Functional role of sodium glucose transporter in high glucose-mediated angiotensin type 1 receptor downregulation in human proximal tubule cells

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Yesudas R, Snyder R, Abbruscato T, Thekkumkara T. Functional role of sodium glucose transporter in high glucose-mediated angiotensin type 1 receptor downregulation in human proximal tubule cells. Am J Physiol Renal Physiol 303: F766–F774, 2012. First published May 30, 2012; doi:10.1152/ajprenal.00651.2011.—Previously, we have demonstrated human angiotensin type 1 receptor (hAT1R) promoter architecture with regard to the effect of high glucose (25 mM)-mediated transcriptional repression in human proximal tubule epithelial cells (hPTEC; Thomas BE, Thekkumkara TJ. Mol Biol Cell 15: 4347–4355, 2004). In the present study, we investigated the role of glucose transporters in high glucose-mediated hAT1R repression in primary hPTEC. Cells were exposed to normal glucose (5.5 mM) and high glucose (25 mM), followed by determination of hyperglycemia-mediated changes in receptor expression and glucose transporter activity. Exposure of cells to high glucose resulted in downregulation of ANG II binding (4,034 ± 163.3 to 1,360 ± 154.3 dpm/mg protein) and hAT1R mRNA expression (reduced 60.6 ± 4.643%) at 48 h. Under similar conditions, we observed a significant increase in glucose uptake (influx) in cells exposed to hyperglycemia. Our data indicated that the magnitude of glucose influx is concentration and time dependent. In euglycemic cells, inhibiting sodium-glucose cotransporters (SGLTs) with phloretin decreased glucose influx by 28.57 ± 0.9123 and 54.33 ± 1.202%, respectively. However, inhibiting SGLTs in cells under hyperglycemic conditions decreased glucose influx by 53.67 ± 2.906%, while GLUT-mediated glucose influx decreased by 9.42 ± 2.162%, while GLUT-mediated glucose influx by 10.220.32.246 on June 23, 2017 http://ajprenal.physiology.org/ Downloaded from http://ajprenal.org

Diabetes and hypertension are two leading contributors to the development of diabetic nephropathy and end-stage renal disease. Several systemic and/or intrarenal networks of growth factors and cytokines may be modulated by diabetes. Activation of the renin-angiotensin system (RAS) is one of several factors believed to be involved in the development of diabetic nephropathy (37). The octapeptide ANG II is the primary effector molecule by which the RAS exerts its physiological actions through membrane receptors; the angiotensin type 1 receptor (AT1R) and type 2 receptor (AT2R) (1, 3). AT1 receptors are classic G protein-coupled receptors and play a critical role in the control of blood pressure and sodium homeostasis (1, 12). A potential link between ANG II and progressive diabetic pathology has been demonstrated in both clinical and experimental studies with angiotensin-converting enzyme (ACE) inhibitors and AT1R blockers (9, 26, 67). Thus it may be presumed that ANG II through binding and activation of the AT1R promotes the progression of diabetes-induced nephropathy in patients and experimental animal models. However, AT1R expression in the kidney is upregulated in the glomerular cells and downregulated in proximal epithelial cells in experimentally induced diabetes mellitus (13, 59) while insulin supplementation normalizes each tissue expression to nondiabetic controls, respectively. More specifically, in the proximal tubule the AT1R is downregulated from early onset to end-stage diabetic nephropathy. The functional significance of this decrease in the AT1R is not well understood, since studies have shown that inhibition of the AT1R prevents the progression of kidney disease. The proximal tubule is a major site of salt and water reabsorption, with up to 67% of the filtered load of sodium reabsorbed in this segment (18). In normal physiology, proximal tubule function is under the control of vasoconstrictor ANG II-stimulated reabsorption (17, 18). Therefore, alterations (increase/decrease) in AT1R expression have significant pathophysiological consequences.

