Blockade of sodium-glucose cotransporter 2 suppresses high glucose-induced angiotensinogen augmentation in renal proximal tubular cells

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INTRODUCTION

Diabetes mellitus (DM) is a common health issue, with increasing prevalence, that often leads to the development of associated end-organ damage, including nephropathy. Type 2 DM (T2DM) is a complex disease where hyperglycemia occurs as a result of the development of insulin resistance. In addition to the problems associated with poor glucose control, T2DM is often accompanied by high blood pressure and associated complications that lead to renal injury and diabetic nephropathy with consequent end-stage renal disease.

An inappropriately activated intrarenal renin-angiotensin system (RAS) promotes angiotensin II (ANG II) formation, causing sodium retention and increased arterial pressure and tissue injury. Intrarenal angiotensinogen (AGT), which is the precursor of angiotensin peptides, is produced predominantly in renal proximal tubular cells (PTCs). The present study investigated the effects of canagliflozin (CANA), a SGLT2 inhibitor, on HG-induced AGT elevation in cultured PTCs. Mouse PTCs were treated with 5–25 mM glucose. CANA (0–10 μM) was applied 1 h before glucose treatment. Glucose (10 mM) increased AGT mRNA and protein levels at 12 h (3.06 ± 0.48-fold in protein), and 1 and 10 μM CANA as well as SGLT2 shRNA attenuated the AGT augmentation. CANA did not suppress the elevated AGT levels induced by 25 mM glucose. Increased AGT expression induced by treatment with pyruvate, a glucose metabolite that does not require SGLT2 for uptake, was not attenuated by CANA. In HG-treated PTCs, intracellular reactive oxygen species levels were elevated compared with baseline (4.24 ± 0.23-fold), and these were also inhibited by CANA. Furthermore, tempol, an antioxidant, attenuated AGT upregulation in HG-treated PTCs. HG-induced AGT upregulation was not inhibited by an angiotensin II receptor antagonist, indicating that HG stimulates AGT expression in an angiotensin II-independent manner. These results indicate that enhanced glucose entry via SGLT2 into PTCs elevates intracellular reactive oxygen species generation by stimulation of glycolysis and consequent AGT augmentation. SGLT2 blockade limits HG-induced AGT stimulation, thus reducing the development of kidney injury in diabetes mellitus.

angiotensinogen; diabetes; proximal tubular cells; sodium-glucose cotransporter 2
blood pressure, intrarenal macrophages, and renal oxidative stress in the animal model (51). Since these pathophysiologica factors have been shown to increase AGT expression levels (14, 31, 33, 34), it is still unclear whether blockade of glucose entry by SGLT2 inhibition can directly attenuate proximal tubular AGT augmentation in DM (2). Indeed, there is evidence that antagonists against ANG II receptors prevent HG-induced AGT augmentation in cultured PTCs (14), suggesting that cofactor(s) including ANG II mediates proximal tubular AGT upregulation under diabetic conditions. Thus, there are multiple factors that could be responsible for the attenuation of renal AGT expression after CANA administration. The present study demonstrates that glucose entry through SGLT2 without cofactors stimulates downstream intracellular mechanisms and consequent AGT expression. Furthermore, the data link the blockade of SGLT2 with the decreased renal AGT expression, providing a molecular mechanism for the antihypertensive effects of SGLT2 inhibition.

