillin and 10 μg/ml streptomycin and plated in six-well plates. Cells were cultured in 37°C at an approximate humidity of 95–98 % with 5 % CO_2 and culture media was changed every 48 h. After 7 days in culture, each well of cells was split (1:3) in the following way. Cells were washed with Ca^{2+} and Mg^{2+}-free PBS pH 7.4 and incubated in 1 ml/well Ca^{2+} and Mg^{2+}-free PBS containing 0.05 % trypsin and 0.02 % EDTA for 1 min at 37°C. Most of the trypsin solution was removed and cells were incubated for another 3 min. Culture medium containing FBS was added to stop the digestion, wells were split, and new culture medium was added. On the third passage cells were seeded on 12 or 18-mm glass coverslips in 12 or 24-well plates for experiments. Cells were characterized using SGLT1 and alpha smooth muscle actin antibodies, indicating that they were MC.

In all experiments using PTC or podocyte cultures, treatment was started on day two or three in culture. MC cultures were used after being passaged three times. Cells were incubated using the following concentrations: 10-30 mM D-glucose and/or 2.5 mg/ml delipidated, endotoxin-free albumin (Sigma-Aldrich) with or without 5 nM ouabain (Sigma-Aldrich), 1 μM dapagliflozin (Selleckchem, Munich, Germany) or 0.2

mM phlorizin (Selleckchem, Munich, Germany) for 2-24 h as indicated in each figure. As controls, 5.6 mM glucose and mannitol (9.4 mM mannitol + 5.6 mM glucose) was used. Dapagliflozin and phlorizin were dissolved in DMSO, an equal amount DMSO was added to all samples in those experiments as control. Cultures were randomly divided between treatment groups for each experiment.

Immortalized murine podocytes

We use a well described and characterized immortalized mouse podocyte cell line (33). Cells were maintained and differentiated as previously described (26) with the following modifications; culture media was glucose free RPMI 1640 medium supplemented with 5.5 mM D-glucose, 10 % FBS, 10 µg/ml penicillin, 10 µg/ml streptomycin and for undifferentiated cells 10 U/ml interferon gamma (Sigma-Aldrich), cell were differentiated for 7-14 days. The differentiated immortalized podocytes were transiently transfected with SGLT2-ires-CFP (GenScript, Piscataway, NJ, USA) or empty vector CFP (Addgene, Cambridge, MA, USA). The DNA plasmids were delivered to the cells using Lipofectamine LTX reagent with plus reagent (ThermoFisher) diluted in Opti-MEM media (ThermoFisher) according to manufacturer´s instructions. Final DNA concentration in each well was 500 ng/ml. Cells were transfected for 48 h and characterized with SGLT2-ires-CFP fluorescence and anti-SGLT2 antibodies.

Immunocytochemical Staining

Following treatment cells were fixed with 4 % paraformaldehyde pH 7.4 and washed three times with PBS. Cells were permeabilized with 0.3 % Triton X-100 for 10 min, washed three times and blocked with 5 % BSA in 0.1 % Triton X-100 for 1 h. Primary antibodies were applied overnight at 4°C. Cells were washed three times and secondary antibodies were applied for 1 h at room temperature. Secondary antibody

controls were subjected to the same treatment, but primary antibodies were omitted. The cells were washed three times, mounted with Immu-Mount (Thermo Shandon, Midland, Canada) and imaged with a confocal microscope. In some experiments, cells were counterstained with 1 µg/ml DAPI (Santa Cruz Biotechnology, Inc., USA) for 1-2 min before mounting.

Glucose uptake

Cells were incubated with 100 µM 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) (Life Technologies, Carlsbad, USA) in Na^+ buffer (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.4 mM K_2HPO_4, 5.5 mM glucose, 20 mM HEPES and 1 mM $CaCl_2$) or Na^+-free buffer (NaCl changed for 135 mM choline chloride) pH 7.4 for 1 h at 37°C. The last 30 min of incubation, 2 drops/ml NucBlue Live ReadyProbes Reagent (NucBlue) (Life Technologies, Carlsbad, USA) were added to the buffer for nuclear stain. Cells were washed once with Na^+ or Na^+-free buffer and imaged with a confocal microscope with fixed settings for all measurements. The glucose uptake was quantified as mean fluorescent intensity of all cells in 5-6 separate areas on each coverslip and expressed as:

Na^+-dependent glucose uptake = (1 – (2-NBDG fluorescence in absence of Na^+ / 2-NBDG fluorescence in presence of Na^+)) * 100%

Average number of cells analyzed from each coverslip was 24 for PTC, 10 for MC and 17 for podocytes.