Previously, we have identified a glucose-mediated transcriptional repressor element and demonstrated that high glucose alters the rate of transcription by interacting with glucose-inducible nuclear trans-acting factor(s) in human proximal tubule cells (54). However, the cellular mechanism by which glucose initializes these repressor effects on the hAT1 gene was not understood. There are two general classes of glucose transporters described in mammalian cells (44, 49), facilitative and sodium coupled. Currently, at least 6 sodium-dependent and 13 facilitative transporters are recognized (61). They exhibit different substrate specificities, transport affinities, developmental regulation, and tissue-specific expression (21, 58). Depending on the concentration gradient, facilitative glucose transporters (GLUTs) transport glucose in either direction across the cell membrane (39), while sodium-glucose cotransporters (SGLTs) transport substrates in a unidirectional manner (36). In particular, SGLT3 is reported as a glucose sensor in the human kidney (62). The kidney plays a major role in glucose homeostasis by reabsorbing filtered glucose. Glucose also influences many aspects of renal function including sodium-glucose cotransport, gluconeogenesis, activation of DNA and protein synthesis, and cellular hypertrophy (49). In the nephron, all filtered glucose is reabsorbed in the proximal tubule. Reabsorption of luminal glucose against a concentration gradient occurs via apical SGLTs (34, 64) and GLUTs (23, 28).
Glucose then diffuses from the cells into the bloodstream via basolateral facilitative glucose transporters (14). SGLT1,2 and GLUT1,2 are the major glucose transporters active in proximal tubule epithelial cells (PTECs) (57, 62, 63). Studies have shown that 90% of filtered glucose is reabsorbed by the SGLT2 and GLUT2 in the proximal tubule (36, 61). Furthermore, diabetic nephropathy is reported to be associated with dysfunction of renal proximal tubular cells and alteration in glucose transporter functions under hyperglycemic condition (20).

The objective of this study was to determine the relationship of glucose transporters with respect to their initiation of a hyperglycemic induction of hAT1R downregulation. We selected hPTECs because 1) in diabetes mellitus hAT1R gene expression downregulates in the proximal tubule; 2) alterations in proximal tubule hAT1R expression results in marked changes in volume reabsorption, glomerular filtration rate, and renal vascular resistance; and 3) these cells are suitable model systems for investigating glucose-specific responses in the absence of insulin-like growth factors and other confounding variables. Our study demonstrates that hAT1R expression does reduce significantly upon exposure to high concentrations of glucose (25 mM). The participation of glucose transporters is not equally distributed to all transporter isoforms, but rather is due principally to increased uptake via SGLT, while the uptake due to GLUT remains roughly the same compared with cells exposed to normal (5.5 mM) concentrations of glucose. The results of this study are important to an understanding of the control of AT1R expression in the proximal tubule and the relationship this receptor may have with cells exposed to normal (5.5 mM) and diabetic condition (25 mM) alterations.

**MATERIALS AND METHODS**

**Materials.** Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). d-[3H]glucose and d-[14C]methyl α-glucopyranoside (αMG) were from PerkinElmer (Boston, MA) and [3H]-ANG II was from Amersham (Piscataway, NJ). Losartan potassium was kindly provided by Merck Research Laboratories (Rahway, NJ). The AT1R (N-10) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), goat-anti rabbit IgG horseradish peroxidase conjugated was from Bio-Rad (Hercules, CA), Alexa Fluor goat anti-rabbit IgG, and 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) were from Invitrogen. Phlorizin and phloretin, were from Sigma (St. Louis, MO).

Real-time PCR reagents and equipment were from Applied Biosystems (Foster City, CA). Oligonucleotides were provided by Integrated DNA Technologies (Coralville, IA). Electrophoresis reagents were from Bio-Rad (Palo Alto, CA). DNA Technologies (Coralville, IA). Western blotting system according to the manufacturer’s instructions (Bioscience, Frederick, MD).

**Cell culture.** Human primary PTECs (hPTECs) were obtained from Lonza (Walkersville, MD). Cells were maintained in renal epithelial cell basal medium (REBM) containing 0.5% FBS and REGM Single-Quots cocktail, at 37°C in 5% CO2 under 100% humidity. Cultures were fed with fresh growth medium every 3 days. For the studies, cells were grown to 75–80% confluence, and the medium was replaced with DMEM with normal glucose (5.5 mM) containing 0.5% FBS and grown for another 24 h. For hyperglycemic conditions, cells were exposed to 25 mM glucose in DMEM containing 0.5% FBS for indicated times. Simultaneously, the control plates were replenished with fresh medium containing normal concentrations of glucose. d-Glucose was used in all experiments unless otherwise stated. In these studies, experiments were performed from passage 3 to passage 7, with no observed change in phenotype or proliferative activity (2).