**MATERIALS AND METHODS**

**Cell culture.** Since 80–90% of filtered glucose is reabsorbed via SGLT2 mainly in the early segment of nephrons (12), an established PTC line, which has been characterized as an early segment PTC line, was used in this study (13). Immortalized mouse PTCs isolated from male mice were used in this study (13). SGLT2 expression in this cell line was confirmed by Western blot analysis (data not shown). PTCs were cultured in accordance with a standard protocol established by the provider. A growth medium (DMEM-F-12, 1:1, Invitrogen) containing 5 mM glucose, 15 mM HEPES, 0.06% NaHCO3, 5 mM sodium pyruvate, 4 mg/ml dexamethasone, 2 mM t-glutamine, 50 mM ascorbic acid 2-phosphate, 20 mM sodium selenite, 1 mM tri-iodothyronine, 5 mg/ml insulin, 10 ng/ml epithelial growth factor, 10 ng/ml mouse interferon-γ, and 5% FBS was used to maintain PTCs at 33°C. The supplements for the medium were purchased from Invitrogen, Sigma-Aldrich, and Fisher Scientific. When cultured PTCs were used in experiments, cells were plated on six-well plates and transferred to a 37°C CO2 incubator. Since some supplements in the growth medium, including dexamethasone, insulin, and interferon-γ, have been shown to alter AGT expression levels in PTCs (14, 42, 48), starving and including dexamethasone, insulin, and interferon-γ in the medium was used as HG in other experiments. CANA, a SGLT2 inhibitor, was provided by Janssen and dissolved in DMSO. When cells were treated with CANA, cells in the control group received the same concentration of DMSO. ANG II type 1 receptor (AT1R) blocker was used to investigate roles of ANG II in ATG regulation.

**Antibodies.** Rabbit anti-mouse AGT antibody from IBL America (catalog no. 28101) was used in this study. Rabbit anti-mouse SGLT2 antibody was purchased from Santa Cruz Biotechnology (catalog no. sc-47402). Mouse anti-β-actin antibody from Abcam (catalog no. ab8226) was used as an internal control. IRDye-labeled anti-mouse IgG and anti-rabbit IgG antibodies were obtained from Li-Cor (catalog nos. 926-32220 and 926-32211, respectively) as secondary antibodies in Western blot analyses.

**Western blot analysis.** AGT protein levels were determined using Western blot analysis. Western blots were performed as previously described (41, 42). Cells were homogenized with 60 μL lysis buffer containing 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM Na3VO4, and 0.25% protease inhibitor cocktail (Sigma). Lysates were sonicated three times for 10 s each. Total protein concentration of the supernatant was quantified using a Micro BCA Protein Assay Kit (Pierce). Then, 20 μg total protein was applied to a precast NuPAGE 4–12% gel (Invitrogen). The separated proteins were transferred to a nitrocellulose membrane (Bio-Rad). After incubation of the membrane with primary and secondary antibodies, detection and analysis were performed using the Odyssey System (Li-Cor). Data were normalized based on mouse β-actin protein expression levels. When experiments required more than one membrane to analyze multiple samples, a positive control cell lysate was used to normalize minor technical variability among the membranes.

**Droplet digital PCR.** Droplet digital PCR (ddPCR) was performed to determine target mRNA copies in PTCs, as previously described (25, 51). Total RNA was isolated using a commercially available RNA isolation kit (Qiagen). RNA concentration was quantified using Nanodrop 2000 (Thermo Scientific). ddPCR was performed using a Bio-Rad ddPCR system. Primers, probes, and reagents for the One-step RT-ddPCR system were purchased from Bio-Rad to generate cDNA and quantify gene expression. After droplet generation and PCR amplification, droplets were analyzed on the QX200 droplet reader, and target cDNA concentration was determined using the QuantaSoft analysis software (Bio-Rad). For each target gene, the amount of total RNA in a PCR reaction was determined with a pilot ddPCR, using serially diluted total RNA, in which the determined amount is in a linear range. Data are expressed as copy numbers of target gene in 1 ng total RNA. Experimental and biological replicates were applied.

**Intracellular ROS measurement in live cells.** Intracellular ROS generation in PTCs cultured on 24-well plates was detected using 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA; Life Technologies); 10 mM working stock was prepared in DMSO and made fresh for each use. When exposed to ROS, H2DCF-DA is oxidized to produce the fluorescent moleculeDCF. After 11 h of treatment in NG or HG, cells were treated with 20 μM H2DCF-DA or DMSO (vehicle control) at 37°C for 1 h. After incubation, cell culture medium was aspirated, and cells were rinsed three times in PBS. Thereafter, fluorescence was detected using a FLUOstar OPTIMA microplate reader (BMG Labtech) using a 492-nm excitation filter and 520-nm emission filter. Live-cell images were captured using an EVOS Fl microscope (AMG).