Detection of apoptotic cells in culture

Cells were fixed in methanol (Solveco AB, Rosersberg, Sweden) for 5 min at 4°C and in ethanol (Solveco AB, Rosersberg, Sweden): acetic acid (2:1) for 5 min at -20°C. After each fixation step the cells were washed with PBS a couple of times. The apoptotic index (AI) was determined with ApopTag Red In Situ Apoptosis Detection

223 kit (TUNEL) (Merk Millipore, Billerica, MA, USA) according to the manufacturer's
224 instructions. The cells were counterstained with 1 µg/ml DAPI for 1-2 min, mounted
225 with Immu-Mount and imaged with a confocal microscope. Cells were considered
226 apoptotic when expressing TUNEL-staining and characteristic apoptotic morphology
227 with condensed nuclei. Total number of cells was determined by DAPI staining and
228 AI was calculated as the percentage of apoptotic cells. For each coverslip 3-5
229 separate areas with at least 100 cells in each image were analyzed except for
230 primary podocytes where approximately 40-50 cells in each image were analyzed. To
231 determine AI of podocytes, podocytes were identified by immunostaining for WT1.
232 Only podocytes outside of a glomerulus and positive for WT1 were included in AI
233 calculations since the total number of podocytes located inside a glomerulus is not
234 possible to determine in this preparation.

235 **SGLT2 knockdown in PTC**

236 SGLT2 gene expression was transiently suppressed using a cocktail of designated
237 siRNAs (Stealth siRNA, cat. no. RSS329982, RSS329983, RSS329984,
238 ThermoFisher). The constructs were delivered into the cells using Lipofectamine
239 RNAiMAX transfection reagent (ThermoFisher) diluted in Opti-MEM media
240 (ThermoFisher) according to manufacturer´s instructions. Briefly, the transfection
241 mixture was added to cells culture medium (10 % FBS) at final concentrations of 30
242 nM for each siRNA. Control cells were transfected with 90 nM of a non-targeting
243 construct (Stealth RNAi™ siRNA Negative Control, Med GC, ThermoFisher). Cells
244 were transfected for 48 h before glucose treatment.

245 **Bax, Bcl-xl abundance and translocation assessment**

246 PTC were incubated with mitochondria-targeted green fluorescent protein CellLight
247 Mitochondria-GFP BacMam (Life Technologies, Grand Island, NY, USA) overnight at

37°C on day two in vitro and treated with glucose and/or albumin on day three in vitro. MC were incubated with BacMam after being seeded on coverslips for 3-4 days and incubated with glucose the following day. At the end of treatment cells were fixed and immunostained for Bax or Bcl-xl. Microscope setting was kept fixed for all measurements. Bax translocation to the mitochondria was analyzed with Matlab (The MathWorks, Inc., Natick, MA, USA) and calculated as the percentage of overlapping Bax (pixels) with mitochondria (pixels) normalized to cell size (pixels). The total abundance of Bax and Bcl-xl was calculated as the percentage Bax or Bcl-xl (pixels) normalized to cell size (pixels). On each coverslip at least three cells were analyzed. The control group was set to 100 %.

Mitochondrial membrane potential detection

The maintenance of mitochondrial membrane potential ($\Delta\psi_m$) was determined with JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanid iodine dye) (Lifetime Technologies, Grand Island, NY, USA). JC-1 dye is a cationic carbocyanine dye which accumulates to the mitochondria. At low concentrations the dye is monomeric causing green (527 nm) fluorescence. As the concentration increase, the dye aggregates causing a fluorescence emission shift from green towards red (590 nm). A depolarization of the mitochondrial membrane is observed as a decrease in red/green fluorescence ratio. Following glucose treatment cells were washed with Krebs-Ringer pH 7.4 and incubated in culture medium containing 2.5 µg/ml JC-1 for 15 min at 37°C. The cells were subjected to live cell imaging using a confocal microscope with fixed settings. The $\Delta\psi_m$ change was quantified as the red (polarized) to green (depolarized) intensity ratio using Matlab. For each coverslip six separate areas were analyzed and all groups were normalized to control.