**Receptor binding assay.** Cells were plated in multiwell plates, and radioligand binding studies were performed as described previously (53). Briefly, cells were seeded in multiwell plates and incubated in respective glucose concentration for 18 h. Cells were rinsed twice with HBSS and incubated in binding buffer (50 mM Tris-HCl pH 7.4, 120 mM NaCl, 4 mM KCl, 1 mM CaCl2, 10 μg/ml of bacitracin, 0.25% BSA, and 2 mg/ml of dextrose) at 22°C. Designated wells were preincubated with 1 μM unlabeled ANG II for 10 min for purposes of calculating nonspecific binding. All samples were then incubated with 0.05 nM [3H]-ANG II for 1 h at 22°C. Nonspecific bound ANG II was removed by washing three times with the ice-cold HBSS. Finally, cells were dissolved in 1 ml 0.25 N NaOH-0.25% SDS lysis solution and transferred to counting vials and analyzed on a Beckman liquid scintillation counter. A parallel subset of each condition was used for protein determination by the Bradford method (11).

**hAT1R mRNA analysis by dual RT-PCR.** Total RNA was prepared from the cells using a Super Array total RNA isolation kit according to the manufacturer’s instructions (Bioscience, Frederick, MD). mRNA was reverse transcribed with oligo (dT) to first-strand cDNA using Superscript II RT (Qiagen, Hilden, Germany). Equivalent concentrations of cDNA were amplified by dual-PCR using the primer sets as follows: AT1R, sense 5'-CATCATCTTTTGTGGTGAGGA-3'; antisense 5'-GCCAGCGACAGCCAAATAA-3' and β-actin, sense 5'-AACCGGAG AAATGACCGCATGCTTT-3'; antisense 5'-AGACCAGTGGCATCTGCTGAAATC-3'. The PCR reactions were run for 35 cycles of 94°C (30 s), 57°C (30 s), and 72°C (30 s) using the ABI 7700 real-time PCR system (Applied Biosys-tems). After amplification, the RT-PCR products were separated in 1.5% (w/v) agarose gels and stained with ethidium bromide. The intensity of bands was captured and analyzed using Quantity One software. For real-time quantitative analysis of AT1R mRNA, two parallel PCRs performed in triplicate, each containing 50 ng total cRNA and either AT1R (sense 5'-ATTTAGGAC GTGCTGATTTAGC-3'; antisense 5'-CACCGGATTCATCATGCTGG-3') or β-actin (sense 5'-TTGACCTGACCATCGTA TAAGA-3'; antisense 5'-GCCGACATCTGATGCTCC-3')-specific primers were performed using 2X SYBR Green Master Mix (Applied Biosystems). Following the reaction, threshold cycles (Ct) were calculated and absolute concentrations were calculated relative to a standard curve for AT1R and β-actin mRNA reactions (51).

**Western blot analysis.** hPTECs were exposed to 25 mM glucose, treated with various agents for the indicated times, and washed with PBS. Cells were scraped in lysis buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 15% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM NaF, and 1 mM PMSF). Protein concentration was determined using Bio-Rad protein assay reagent based on the Bradford method (11). Equal amounts of proteins were resolved by 8% PAGE, transferred to a nitrocellulose membrane, and incubated with their respective primary antibodies. Immunoreactive bands were visualized using a chemiluminescent Western blotting system according to the manufacturer’s instructions (Amersham). The intensity of bands was captured by the Bio-Rad VersaDoc and quantitated using Quantity One software.

**Glucose uptake studies.** Cells plated in a 24-well plate with REBM (Lonza) and grown to 70–80% confluence and were then incubated in serum-free DMEM for 18–24 h. The cells were then incubated with 0.05 nM (1 μCi/well) of d-[3H]glucose/[14C]jodoglucose along with medium for the desired time points. A physiologically normal glucose concentration (5.5 mM) and diabetic condition (25 mM) were the two conditions studied. Glucose transport studies were conducted using glucose-free medium as a control (56). Phlorizin- and phloretin-sensitive r-glucose uptake was assessed in the presence of these inhibitors in the uptake medium. After the incubation period, cells were washed twice in ice-cold HBSS, solubilized in 1 ml NaOH/SDS solution, and aliquots were submitted for liquid scintillation counting or protein determination.
Immunofluorescence microscopy. Proximal tubule cells were seeded in chamber slides and treated under indicated conditions. Cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde in PBS, pH 7.4, for 22°C for 20 min, then permeabilized with 0.1% Triton X-100 in PBS for 5 min. Cells were blocked in 5% goat serum at 22°C for 1 h and incubated in hAT1R-specific primary antibody in 2.5% goat serum overnight at 4°C. After washing, cells were incubated with a fluorescence-tagged secondary antibody for 1 h at 22°C in the dark to prevent photodecay of the fluorophore. Nuclei were stained with 10 nM DAPI, and samples were mounted using ProLong Gold antifade mounting medium (Invitrogen, Eugene, OR). Image capture was performed at 22°C using an Olympus IX-81 microscope equipped with an Olympus SpectraMaster II monochromator light source and UltraPix camera (PerkinElmer) under a PlanApo 60x/1.40 oil-immersion objective. Images were analyzed using UltraView software (PerkinElmer). Other analyses were performed using an Olympus IX-81 microscope equipped with an Olympus U-CMAD3 camera under a PlanApo 60x/1.40 oil-immersion objective and analyzed using Slidebook software.