**SGLT2 gene knockdown.** SGLT2 knockdown was performed in PTCs using shRNA plasmid (Creative Biogene) targeting base 628 of the mouse SGLT2 gene using the following sequence: 5′-GC-CTTCATCCTCCTACTGGTTAT-3′. A negative control shRNA plasmid was also used. *Escherichia coli* cells containing the plasmid were grown in LB broth supplemented with kanamycin (50 μg/mL). Plasmid DNA was harvested using a QIAprep Spin Miniprep Kit (Qiagen) and eluted in ddH2O to a final concentration of 1.1 μg/mL. The purified plasmid was transfected into PTCs using electroporation (Neon Transfection System, Invitrogen), and the cells were cultured on 24-well plates. After transfection with anti-SGLT2 or negative control plasmid, cells were serum starved for 48 h at 37°C. Cells were then treated with NG or HG.

**Statistical analysis.** Data are expressed as means ± SE. Data were analyzed using Student’s t test or one-way ANOVA followed by a post hoc Bonferroni-Dunn multiple comparison test. P values of <0.05 were considered statistically significant.
Fig. 1. Effects of canagliflozin (CANA) on high-glucose (HG)-induced angiotensinogen (AGT) protein augmentation in proximal tubular cells (PTCs). Temporal changes in AGT protein levels by treatments of PTCs with 5 mM glucose (A), 10 mM glucose (B), and 25 mM glucose (C) were evaluated by Western blot analyses. A pooled cell lysate sample was applied to all Western blot gels used in the experiments, and band intensities of AGT and β-actin in the pooled sample were used to normalize minor technical variability among membranes. Dotted lines indicate AGT levels in PTCs that received 10 mM CANA treatment.

D: merged data without error bars. AGT and sodium-glucose cotransporter 2 (SGLT2) mRNA levels in normal glucose (NG; 5 mM glucose)-treated and HG (10 mM glucose)-treated cells are shown in E (n = 6) and F (n = 6), respectively. Data are expressed as means ± SE. *P < 0.05, significant difference vs. the NG group; †P < 0.05, significant difference vs. the HG (non-CANA) group.
RESULTS

Effects of CANA on HG-induced AGT protein augmentation in PTCs. We tested whether direct treatment with CANA attenuates HG-induced AGT upregulation. A positive control cell lysate sample was applied to all Western blot gels, and band intensities of AGT and β-actin in the pooled sample were used to normalize minor technical variability among membranes. Incubation in 5 mM glucose did not alter AGT protein levels at 6, 12, and 24 h compared with levels at 3 h (n = 4; Fig. 1A). Furthermore, 10 μM CANA treatment did not alter expression levels at all tested time points. However, incubation in 10 mM glucose stimulated AGT expression for up to 12 h and was sustained for up to 24 h (Fig. 1B). CANA treatment of cells treated with 10 mM glucose significantly attenuated the AGT augmentation at 12 and 24 h (54.9 ± 0.02% suppression by CANA at 12 h). In contrast, CANA treatment did not inhibit the augmentation of AGT expression in PTCs treated with 25 mM glucose (Fig. 1C), suggesting competitive interaction between glucose and CANA and/or glucose uptake facilitated by other glucose transporters at very high glucose levels. Treatment of PTCs with a high concentration of D-mannitol (5 mM glucose + 20 mM mannitol) did not show greater AGT expression levels compared with the NG-treated group (data not shown), suggesting that increased osmolality by HG does not affect AGT expression in PTCs. Since these results indicate that maximal induction of AGT expression was achieved by 10 mM glucose (Fig. 1D), 10 or 15 mM glucose was used as the HG treatment in the following experiments.

Changes in AGT mRNA expression in PTCs were also investigated using ddPCR (n = 6; Fig. 1E). Treatment of PTCs with HG (10 mM) markedly increased AGT mRNA levels at 12 h (30.9 ± 5.3 copies/ng RNA in the NG group vs. 329.4 ± 24.0 copies/ng RNA in the HG group). CANA treatment at 10 μM attenuated HG-induced AGT mRNA augmentation in PTCs (100.5 ± 10.7 copies/ng RNA), supporting the protein data.