Reactive oxygen species detection

ROS was measured with Di(Acetoxymethyl Ester) 6-Carboxy-2',7'-Dichlorodihydrofluorescein Diacetate (DCFDA) (ThermoFisher Scientific), where intracellular ROS causes nonfluorescent DCFDA molecules to convert to a green fluorescent form. Following glucose treatment cells were incubated with 10 μM DCFDA and counterstained with 2 drops/ml of NucBlue for 30 min at 37ºC. Cells were rinsed twice with PBS before being subjected to live cell imaging using a confocal microscope with fixed settings for all measurements. ROS was quantified as mean DCFDA intensity in each image. For each coverslip at least 8 individual areas were analyzed. All groups were normalized to control.

Polymerase chain reaction

Cells and tissue samples were collected, and mRNA extracted and purified with RNeasy mini kit (Qiagen, Hilden, Germany) following manufacturer's instructions. PTC were collected as positive control for SGLT2, rat intestine tissue was collected as positive control for SGLT1 and Cos7 were collected as negative control for SGLT1 and SGLT2. Reverse transcription was performed using Iscript cDNA synthesis kit (Bio-Rad Laboratories AB, Solna, Sweden) following manufacturer's instructions using 1 μg sample as starting material. PCR mix was as follows: 1X Phusion GC buffer, 0.2 mM dNTPs, 1.2 mM $MgCl_2$, 0.5 μM each for forward and reverse primer, 5 % glycerol, 0.02 U/μl Phusion polymerase, 2 μl reverse transcription product for each 50 μl preparation and sterile water. Glycerol was used to prevent aggregation of primers, PCR template and PCR product.

Great care was taken to identify specific primers that yielded only one possible PCR product according to Primer Blast. The following primers were used: forward SGLT1 *AGTCTACGTAACAGCACAGAAGAGC*, reverse SGLT1 *CTTCCTCCTCTTCCTTAG*

TCATCTT, forward SGLT2 *CTCTAACATCGCCTACCCACG* and reverse SGLT2 *AGAAAGCACCCTTCTCATTAACAC*.

PCR program was as follows: 98°C for 30 s hot start, 35 cycles: 98°C 10 s, 63°C 15 s and 72°C 30 s, finally 72°C 3 min and 4°C hold.

PCR products were separated on agarose gel and visualized using SYBR green I nucleic acid gel stain. The expected PCR product for SGLT1 is 199 bp and for SGLT2 377 bp. The PCR products were purified using GeneJET PCR purification kit following manufacturer's instructions, sequenced (KI-gene, Solna, Sweden) and matched with expected product (Nucleotide Blast) for verification.

Quantitative real-time reverse transcription polymerase chain reaction

Cells were transfected with SGLT2 siRNA (cat. no. RSS329983, Thermo Fisher) or non-targeting construct for 48 h, collected and mRNA were extracted and purified with RNeasy mini kit following manufacturer's instructions. RNA concentration was determined using the Qubit™ RNA HS assay kit and Qubit™ 3.0 fluorometer. The samples were subjected to one-step quantitative real-time reverse transcription polymerase chain reaction measurements using the Quant-X One Step qRT-PCR SYBR Kit (Clontech Laboratories Inc., Mountain View, CA, USA) on a C1000 Touch™ Thermal Cycler (Bio-Rad). Each sample was analyzed in duplicates and the SGLT2 expression was analyzed using the ΔΔCt method described by Pfaffl (39) and GAPDH as house-keeping gene. The following primers were used: Rn_Gapd_1_SG QuantiTect Primer Assay (Qiagen, Hilden, Germany) for GAPDH and forward *CTCTAACATCGCCTACCCACG* and reverse *AGAAAGCACCCTTCTCATTAACAC* for SGLT2.

Statistical analysis

All data were expressed as mean ± SEM. Significance was determined with a one-way ANOVA (single treatment) or a two-way ANOVA (multiple treatments) followed by t-test when applicable. In experiments with only two sample groups a t-test was used. The statistical significance levels are represented as *$p<0.05$, **$p<0.01$ or ***$p<0.001$ as indicated.