Cell viability assay. For the viability assay, high glucose- and phlorizin-treated cells were harvested by 2-min trypsinization followed immediately by anti-trypsin inactivation. Trypan blue solution (0.4%) was added to aliquots of cell suspension in a 1:1 ratio and allowed to stand for 5 min at room temperature. A hemocytometer was used to count live and dead cells, and the number of viable cells was calculated and expressed as a percentage.

Data analysis. Results presented in the study are triplicate values and representative of three or more independent experiments. Gel images and X-ray films were scanned on an Epson Expression 1600 Photoscanner using Adobe Photoshop CS3. Statistical significance was analyzed with GraphPad Prism software. Where appropriate, statistical differences were compared by use of ANOVA with post hoc Bonferroni tests or appropriate $t$-tests for parametric or nonparametric comparison. The values presented are means ± SE, and $P < 0.05$ was considered to be significant.

RESULTS

High glucose downregulates the hAT1R. Cell were exposed to normal-glucose and high-glucose medium for 48 h, and we measured the hAT1R-specific ANG II binding. The results show that cells exposed to the hyperglycemic condition downregulated hAT1R binding (Fig. 1A). After 48 h, there was a 53.28 ± 2.212% ($P < 0.0001, n = 3$) reduction in the [3H]ANG II binding on cells grown under high glucose compared with the cells exposed to a normal concentration of glucose. To determine whether these changes in ANG II binding were hAT1R specific, we investigated binding with the AT1R antagonist losartan. The results showed that the hAT1R is the major subtype downregulated by high glucose treatment (hAT1R blockade yielded 55.00 ± 2.331% reduction in ANG II binding) ($P < 0.0001, n = 3$) in normal glucose losartan-treated cells and 51.66 ± 4.070% reduction ($P = 0.0002, n = 3$) in high glucose losartan-treated cells compared with normal glucose control (Fig. 1A). The high glucose-induced decrease in [3H]ANG II binding does not represent proximal tubule cell toxicity or accelerated cell death, as trypan blue cell viability assays showed that the viable cell count at 48-h high glucose exposure was similar to the control ($P = 0.7725, n = 3$) (Fig. 1B). To evaluate whether the observed changes are due to $d$-glucose itself, or rather a change in cellular osmolarity, the cells were exposed to 25 mM $d$-glucose, $l$-glucose, or mannitol. A binding study was then performed after 48-h incubation in these conditions (Fig. 1C). The results show that only $d$-glucose is capable of downregulating [3H]ANG II binding (binding reduced 55.34 ± 5.255%, $P = 0.0005, n = 3$), while $l$-glucose and mannitol both had no effect ($P = 0.6718, n = 3$; $P = 0.6218, n = 3$, for $l$-glucose and mannitol, respectively). Furthermore, an immunofluorescence study using a specific antibody directed against the hAT1R showed significantly less immunoreactivity at the cells’ surface when exposed to high...
glucose compared with normal glucose (Fig. 2A). We confirmed this observation by Western blot analysis, which displayed a 45.5 ± 8.242% decrease in hAT1R protein expression compared with control (P = 0.0005, n = 3) (Fig. 2, B and C).

In a previous study (54), we have demonstrated that hAT1R promoter sensitivity to hyperglycemia results in transcriptional repression of hAT1R gene expression. To further validate the promoter studies and to correlate the high glucose-mediated downregulation of hAT1R binding and protein, we determined the hAT1R mRNA expression in normal glucose and high glucose conditions using dual RT-PCR. The results showed downregulation of hAT1R mRNA in hyperglycemic conditions compared with control (Fig. 3A). In total RNA from hPTECs under hyperglycemic conditions, AT1R mRNA expression decreased by 60.6 ± 4.643% (P < 0.0001, n = 5) (Fig. 3B). The above results demonstrate that cells exposed to high glucose showed downregulation of hAT1R protein and mRNA expression at 48 h, which is consistent with transcriptional repression.