Moreover, SGLT2 expression levels were determined using ddPCR (n = 6; Fig. 1F). SGLT2 mRNA levels were higher in the HG-treated group than in the NG-treated group (47.2 ± 2.7 copies/ng RNA in the NG group vs. 100.4 ± 12.9 copies/ng RNA in the HG group). The elevated SGLT2 expression in the HG-treated group was normalized by CANA treatment.

Dose dependency of CANA on HG-induced AGT augmentation in PTCs. CANA attenuated the augmentation of AGT by 10 mM glucose in a dose-dependent manner (n = 4; Fig. 2). CANA (1 μM) partially, but significantly, suppressed AGT augmentation at 12 h. CANA (10 μM) exhibited further inhibition on AGT upregulation (35.1 ± 0.07% suppression by 1 μM CANA and 54.1 ± 0.08% suppression by 10 μM CANA).

Effects of SGLT2 knockdown on HG-induced AGT augmentation in PTCs. Since CANA attenuated HG-induced AGT augmentation in PTCs, as shown in Figs. 1 and 2, we confirmed roles of SGLT2 using a specific shRNA targeting the SGLT2 gene (n = 3; Fig. 3). PTCs were transfected with plasmid DNA expressing SGLT2 shRNA or negative control shRNA. Transfection with SGLT2 shRNA resulted in a 47.2% suppression of SGLT2 expression compared with PTCs that received negative control shRNA (Fig. 3A). AGT protein levels were increased by HG (15 mM) in cells that received negative control plasmid (5.65 ± 0.62-fold, ratio to NG group; †P < 0.05, significant difference vs. the normal glucose (NG) group). The elevated AGT protein expression in HG groups was significantly reduced by SGLT2 shRNA treatment (47.2% reduction; †P < 0.05, significant difference vs. HG group). Since CANA attenuated HG-induced AGT augmentation in PTCs, SGLT2 knockdown partially, but significantly, suppressed AGT expression in HG groups (Fig. 3B).

Fig. 3. Effects of sodium-glucose cotransporter 2 (SGLT2) knockdown on high glucose (HG)-induced angiotensinogen (AGT) augmentation in proximal tubular cells (PTCs). In addition to experiments using canagliflozin (CANA), SGLT2 gene knockdown was performed. Cells received negative control shRNA (Nega) or SGLT2-specific shRNA (SGLT2). HG-treated groups received 15 mM glucose for 12 h. SGLT2 (A) and AGT (B) protein levels were determined by Western blot analyses (n = 3). Data are expressed as means ± SE. *P < 0.05, significant difference vs. the normal glucose (NG) group; †P < 0.05, significant difference vs. the HG (non-CANA) group.

Nega SGLT2 Nega SGLT2 shRNA

0 0 0.01 0.1 1 10

AGT/β-actin (AU)

0 0.5 1.0 1.5

0 0 0.01 0.1 1 10

CANA (μM)

0 0.5 1.0 1.5

AGT/β-actin (AU)

0 0 0.01 0.1 1 10

CANA (μM)

Fig. 2. Dose dependency of canagliflozin (CANA) on high glucose (HG)-induced angiotensinogen (AGT) augmentation in proximal tubular cells (PTCs). AGT protein levels by treatment with different concentrations of CANA at 12 h were evaluated by Western blot analyses (n = 4). HG-treated groups received 10 mM glucose. Data are expressed as means ± SE. *P < 0.05, significant difference vs. the normal glucose (NG) group; †P < 0.05, significant difference vs. the HG (non-CANA) group.

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the control group; Fig. 3B). However, HG did not augment AGT protein in cells expressing the shRNA sequence, suggesting that SGLT2 knockdown prevented the enhancing effects of HG.