RESULTS

Our study is performed on primary cells from rat kidneys. Preparation of cells is described in Figure 1a. PTC and podocytes were studied within 3 days after plating and MC in passage 3, after other glomerular cells had been eliminated. All cells were cultured in a medium containing 5.6 mM glucose prior to the study. Primary PTC culture were prepared from the outer 150 µm of the renal cortex, which has approximately 90 % proximal tubule volume (3) and generates a culture where 99 % of the cells express the SGLT2 isoform during the first three days after plating (11). The late proximal tubular segments express the SGLT1 isoform. MC cells are reported to express either the SGLT1 or the SGLT2 isoform (44, 45). PTC cultured for 3 days stained for SGLT2 and MC stained for SGLT1 (Supplemental Figure S1a, b). A PCR study demonstrated the presence of SGLT1 and 2 mRNA in PTC sample and of SGLT1 mRNA, but not SGLT2 in MC sample (Figure 1b). PCR products were sequenced for verification. To verify that cells express functional SGLT, the basal glucose uptake was determined in all cell types in presence and absence of sodium. Studies were performed in triplicate and repeated three times. Approximately 60 % of PTC glucose uptake and approximately 40 % of MC glucose uptake is sodium dependent. Podocyte glucose uptake is sodium independent. (Figure 1c, d)

We first tested the apoptotic effect of short-term exposure to a moderately increased glucose concentration (10-15 mM) in PTC. Cells were TUNEL-stained for determination of apoptotic index (AI). A time and concentration dependent increase in AI was recorded (Figure 2 and Supplemental Figure S2). Since PTC can take up glucose via SGLT as well as GLUT, we questioned whether preventing SGLT-mediated glucose uptake could protect from apoptosis. The apoptotic effect of high glucose exposure to increased extracellular glucose concentration was almost completely abolished in PTC co-incubated with glucose and the SGLT2 inhibitor dapagliflozin compared to glucose alone (Figure 3a). To further validate the role of SGLT2 in glucose triggered apoptosis, we downregulated the SGLT2 expression in PTC with siRNA for 48 h. The siRNA treatment reduced SGLT2 mRNA levels (Figure 3c) and prevented glucose induced apoptosis (Figure 3b). The level of AI in control cells with SGLT2 siRNA and negative control was comparable. The possibility that increased osmotic pressure was responsible for the apoptotic effect was excluded by parallel studies using mannitol instead of glucose (Supplemental Table S1). Since few studies have documented the relevance of apoptosis in human DKD, we re-examined biopsy specimens from five male patients with DKD with regard to apoptosis and compared them with biopsies from three male healthy kidney donors in corresponding age (Supplemental Figure S3b). The number of tubules with apoptotic cells in DKD patients was 3 fold higher than in control individuals (Supplemental Figure S3a, c). An aggregation of apoptotic cells in cross-sections of tubular lumen was often observed in the biopsies from DKD patients (Supplemental Figure S3d).

Several lines of evidence suggest that hyperglycemic toxicity is associated with activation of the mitochondrial apoptotic pathway, controlled by the Bcl family of

370 proteins to which the apoptotic protein Bax and the anti-apoptotic protein Bcl-xl
371 belong (12, 16). Under healthy condition, Bcl-xl mainly resides on the mitochondria,
372 whereas Bax is located both in cytosol and on mitochondria. During the course of
373 apoptosis, the abundance of Bcl-xl decreases and the abundance of Bax increases
374 allowing Bax to translocate from cytosol to mitochondria, where it will ultimately
375 permeabilize the mitochondrial membrane, which marks the point of no return in the
376 apoptotic process (Figure 4a). Figure 4b and c show PTC immune-stained for Bcl-xl
377 and Bax, respectively. Mitochondria are visualized with mitochondrial targeted GFP.
378 Quantification of the fluorescent signals show decreased expression of Bcl-xl and
379 increased expression and mitochondrial location of Bax in PTC after 4-8 h exposure
380 to 15 mM glucose (Figure 4d-f). The ongoing apoptotic process was accompanied by
381 a decrease in mitochondrial membrane potential and an increase of reactive oxygen
382 species (Figure 4g, h and Supplemental Figure S4).

383 Proteinuria is a hallmark of DKD. Since proteinuric kidney disease is known to be
384 associated with PTC apoptosis (11), we next examined if co-exposure to threshold
385 concentrations of glucose and albumin would have an additive effect. Cells were
386 exposed to glucose (10 mM) and albumin (2.5 mg/ml) either alone or in combination
387 for 8 h (Figure 5a). Albumin concentration was selected based on our previous study
388 (11), where we show that albumin triggers apoptosis in a dose-dependent manner in
389 PTC. Cells co-exposed to glucose and albumin had significantly higher AI than cells
390 exposed to glucose or albumin alone (Figure 5b). Cells co-exposed to glucose and
391 albumin, in contrast to cells exposed to either substance alone, had a significant
392 increase in Bax abundance and Bax translocation to the mitochondria compared to
393 control (Figure 5c, d).