Under hyperglycemia, glucose uptake is concentration and time dependent. To determine the magnitude of glucose influx in normal and hyperglycemic hPTECs, we performed dose-response and time course studies. Glucose uptake studies indicated that the glucose influx is concentration and time dependent (Fig. 4). The dose-response study with concentrations ranging from euglycemic (human) plasma glucose level of 5.5 mM to hyperglycemic concentration (25 mM) gave a linear plot (Fig. 4A). To determine the time course, cells were exposed to D-[3H]glucose for up to 30 min. Upon comparison of normal and hyperglycemic cells, the uptake of glucose reached V_{max} between 15 and 20 min in hyperglycemic cells.

Fig. 2. A: immunofluorescent study shows hAT1R is downregulated in cells exposed to 25 mM glucose. Immunofluorescent staining used primary rabbit anti-hAT1R IgG followed by secondary anti-rabbit IgG conjugated with Alexa Fluor 488. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Left: native hAT1R in hPTECs in euglycemic condition (5.5 mM). Right: hAT1R expression after treatment with 25 mM glucose for 48 h. B: Western blot analysis using total cell lysates indicates hAT1R protein is downregulated during hyperglycemic conditions (HG). Total cell lysates were prepared from control (5.5 mM) and hyperglycemic (25 mM)-treated cells and immunoblotted with hAT1R antibody (top). Blots were stripped and reprobed with anti-β-actin antibody to demonstrate equal loading (bottom). A representative blot is shown (n = 3). C: densitometric analysis of hAT1R Western blots normalized to β-actin control (n = 3). Values are means ± SE. ***P < 0.001 vs. untreated control.

Fig. 3. A: hyperglycemic conditions downregulate mRNA expression in hPTECs. Shown in a representative dual PCR-ethidium bromide gel of exposed cells that were euglycemic (NG, left) or hyperglycemic (HG, right) for 48 h. Bands were captured and quantified using Bio-Rad Quantity One software. hAT1R bands are normalized to β-actin control. Bands detected are at 296 and 250 bp for β-actin and hAT1R mRNAs, respectively. B: quantitation of hAT1R mRNA expression determined by densitometric analysis after normalization to β-actin and data are expressed as means ± SE; n = 3. *P < 0.001 compared with untreated control.
compared with <5 min for euglycemic conditions. Under hyperglycemic conditions, glucose uptake was significantly increased (290.3 ± 17.32% relative to normal glucose control at 30 min; \( P < 0.0001, n = 3 \)) (Fig. 4B).

**Increased glucose uptake in hyperglycemic hPTECs is mediated by SGLTs.** To further investigate the role of glucose transporters in the increased glucose uptake in cells exposed to hyperglycemic conditions, we conducted glucose uptake studies while selectively inhibiting different glucose transporters. Phlorizin is reported as a potent competitive inhibitor for SGLT1, -2, and -3 with varying inhibitor constants (63). In previous studies, 0.5 mM phlorizin inhibited more than 95% of \( \alpha \)-MG (a glucose analog) uptake in rabbit proximal tubule cells (48) and 88 ± 9% of \( \alpha \)-MG uptake in hPTECs (56). Phlorizin has no known affinity or inhibitor effect on GLUT1–12 (5). Phloretin is a potent inhibitor of facilitative glucose transporters (32). Therefore, we used 0.5 mM phlorizin or 150 \( \mu \)M phloretin to inhibit D-glucose uptake, each concentration confirmed as effective in vitro to specifically inhibit respective transporters according to the literature. At a normal (5.5 mM) glucose concentration, cells incubated with phlorizin or phloretin showed decreased glucose uptake by 28.57 ± 0.9123 (\( P < 0.0001, n = 3 \)) and 54.33 ± 1.202% (\( P < 0.0001, n = 3 \)), respectively. In contrast, in high glucose (25 mM)-treated cells, phlorizin treatment decreased glucose influx by 53.67 ± 2.906% (\( P < 0.0001, n = 3 \)). However, GLUT-mediated glucose uptake remained the same in 25 mM glucose-treated cells as phloretin reduced glucose uptake by 57.67 ± 3.180% (\( P < 0.0001, n = 3 \)), or at a level not significantly different from euglycemic cells undergoing GLUT inhibition (\( P = 0.2066, n = 3 \)) (Fig. 5A). Additionally, we performed an uptake study using \( \alpha \)-MG as a selective substrate for SGLT transport. The results show that under hyperglycemia, there is a significant increase in \( \alpha \)-MG uptake (203.3 ± 9.132%, \( P < 0.0001, n = 3 \)) indicating an increased capacity for glucose uptake is mediated by SGLT (Fig. 5B).