**Effects of pyruvate on AGT mRNA expression in PTCs.** Increased glucose uptake results in elevations in intracellular pyruvate. To test whether increased intracellular glucose metabolism promotes AGT expression in PTCs, we treated PTCs with sodium pyruvate (n = 4; Fig. 4). Pyruvate (5 mM) treatment enhanced AGT expression in PTCs (16.5 ± 1.8 copies/ng RNA in the NG group and 387.9 ± 59.4 copies/ng RNA in the NG + pyruvate-treated group). CANA treatment did not attenuate pyruvate-induced AGT augmentation in PTCs (501.2 ± 115.1 copies/ng RNA in the NG + pyruvate + CANA-treated group).

**Effects of CANA on intracellular ROS generation in PTCs.** In vivo and in vitro studies have demonstrated that elevated ROS mediates HG-induced AGT upregulation in PTCs (5, 14). Thus, effects of CANA on intracellular ROS generation in PTCs were investigated (n = 5; Fig. 5). Treatment of PTCs with HG (10 mM) resulted in elevation of intracellular ROS generation (4.24 ± 0.23-fold, ratio to baseline). In HG-treated cells, CANA treatment suppressed intracellular ROS levels to lower than the baseline. Furthermore, CANA decreased intracellular ROS levels even under the NG condition.

A previous study reported that ROS inhibition prevents enhancement of AGT expression in HG-treated PTCs (14). Thus, we also evaluated whether tempol, a superoxide dismutase mimetic, attenuates HG-induced AGT upregulation in our model. Both AGT mRNA (n = 4; Fig. 5B) and protein (n = 6; Fig. 5C) levels were higher in HG-treated PTCs compared with NG-treated PTCs. Pretreatment with 2.5 mM tempol prevented the augmentation of AGT expression.

**Effects of an Ang II receptor inhibitor on HG-induced AGT augmentation in PTCs.** Antagonizing Ang II receptors has also been shown to suppress HG (25 mM)-induced AGT upregulation in PTCs (14), suggesting that activation of endogenous ANG II receptors mediates the AGT elevation. In the present study, PTCs were pretreated with 1 mM active olmesartan, an inhibitor against AT1Rs. Furthermore, 25 mM glucose was used in this experiment to induce the maximum AGT augmentation and consequent ANG II formation as in the previous study (14). Although HG treatment did not alter AT1R expression levels (n = 4; Fig. 6A), HG treatment enhanced AGT expression in PTCs (128.3 ± 16.8-fold, ratio to the control group; Fig. 6B). The increased AGT levels in HG-treated PTCs were not attenuated by olmesartan (124.9 ± 11.7-fold in the HG + olmesartan-treated group, ratio to the control group).

**DISCUSSION**

Our findings demonstrate that CANA attenuates HG-induced ROS generation and AGT upregulation in PTCs. Our previous report described the ability of CANA treatment to reduce renal AGT expression and associated hypertension and related kidney injury in vivo. The present study elucidates the molecular mechanism underlying these effects.

Kidney disease leading to end-stage renal disease is a major complication in DM, occurring in ~40% of patients (46). Many basic and clinical investigations have demonstrated renoprotective effects of RAS blockers in the development of diabetic nephropathy (52). Thus, intrarenal RAS has been a target to treat diabetic nephropathy. As a key mechanism in the activation of the intrarenal RAS under diabetic conditions, elevation of proximal tubular AGT production has been shown in both type 1 and type 2 DM (5, 21, 29, 36). Selective SGLT2 inhibitors, which reduce glucose reabsorption in renal proximal tubules, have been developed to improve glycemic control in patients with T2DM (47). Our previous study showed that the augmentation of intrarenal AGT expression in T2DM mice is prevented by CANA treatment (51). The results of the present study show that CANA attenuates the enhanced AGT expression in PTCs under HG conditions, indicating that glucose entry via SGLT2 directly contributes to AGT augmentation in diabetic kidneys. Results obtained from PTCs with suppressed SGLT2 expression caused by shRNA provide further support for this finding. Although CANA prevented the effect on AGT augmentation of 10 mM glucose, CANA did not suppress the increased AGT levels stimulated by 25 mM glucose. A previous study demonstrated augmentation of glucose transporter 2 in patients with DM (39). Thus, other glucose transporters may facilitate glucose uptake under hyperglycemic conditions. Alternatively, there may be a competitive interaction between CANA and glucose underlying PTC uptake of glucose that is overwhelmed by excessive glucose concentrations. To put these glucose effects in perspective, a continuous glucose monitoring study has demonstrated that blood glucose concentrations in patients with controlled T2DM rarely exceed 15 mM (3). Therefore, SGLT2 inhibition can still be an effective therapy to prevent and/or mitigate the development of diabetic nephropathy. In fact, not only intrarenal AGT augmentation but also the degree of hypertension and kidney injury were attenuated by CANA treatments in T2DM (51, 53).