MC also exhibited high sensitivity to short-term high glucose exposure. A significant increase in AI was recorded in cells exposed to 15 mM glucose for 8 h (Figure 6a). Activation of the mitochondrial apoptotic pathway was confirmed by decreased Bcl-xl abundance, increased Bax abundance and translocation of Bax to the mitochondria (Figure 6b-f). No apoptotic response was observed in MC exposed to 15 mM glucose and co-incubated with phlorizin, a non-selective inhibitor of SGLT 1 and 2 (Figure 6g).

We have previously shown that sub-saturating concentrations of ouabain activates a Na,K-ATPase/IP3 receptor signaling pathway (2) and that down-stream effects involve protection from apoptosis in rat PTC exposed to excessive concentrations of albumin (11) and Shiga toxin (10). Here we show that ouabain 5 nM, which has no effect on intracellular sodium concentration (28), also protects from glucose-triggered apoptosis in SGLT expressing cells (Table 1 and 2). Ouabain 5 nM rescued from apoptosis and changes in Bax and Bcl-xl expression in PTC and MC exposed to 15 mM glucose for 8 h, and in PTC co-exposed to glucose and albumin for 8 h. Ouabain 5 nM also protected from mitochondrial depolarization and increased ROS formation in PTC exposed to 15 mM glucose for 4 and 8 h.

The podocytes were cultured for 3 days and did at that time express the podocyte specific proteins; nephrin, synaptopodin and WT1 (Figure 7a) as well as GLUT4 (Supplemental Figure S1c). The culture medium contained 0.85 μM insulin. The podocytes were first exposed to 15 mM glucose for 8 h. Surprisingly, we found no increase in AI (Figure 7b, f). Nor was there any increase in ROS (Figure 7c). The AI for podocytes was, under control conditions, in the same range as the AI for proximal tubular and mesangial cells studied under control conditions (Supplemental Table

S1). In the majority of previous studies on the response of immortalized podocytes to high glucose exposure, the glucose concentration has been around 25 mM or higher (25, 29, 42). Therefore, we next tested whether 8 or 24 h exposure to 30 mM glucose would provoke apoptosis in podocytes. This was not the case (Figure 7d-f). Immortalized podocytes transfected with SGLT2 vector expressed the protein (Figure 8a, b), but did not display sodium-dependent glucose uptake (Figure 8c, d) and did not respond with apoptosis following exposure to 15 mM glucose for 8 h (Figure 8e).

DISCUSSION

This is the first ex vivo study comparing the early response to a moderate increase in glucose concentrations in three DKD target cells. Our study highlights the importance of using primary cells for understanding the disease process and provides experimental evidence for the hypothesis that cells that are less efficient in adapting their glucose uptake are particularly vulnerable to the acute effects of hyperglycemia.

The majority of our studies were performed on PTC, which mainly express the high capacity, low affinity SGLT2 isoform. Even modest increases in extracellular glucose evoked an SGLT dependent prompt apoptotic response in a small but significant fraction of PTC and MC. The apoptotic response is directly related to excessive glucose uptake via the SGLT transporters, since apoptosis was not observed following inhibition or down-regulation of SGLT. The SGLT glucose uptake is energized by the sodium gradient generated by Na,K-ATPase transporting 3 Na^+ out of the cell and 2 K^+ into the cell, at the cost of 1 ATP. We showed several years ago that exposure of rat proximal tubules to high glucose concentrations results in increased Na,K-ATPase activity and Na,K-ATPase dependent energy consumption (27) (Supplemental Figure S5). This implies that sodium-dependent glucose transport

via SGLT lacks a robust negative feedback control to protect against excessive glucose uptake. Primary podocytes had no measurable sodium-dependent glucose uptake and were resistant to the short-term apoptotic effects of high glucose exposure. Podocytes express GLUT4, which is located in intracellular vesicles that are translocated to the plasma membrane in an insulin-dependent manner. The insulin-signaling pathway has a well-developed negative feedback control via the state of activity of several signaling proteins, including the Rab-GTPase-activating. Podocytes transfected with SGLT2 vector did not exhibit a sodium-dependent glucose uptake and did not respond to high glucose exposure with apoptosis, suggesting a more complex relationship between SGLT and Na,K-ATPase than what was previously believed.