**Hyperglycemia-induced hAT1R downregulation is mediated by SGLT.** To determine the transporter-mediated effect of high glucose on native hAT1R expression, cells were exposed to phlorizin and phloretin for 48 h and measured for hAT1R-specific ANG II binding. Cell viability studies indicated that treatment with phlorizin or phloretin and 25 mM glucose did not significantly affect cell survival (data not shown). SGLT inhibition for 48 h displayed restoration to normal glucose hAT1R binding (mean difference between normal glucose

![Fig. 4. Time- and dose-dependent effect of glucose uptake. A: time course of D-[3H]glucose uptake for 30 min in the presence of NG (●) and HG (○). Data are expressed as nmol/mg protein; \( n = 3 \). B: D-[3H]glucose uptake studies with increasing concentration of glucose (from 0 to 25 mM) for 20 min. Data are expressed nmol/mg protein/20 min; \( n = 3 \).](http://ajprenal.physiology.org/)

![Fig. 5. Effects of sodium-glucose cotransporter (SGLT)- and glucose transporter (GLUT)-specific inhibitors in the presence of NG and HG. A: 20-min (time to reach maximum glucose uptake) D-[3H]glucose uptake study demonstrating SGLT and GLUT inhibition with phlorizin (Pzin) and phloretin (Ptin). B: \( [\text{14C}]\)methyl \( \alpha \)-glucopyranoside (\( \alpha \)-MG) uptake studies with euglycemic and hyperglycemic conditions at 20 min. Values are means ± SE expressed as nmol·mg protein⁻¹·20 min⁻¹; \( n = 3 \). ***\( P < 0.001 \) compared with untreated control.](http://ajprenal.physiology.org/)
control and 25 mM glucose combined with 0.5 mM phlorizin
8.661 ± 5.102%; \( P = 0.1089, n = 8 \), while treatment with
phloretin had no significant effect on ANG II binding (mean
difference normal glucose control and 25 mM glucose com-
bined with 0.15 mM phloretin, 51.41 \pm 4.321%; \( P < 0.0001, 
\) \( n = 8 \) ) (Fig. 6). To validate this observation, immuno- 
fluorescent microscopy was performed after 48-h phlorizin/phloretin
treatment in hyperglycemic conditions. The results of this 
study demonstrated that hAT1R expression was restored to
near control levels with treatment with 0.5 mM phlorizin but
not with 0.15 mM phloretin (Fig. 7). We also observed that
phlorizin-mediated restoration of hAT1R protein expression
correlated with a restoration of hAT1R mRNA expression
(mean difference between control and 25 mM glucose com-
bined with 0.5 mM phlorizin, 7.777 \pm 9.280%; \( P = 0.4161, 
\) \( n = 3 \) ), while phloretin-treated cells still showed significant
downregulation of hAT1R mRNA (mean difference between
normal glucose control and 25 mM glucose combined with
0.15 mM phloretin, 51.39 \pm 4.604%; \( P < 0.0001, n = 3 \))
(Fig. 8).

**DISCUSSION**

The RAS plays a major role in blood pressure regulation and
electrolyte/extracellular fluid volume control. As a result, fac-
tors that influence this system have significant potential for 
pathological developments, particularly in the kidney (27).
Diabetic nephropathy is a disease affecting approximately
one-third of patients with type 1 diabetes as well as 20–40% of

![Fig. 6. SGLT inhibition restores [3H]ANG II binding in cells exposed to hyperglycemic conditions. Radioligand binding assay was performed after 48-h 25 mM glucose (HG) treatment, HG with SGLT inhibition (0.5 mM Pzin), and HG with GLUT inhibition (0.15 mM Ptin). Values are means ± SE; \( n = 8 \). *** \( P < 0.001 \) compared with NG control.](image1)