In our experiments, 1 and 10 μM CANA significantly attenuated 10 mM HG-induced AGT elevation in PTCs. The IC50 values of CANA on glucose uptake via human SGLT2 is less than 10 nM (23), which is much lower than the effective

![Fig. 4. Effects of pyruvate on angiotensinogen (AGT) mRNA expression in proximal tubular cells (PTCs). PTCs were treated with 5 mM pyruvate under normal glucose (NG) conditions. AGT mRNA levels were evaluated by droplet digital PCR (n = 4). Data are expressed as means ± SE. *P < 0.05, significant difference vs. the NG group.](image-url)
concentrations of CANA on AGT upregulation determined in the present study. It has been reported that 99% of CANA is absorbed by albumin contained in FBS for cell cultures (40). This may be a reason that high doses of CANA, such as more than 1 \( \mu \text{M} \), are required to exhibit significance in many in vitro investigations, including in the present study (15, 18, 27).

SGLT2 is expressed mainly in S1 and S2 segments of proximal tubules (12). Therefore, SGLT2 inhibition may not directly attenuate hyperglycemia-induced AGT elevation in the S3 segment. Recent studies have revealed that there is much greater AGT expression in the S2 and S3 segments than in the S1 segment in the kidneys (19, 37). Taken together, SGLT2 inhibitors can directly prevent or attenuate AGT augmentation in the early segments of proximal tubules in DM, as shown in the present study, and blunt enhanced AGT expression in the S3 segment due to reduced blood glucose levels. In fact, several studies have attempted to show suppressive effects of SGLT2 inhibitors on elevated intrarenal AGT in DM. Administration of dapagliflozin, a SGLT2 inhibitor, in T2DM rats attenuated elevated urinary AGT excretion (43). In patients with T2DM, SGLT2 inhibition exhibited a decreasing, but not significant, trend in urinary AGT excretion rates (54). In contrast, the \( \text{db/db} \) mouse model of T2DM exhibited decreased renal AGT mRNA expression that was enhanced by treatment with a SGLT2 inhibitor (49). Furthermore, elevated urinary AGT levels in response to empagliflozin treatment of the Otsuka Long-Evans Tokushima fatty DM rat (44) have been reported. Therefore, the effects of SGLT2 inhibition on intrarenal and/or urinary AGT augmentation in T2DM may be dependent on the animal model used. It has been proposed that excessive delivery of glucose to the S3 segment by inhibition of glucose reabsorption in the S1 and S2 segments transiently results in stimulation of AGT expression in the S3 segment, explaining the inconsistent findings on effects of SGLT2 inhibi-
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Since SGLT2 is a sodium-glucose cotransporter, SGLT2 inhibition may finally attenuate the AGT augmentation in the S3 segment.

Changes in SGLT2 expression in PTCs under diabetic conditions are inconsistent. A previous study using the HK-2 cell line, a human PTC line, reported that excessive HG (30 mM) treatment did not alter SGLT2 expression levels (35). On the other hand, our previous animal study demonstrated that renal cortical SGLT2 expression was greater in T2DM mice compared with control mice (51). In the present study using an in vitro setting, HG (10 mM) increased levels of SGLT2 transcripts in PTCs, and CANA attenuated the augmentation, supporting findings in the previous animal study (51). Thus, CANA may help reduce blood glucose levels in T2DM by blockade of glucose reabsorption via SGLT2 and normalizing SGLT2 expression in PTCs.