The high sensitivity of PTC to moderately increased extracellular glucose concentrations raises the question whether PTC are targeted already at the onset of diabetes. DKD is rarely diagnosed during the early phase of diabetes, but it is conceivable that DKD exists for a long time as an incipient disease, since the kidney has a high reserve capacity and renal epithelial cells have a relatively high regenerative capacity (17). Albuminuria is both a biomarker and a risk factor in CKD, and excessive albumin uptake in renal epithelial cells is accompanied by a time- and dose-dependent activation of the mitochondrial apoptotic pathway (11). Exposure of PTC to both albumin and glucose resulted in more extensive apoptosis than exposure to albumin or glucose alone. This finding may offer an explanation to the common clinical observation that onset of micro-albuminuria is associated with accelerated decay of renal function (22). Since apoptosis is accompanied by increased secretion of TGF-beta and other pro-inflammatory products that drive a fibrotic process (32, 40), we propose that acute apoptotic responses of PTC to

repeated episodes of hyperglycemia is a major cause of the progressive interstitial fibrosis in DKD. Podocytes do not regenerate and are generally considered the weak link in DKD (30, 41). Our study suggests that hyperglycemia does not exert an immediate toxic effect on the podocytes, and that other factors, such as exposure to glycated proteins and lack and/or resistance to insulin are more likely to be the primary cause of podocyte injury (13, 41).

Ouabain has been demonstrated to rescue PTC from apoptosis in animal models of proteinuric disease (11) and hemolytic uremic syndrome (10) when given in sub-saturating non-toxic concentrations. Here we showed that ouabain 5 nM rescued both PTC and MC from the onset of high glucose triggered apoptosis. These findings have implications for the future design of a therapy that aims to halt the progressive course of DKD by preventing apoptosis.

SGLT2 inhibitors are widely used as blood glucose lowering agents in diabetic treatment (46, 49). Their protective effect with regard to cardiovascular outcome is well documented, while the effect on DKD is still being evaluated (15, 35, 48, 50). Since SGLT2 inhibitors should increase the glucose load to the SGLT1 expressing late PTC, the overall reno-protective effect is difficult to predict and need further studies. Our study points to a previously unappreciated role of SGLT transporters for diabetic complications. The vulnerability of SGLT 1 and 2 expressing cells to short-term exposure to increased extracellular glucose concentrations should have implications for many SGLT expressing cell types, including cardiomyocytes, endothelial cells and pancreatic alpha islet cells (4, 5, 18). Currently there is little information available about the acute response of these cells to high glucose exposure.

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Variable	Cell type	Control	Glucose	Glucose Ouabain	n (#prep)
AI (%)	PTC	3.1 ± 0.1	7.5 ± 0.3	2.9 ± 0.2	9 (3)
Bcl-xl expression (%)	PTC	100	68 ± 4	97 ± 7	15 (5)
Bax expression (%)	PTC	100	155 ± 14	124 ± 9	15 (5)
Bax/mito overlap (%)	PTC	100	144 ± 14	118 ± 9	15 (5)
$\Delta\psi_m$ (%)	PTC	100	66 ± 6	89 ± 5	3 (3)
ROS (%)	PTC	100	143 ± 9	118 ± 11	8 (2)
AI (%)	MC	3.1 ± 0.3	5.3 ± 0.7	3.3 ± 0.5	12 (4)
Bcl-xl expression (%)	MC	100	62 ± 3	97 ± 6	12 (4)
Bax expression (%)	MC	100	162 ± 19	118 ± 19	13 (5)
Bax/mito overlap (%)	MC	100	153 ± 24	96 ± 14	13 (5)

Table 1. Ouabain rescues PTC and MC from high-glucose induced apoptosis

Quantification of AI, Bcl-xl abundance, Bax abundance and accumulation on mitochondria, mitochondrial membrane potential ($\Delta\psi_m$) and ROS in PTC and MC incubated with control (5.6 mM), 15 mM glucose or 15 mM glucose + 5 nM ouabain containing medium for 8 h as indicated. Table shows mean ± SEM. n = number of coverslips, #prep = number of cell preparations.