![Fig. 7. hAT1R-specific immunofluorescent studies in cells exposed to NG (A), HG (B), HG with 0.5 mM Pzin (C), and HG with 0.15 mM Ptin (D). Immunofluorescent staining was done using primary polyclonal anti-hAT1R IgG followed by secondary IgG conjugated with Alexa Fluor 488. Nuclei were stained with DAPI. Images are representative of experiments performed; \( n = 3 \).](image2)

![Fig. 8. SGLT inhibition (Pzin) restores hAT1R mRNA in hyperglycemic conditions (HG), while Ptin remains ineffective. hAT1R expression was quantified by real-time PCR analysis after normalization to \( \beta \)-actin. Values are means ± SE relative to NG; \( n = 3 \). *** \( P < 0.001 \) compared with NG control.](image3)
patients with type 2 diabetes (50, 66). Diabetic nephropathy leading to end-stage renal disease is also associated with a significantly increased risk for the development of cardiovascular disease (CVD) (8, 60). In the present study, we demonstrated that hyperglycemic conditions in hPTECs downregulated ANG II binding, which correlated with a reduction in both hAT1R protein and mRNA, and thus the study elucidates at least one mechanism of how glucose regulates endocrine signaling within the kidney. In previous studies, when mesangial cells were grown under hyperglycemic conditions, the AT1R-mediated increase in intracellular calcium and the contractile response were diminished (25). Similarly, in the renal microvasculature innervating the nephron, the effects of ANG II were significantly reduced in diabetic rats (24, 30). The current study’s findings were supportive of previous reports demonstrating in animal models that downregulation of ANG II binding was associated with AT1R mRNA and protein in diabetic rat proximal tubule cells (13, 40) as well as primary rabbit proximal tubule cells (45). By using the respective inhibitors of transcription and translation, actinomycin D and cycloheximide, Park and Han (45) suggested that there was a role for transcription and translation on glucose-mediated downregulation of ANG II binding in rabbit proximal tubule cells. Although we have not correlated transcriptional activity with the detected mRNA downregulation presented in this study, in a previous study we have shown the promoter of the hAT1R gene has a cis-acting glucose repressor element (GluRE) acting through trans-acting factors (54). Furthermore, there is significant evidence in support of hAT1R downregulation in diabetes. In humans, there is a significant reduction in ANG II-induced renal vascular resistance and mean arterial blood pressure in early type 1 diabetes mellitus (42).

The larger question posed by these studies and the present study is why the downregulation of a receptor, known to be upregulated during the pathogenesis of cardiovascular disease, should be inversely downregulated in the kidneys of hyperglycemic subjects, thereby exacerbating the nephropathy and eventually the progression of cardiovascular disease. One possible explanation may be simultaneous downregulation of the AT1R in the glomerulus, thereby removing the endogenous antagonistic effect that this receptor has with AT1R signaling (59). It was found that in streptozotocin-induced diabetic rats, AT1R and AT3R expression were significantly downregulated, with downregulation of the AT1R in the tubule epithelium and AT3R downregulation in the glomerulus, leading the authors to conclude that the reduced AT1R expression may be partially offset by the concomitant reduction in the AT3R and its counteracting effects, resulting in an AT1R-dominant response. Alternatively, the AT1R may have variable responsivity to ANG II among different cell types in the kidney. Patients have generally been found to be hyperresponsive to ANG II infusion (7), but the hemodynamic responsiveness to ANG II in the kidney has been reported as normal or even reduced (10, 16, 22), indicating that the receptor itself is either downregulated or becoming less sensitive to ligand stimulation. The origin of deregulated AT1R production is not resultant from a single stimulus during type 2 diabetes mellitus; hyperinsulinemia is known to act as a potent upregulator of hAT1R expression (4, 19, 29), but the overexpression of the receptor is believed to be consequent to reactive oxygen species resultant in insulin resistance, rather than the direct signaling by insulin itself (43).

In the study above, the authors found that fructose-fed rats were indeed hyperinsulinemic, hypertensive, and showed enhanced expression of the AT1R. However, upon exogenous infusion of insulin, AT1R expression and hypertension were entirely alleviated. It is possible that upon more efficient insulin signaling in an otherwise insulin-resistant system, reactive oxygen species production was minimized, leading to no further increase in AT1R production, but rather a return to homeostasis with the exception of circulating insulin titers.