Since SGLT2 is a sodium-glucose cotransporter, SGLT2 inhibition also lowers sodium reabsorption in proximal tubules (4). This manipulation of sodium uptake may influence AGT regulation. To test whether it was the increased glycolysis associated with increased glucose uptake that promotes increases in AGT expression, we treated cells with pyruvate, a glucose metabolite that does not require SGLT2 to enter the cell. Applying pyruvate instead of HG also augmented AGT expression in PTCs, but CANA treatment did not influence the pyruvate effect. These findings suggest that glucose entry via SGLT2 leading to enhanced glycolysis, but not sodium influx via SGLT2, plays important roles in proximal tubular AGT augmentation in DM.

Elevated ROS has been identified as a crucial contributor to intrarenal AGT upregulation under diabetic conditions (5, 14, 29). Treatment of PTCs with a ROS scavenger also resulted in normalizing AGT expression in HG-treated PTCs in this study, indicating that elevated ROS mediates the AGT augmentation. These findings indicate that CANA treatment suppressed intrarenal AGT levels by lowering or preventing oxidative stress in T2DM mice (51). A recent study has identified downstream factors of ROS, including nuclear factor erythroid 2-related factor 2, in proximal tubular AGT regulation in DM (1). Further investigations will elucidate intracellular mechanisms participating in SGLT2-mediated AGT regulation in renal proximal tubules.

Numerous pathophysiological factors are altered in diabetic conditions. SGLT2 inhibition also reduces blood pressure, renal immune cell infiltration, fibrotic factors, and advanced glycation end product levels in DM (10, 24, 26, 50, 51); these factors may potentiate AGT expression in PTCs (11, 14, 31, 33, 34). Moreover, renal ANG II, which is increased further by elevated AGT production in PTCs or by intracellular de novo mechanisms, also contributes to proximal tubular AGT augmentation (22, 41). A previous study has shown that AT₁R inhibition prevents HG-induced AGT augmentation in cultured PTCs (14), suggesting that cofactor(s) including ANG II mediate proximal tubular AGT upregulation under diabetic conditions. In the present study, 1 μM olmesartan, an AT₁R blocker, did not limit AGT augmentation in HG-treated PTCs, whereas that dose of olmesartan has been shown to inhibit AGT augmentation by ANG II and a cytokine in PTCs (41). Accordingly, AT₁Rs may not be required for HG-induced AGT augmentation in PTCs. The inconsistency between the previous finding and the results in the present study may be due to the cell lines used in the studies. We used mouse PTCs, which were characterized as cells in the early proximal tubular segment (S1 segment). However, expression of renin, which processes AGT to produce angiotensin, mRNA, and its activity were detected in the S2 segment (30). Thus, AGT may be regulated by HG, ANG II, and other factors in more distal proximal tubular segments. However, factors such as proinflammatory cytokines, fibrotic factors, and advanced glycation end products are unlikely to be participating in HG-induced AGT augmentation in our in vitro setting. In the present study, augmentation of AGT expression by HG was already observed at 6 h and later time points. Thus, even though PTCs produce and secrete these pathogenic factors during short-term HG treatments, they would have been diluted by the medium. These results suggest that glucose entry through SGLT2 without cofactors stimulates AGT expression in PTCs derived from the early segment.

In conclusion, this study demonstrated that enhanced glucose entry via SGLT2 into PTCs elevates intracellular ROS...
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generation by stimulation of glycolysis and consequent AGT augmentation. Thus, SGLT2 inhibition limits HG-induced up-regulation of renal AGT stimulation. These findings provide a basis for the mitigation of the development of hypertension and kidney injury in DM by CANA and suggest possible mechanistic explanations of the clinical results of trials like the Canagliflozin Cardiovascular Assessment Study (CANVAS) and Canagliflozin and Renal Events in Diabetes with Established Nephropathy Clinical Evaluation (CREDENCE), in which CANA improved cardiovascular and renal outcomes to an extent that cannot be explained by the glucose-lowering properties of the drug (17, 28).

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AUTHOR CONTRIBUTIONS


REFERENCES