Variable	Cell type	Control	Glucose Albumin	Glucose Albumin Ouabain
AI (%)	PTC	3.0 ± 0.2	7.4 ± 0.4	3.4 ± 0.4
Bax expression (%)	PTC	100	149 ± 12	104 ± 8
Bax/mito overlap (%)	PTC	100	170 ± 22	124 ± 16

Table 2. Ouabain rescues PTC from high glucose and albumin induced apoptosis Quantification of AI, Bax abundance and accumulation on mitochondria in PTC incubated with control (5.6 mM), 15 mM glucose + 2.5 mg/ml albumin or 15 mM glucose + 2.5 mg/ml albumin + 5 nM ouabain containing medium for 8 h as indicated. Table shows mean ± SEM. n = 9 coverslips from 3 individual cell preparations.

LEGENDS

Figure 1. Cell preparation; Documentation of SGLT expression in PTC and MC (a) PTC (left) were prepared by digesting the outer cortex (150 µm) of rat kidneys into single cells, allowing the cells to culture for 2-3 days before characterizing the cells with SGLT2 antibodies. MC (middle) were prepared by perfusing rats with magnetic beads, extracting glomeruli containing beads with a magnetic collector and digesting the glomeruli to single cells. Following, passage 3 MC were characterized with alpha-smooth muscle actin (α-SMA) antibodies. Podocytes (right) were prepared from extracted glomeruli as for MC. Glomeruli were plated for 3 days letting the podocytes move out from the glomerulus. Podocytes were characterized with WT1 antibodies. (b) PCR for SGLT1 (left) and SGLT2 (right) in PTC, MC, Cos7 and intestine tissue as indicated. Arrows show 199 bp for SGLT1 and 377 bp for SGLT2. (c) Glucose uptake in PTC (left), MC (middle) and podocytes (right) measured with 2-NBDG (green) in Na^+ (upper panel) or Na^+-free (lower panel) buffer (5.6 mM glucose). Cells were counterstained with NucBlue (blue). Scale bars 20 µm. (d) Quantification of Na^+-dependent glucose uptake in PTC, MC and podocytes. Data are expressed as mean ± SEM. n=9 coverslips for PTC and n=8 coverslips for MC from 3 individual cell preparations. n=5 coverslips from 2 individual cell preparations for podocytes. *$p<0.05$, ***$p<0.001$

Figure 2. Short-time apoptotic response of PTC to increased glucose concentration (a) PTC stained with TUNEL (red) and DAPI (blue). PTC were incubated with control (5.6 mM) or 15 mM glucose containing medium for 2, 4 and 8 h. Scale bars 40 µm. (b, c) Quantification of AI in PTC incubated with control, 10 mM glucose in (b) or 15 mM glucose in (c) containing medium for 2, 4 and 8 h. Approximately 100-200 cells in 5 separate areas of each coverslip were counted.

Data are expressed as mean ± SEM. n=9 coverslips from 3 individual cell preparations. *p<0.05, ***p<0.001

Figure 3. SGLT2 inhibition with dapagliflozin or knockdown with siRNA protects from high glucose induced apoptosis in PTC (a) Quantification of AI in PTC incubated with control (5.6 mM), 15 mM glucose or 15 mM glucose + 1 μM dapagliflozin containing medium for 8 h. Dapagliflozin was dissolved in DMSO, an equal amount DMSO was added to all samples as control. Data are expressed as mean ± SEM. n=9 coverslips from 3 individual cell preparations. (b) Top panel: Time-line for siRNA silencing. Bottom panel: Quantification of AI in PTC transfected with SGLT2 or negative control (nc) siRNA for 48 h and incubated with control or 15 mM glucose for 8 h. Data are expressed as mean ± SEM. n=6 coverslips from 2 individual cell preparations. (c) Quantification of SGLT2 mRNA expression following siRNA exposure for 48 h. Data are expressed as mean ± SEM. n=3 cell preparations. **p<0.01, ***p<0.001

Figure 4. High glucose triggers apoptosis via the mitochondrial pathway in a time-dependent manner in PTC (a) Cartoon illustrating activation of the mitochondrial apoptotic pathway. In normal condition, there is a balance between Bcl-xl and Bax preventing apoptosis. When an apoptotic stimulus, i.e. high glucose, activates the intrinsic apoptotic pathway, the balance between Bax and Bcl-xl is disrupted which leads to mitochondrial dysfunction (decreased $\Delta\psi_m$) and apoptosis. (b, c) Immunofluorescence staining for Bcl-xl (b) and Bax (c) expression (red) in PTC incubated with control (5.6 mM) or 15 mM glucose containing medium for 8 h. Mitochondria are shown in green. Scale bars 10 μm. (d-f) Quantification of Bcl-xl abundance (d), Bax abundance (e) and Bax accumulation on mitochondria (f) in PTC incubated with control or 15 mM glucose containing medium for 2, 4 and 8 h. Data