However, insulin responsiveness is important to facilitative transport of glucose across different cell membranes, but as this study demonstrates, the increase in glucose uptake is mediated by sodium-coupled glucose transporters (i.e., non-GLUT) in hPTECs. In these cells, the distribution of SGLTs and GLUTs are site specific, with the GLUTs expressed basolaterally while the SGLTs are expressed in the brush border or luminal side, and the SGLTs mediating glucose influx from the renal filtrate and the GLUTs located at the basolateral border facilitating efflux to the surrounding tissues and vasculature (52). However, in diabetes it has been shown that GLUTs translocate to the brush border of the proximal tubule cells, diminishing their ability to efflux glucose to the basolateral tissues (41). In the present study, the hyperglycemic cells showed a 290.3% increase in glucose uptake compared with the euglycemic control. This result is consistent with a report in which it was demonstrated that SGLTs were upregulated in human exfoliated proximal tubular epithelial cells of patients with type 2 diabetes and markedly increased glucose uptake (46). However, in the current study SGLT-mediated glucose influx in hyperglycemia occurs within minutes of initial exposure to elevated glucose, suggesting an increased activity of the transporter without a significant change in the expression profile. The dose-dependent relationship between glucose concentration and observed uptake was linear within the time observed, and inhibiting the SGLTs reversed the downregulatory effect on the hAT1R. Based on the findings of the present study and previous observations in the literature, the proximal tubule cells in diabetes may take in more glucose, but the facilitative transporters requisited to remove the absorbed glucose to the basolateral tissues may be insufficient. With regard to insulin and expression of the hAT1R, our laboratory previously conducted gene promoter analysis and determined that insulin treatment of hPTECs resulted in a direct upregulation of hAT1R expression through a cis-acting element (64), which was consistent with other studies indicating that hyperinsulinemia upregulates hAT1R expression in hPTECs. However, the present study’s findings suggest that glucose uptake by sodium-coupled transport predominates in regulating hAT1R expression. Future studies in other tissue types should characterize insulin’s mixed effects in the regulation of the hAT1R with respect to its actions as a potentiator of glucose uptake and direct effect as a growth factor.

Human PTECs isolated from patients with type 2 diabetes showed a significant increase in glucose uptake, up to threefold, compared with those from healthy volunteers (46). One of the principal findings of the present study was that the increase in glucose uptake is primarily mediated by SGLTs, rather than the facilitative glucose transporters (GLUTs). Glucose transport in the diabetic kidney is upregulated in response to hyperglycemia and glycosuria (28). Sakhraei et al. (48) reported that 25% of d-glucose uptake by rabbit PTECs was
mediated by the sodium-glucose transport process (48). Other researchers have found that phlorizin-induced inhibition of SGLT mediated a 28.8 ± 3.5% reduction in αMG uptake in hPTECs (31). SGLTs serve diverse purposes in human cells, acting as cotransporters, uniporters, glucosensors, water channels, and water transporters (63). ANG II is reported by Kawano et al. (33) to inhibit SGLT in renal proximal tubule epithelial cells; therefore, we may see how the respective receptor and transporter play a complex self-regulatory role, with each participant in the cascade having a positive feedback mechanism on the ligand or transport molecule involved and a negative feedback mechanism on the opposing strata. The transporter facilitates the high glucose transport to downregulate the receptor, whereas receptor stimulation results in downregulated glucotransporter expression. There are numerous factors that may affect the expression of SGLT in a given tissue, including proteinuria (35), hyperammonemia (47), and hyperglycemia itself (6, 46). While this is the first report of the role of SGLTs in the regulation of hAT1R expression, due to the inherent complexity of this system, the role of specific SGLTs and their respective long-term regulation of hAT1R expression require further investigation.

In conclusion, our study found that exposure of hPTECs to hyperglycemia downregulated the hAT1R. The observed effect of hyperglycemia on hAT1R gene expression was mediated through increased glucose transport via sodium-glucose transporters, which eventually has a downregulatory effect on through increased glucose transport via sodium-glucose transport. Glucose transport may result in a significantly different hAT1R expression profile.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: R.Y. and R.S. performed experiments; R.Y., R.S., T.A., and T.T. analyzed data; R.Y., R.S., T.A., and T.T. interpreted results of experiments; R.Y., R.S., T.A., and T.T. conceived and designed research; R.Y., R.S., T.A., and T.T. prepared figures; R.Y., R.S., T.A., and T.T. wrote the initial draft; T.T. provided critical revisions; T.T. approved final version of manuscript.

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