are expressed as mean ± SEM. n=15 coverslips from 5 individual cell preparations. (g) Quantification of $\Delta\psi_m$ in PTC incubated with control or 15 mM glucose containing medium for 2, 4 and 8 h. Data are expressed as mean ± SEM. n=3 coverslips from 3 individual cell preparations. (h) Quantification of ROS in PTC incubated with control or 15 mM glucose containing medium for 4 h. Data are expressed as mean ± SEM. n=8 coverslips from 2 individual cell preparations. *p<0.05, **p<0.01 ***p<0.001

Figure 5. Short-time apoptotic response of PTC co-incubated with high glucose and albumin (a) Cartoon illustrating uptake of high glucose (red arrow) and albumin (purple arrow) in PTC. (b-d) Quantification of AI (b), Bax abundance (c) and Bax accumulation on mitochondria (d) in PTC incubated with control (5.6 mM), 10 mM glucose, 2.5 mg/ml albumin or 10 mM glucose + 2.5 mg/ml albumin containing medium for 8 h. Data are expressed as mean ± SEM. n=9 coverslips from 3 individual cell preparations. *p<0.05, **p<0.01, ***p<0.001

Figure 6. Short-time apoptotic response of MC to increased glucose concentration (a-d) Quantification of AI (a), Bcl-xl abundance (b), Bax abundance (c) and Bax accumulation on mitochondria (d) in MC incubated with control (5.6 mM) or 15 mM glucose containing medium for 8 h. n=12 coverslips from 4 individual cell preparations for AI and Bcl-xl. n=13 coverslips from 5 individual cell preparations for Bax. (e, f) Immunofluorescence staining for Bcl-xl (e) and Bax (f) expression (red) in MC incubated with control or 15 mM glucose containing medium for 8 h. Mitochondria are shown in green. Scale bars 10 μm. (g) Quantification of AI in MC incubated with control, 15 mM glucose or 15 mM glucose + 0.2 mM phlorizin containing medium for 8 h. Phlorizin was dissolved in DMSO, an equal amount DMSO was added to all samples as control. n=9 coverslips from 3 individual cell preparations. Data are expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001

Figure 7. Primary podocytes do not exhibit short-time apoptotic response to increased glucose concentration (a) Immunostaining for podocyte specific markers in primary podocytes; left synaptopodin (green) and DAPI (blue), right nephrin (red) and WT1 (green). Scale bars 10 μm. (b) Quantification of AI in podocytes incubated with control (5.6 mM) or 15 mM glucose containing medium for 8 h. n=12 coverslips from 4 individual cell preparations. (c) Quantification of ROS production in podocytes incubated with control or 15 mM glucose containing medium for 8 h. n=8 coverslips from 2 individual cell preparations. (d, e) Quantification of AI in podocytes incubated with control or 30 mM glucose containing medium for 8 h (d) or 24 h (e). n=12 coverslips from 4 individual cell preparations. (f) Podocytes stained with TUNEL (red), WT1 (green) and DAPI (blue). Podocytes were incubated with control, 15 mM glucose or 30 mM glucose for 8 or 24 h as indicated. Scale bars 40 μm. Data are expressed as mean ± SEM.

Figure 8. Immortalized podocytes transfected with SGLT2 do not have a sodium-dependent glucose uptake or increased apoptosis (a) Immortalized podocytes transfected with SGLT2-ires-CFP (green). Nuclei were counterstained with DRAQ5 (red). Scale bar 40 μm. (b) Immunostaining for SGLT2 (green) in immortalized podocytes transfected with SGLT2. Scale bar 40 μm. (c) Glucose uptake in immortalized podocytes measured with 2-NBDG (green) in Na^+ or Na^+-free buffer (5.6 mM glucose). Scale bars 40 μm. (d) Quantification of Na^+-dependent glucose uptake in immortalized podocytes. n=8 coverslips. (e) Quantification of AI of immortalized podocytes transfected with empty vector CFP or SGLT2, incubated with control (5.6 mM) or 15 mM glucose containing medium for 8 h. n=6 coverslips. Data are expressed as mean ± SEM.