Insulin stimulates SGLT2-mediated tubular glucose absorption via oxidative stress generation

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Abstract

Background: Ninety percent of glucose filtered by the glomerulus is reabsorbed by a sodium-glucose cotransporter 2 (SGLT2), which is expressed mainly on the apical membrane of renal proximal tubules. Since SGLT-2-mediated glucose reabsorption is enhanced under diabetic conditions, selective inhibition of SGLT2 has been proposed as a potential therapeutic target for the treatment of patients with diabetes. However, it remains unclear which diabetes-associated factors are involved in overexpression of SGLT2.

Methods: Therefore, in this study, we examined whether insulin, high glucose, advanced glycation end products (AGEs), or H2O2 stimulated SGLT2 expression in human cultured proximal tubular cells, and then investigated the underlying molecular mechanisms.

Results: High glucose or AGEs did not affect SGLT2 expression in tubular cells. Insulin significantly increased tubular SGLT2 level in a dose-dependent manner, whereas bell-shaped dose-response curves were observed for H2O2-treated cells. An anti-oxidant, N-acetylcysteine completely blocked insulin-induced up-regulation of SGLT2 as well as increase in glucose absorption by tubular cells. Furthermore, insulin dose-dependently increased reactive oxygen species generation in tubular cells.

Conclusions: Our present study demonstrated that insulin could stimulate SGLT-2-mediated glucose entry into cultured proximal tubular cells via oxidative stress generation. Suppression of the insulin-induced overexpression of SGLT2 in tubular cells might be a novel therapeutic strategy for the treatment of diabetic nephropathy.

Keywords: SGLT2, Oxidative stress, Insulin, Diabetic nephropathy, AGEs

Introduction

Diabetic nephropathy is a leading cause of end-stage renal disease, which could account for disabilities and high mortality rates in patients with diabetes [1, 2]. Diabetic nephropathy is characterized by functional and structural changes in the glomerulus such as glomerular hyperfiltration, thickening of glomerular basement membrane, and an expansion of extracellular matrix in the mesangial areas, which could ultimately progress glomerular sclerosis associated with an increased urinary excretion rate of albumin and renal dysfunction [2, 3]. Indeed, characteristic histological changes of diabetic nephropathy are diffuse and nodular glomerulosclerosis [2, 3]. However, it is supposed that changes within the tubulointerstitium are more important than glomerulopathy in terms of renal dysfunction in diabetic nephropathy [4, 5].

Ninety percent of glucose filtered by the glomerulus is reabsorbed by a low-affinity/high capacity sodium-glucose cotransporter 2 (SGLT2), which is expressed mainly on S1 and S2 segment of renal proximal tubules [6–8]. Since blockade of SGLT2 promotes urinary glucose excretion and resultantly improves hyperglycemia in an insulin-independent manner, SGLT2 inhibitors are now one of the widely used agents for the treatment of diabetes [9–11]. Furthermore, we have previously shown that increased glucose uptake into cultured renal proximal tubular cells via SGLT2 stimulates oxidative stress generation and resultantly potentiates the pro-apoptotic effects of advanced glycation end products (AGEs), senescent macroprotein derivatives formed acceleratedly under diabetes, on tubu-
lar cells [12,13]. Therefore, blockade of SGLT2 could also be a therapeutic target for preventing tubular apoptosis and atrophy in diabetic nephropathy. SGLT2 levels in tubular cells harvested from the urine of diabetic subjects are increased compared with non-diabetic subjects [14]. However, which diabetes-associated factors are involved in SGLT2 overexpression in diabetic kidney remains unclear. Therefore, in this study, we examined whether insulin, high glucose, AGEs, or H2O2, stimulated SGLT2 expression in human cultured proximal tubular cells, and then investigated the underlying molecular mechanisms.

Materials and methods

Materials
Insulin, bovine serum albumin (BSA) (essentially fatty acid free and essentially globulin free, lyophilized powder), N-acetylcysteine (NAC), and poly-L-lysine were purchased from Sigma (St. Louis, MO, USA). D-Glucose and H2O2 were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). D-glyceraldehyde from Nakalai Tesque (Kyoto, Japan). Antibodies (Abs) directed against human SGLT2 and β-actin from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Cells
Proximal tubular epithelial cells from human kidney were maintained in complete medium (basal medium supplemented with 5% fetal bovine serum, 0.5 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 0.5 μg/ml epinephrine, 6.5 ng/ml triiodo-L-thyronine, 10 μg/ml transferrin, 5 μg/ml insulin, and GA-1000) according to the supplier’s instructions (Lonza Walkersville, Inc. Walkersville, MD, USA) [15]. Cells at 3-5 passages were used for the experiments. Insulin, H2O2, or other treatments were carried out in a serum-free basal medium containing 10 μg/ml transferrin and GA-1000.

Preparation of AGEs-BSA
AGEs-BSA was prepared as described previously [16]. In brief, BSA (25 mg/ml) was incubated under sterile conditions with 0.1 M D-glyceraldehyde in 0.2 M NaPO4 buffer (pH 7.4) for 7 days. Then unincorporated sugars were removed by PD-10 column chromatography and dialysis against phosphate-buffered saline. Control non-glycated BSA was incubated in the same conditions except for the absence of reducing sugars.

Western blot analysis
Tubular cells were treated with or without the indicated concentrations of insulin and H2O2, 11, 22 or 33 mM glucose, 100 μg/ml AGEs-BSA or non-glycated BSA in the presence or absence of 1 mM NAC. After 24 h, proteins were extracted from tubular cells with lysis buffer, and then separated by SDS-PAGE and transferred to nitrocellulose membranes as described previously [13]. Membranes were probed with Abs against SGLT2 or β-actin, and then immune complexes were visualized with an enhanced chemiluminescence detection system (Amer sham Bioscience, Buckinghamshire, United Kingdom). Data were normalized by the intensity of β-actin-derived signals and related to the value of non-treated control cells.

Assay for sodium-dependent glucose uptake
Tubular cells were treated with or without 50 ng/ml insulin in the presence or absence of 1 mM NAC for 24 h. Tubular cells were incubated with complete medium containing 100 μM 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG, Peptide Institute Inc., Osaka, Japan), fluorescent derivative of glucose for 15 min. Then culture medium was removed and replaced with Hanks’ balanced salt solution, and fluorescence intensity in the cells was analyzed in an ARVO fluorescent plate reader (PerkinElmer, Inc., Winter Street Waltham, MA, USA) as described previously [13].

Measurement of reactive oxygen species (ROS) generation
Intracellular formation of ROS was detected using a fluorescent probe carboxy-H2DFFDA (Life Technologies Japan, Tokyo, Japan) as described previously [17]. In brief, 96-well plates (FALCON, New York, NY, USA) were coated with 0.01% poly-L-lysine for 30 min at room temperature. Then tubular cells were seeded into the well, and incubated with 0.1% dimethyl sulfoxide (DMSO) in the presence or absence of 10 μM carboxy-H2DFFDA for 1 h. Then the cells were washed with phosphate-buffered saline, and treated with or without the indicated concentrations of insulin. After 24 min, intracellular ROS generation was measured with an ARVO X3 fluorescent plate reader (PerkinElmer Japan, Yokohama, Japan). ROS production was calculated by subtracting the fluorescence for cells pre-incubated with DMSO only from that with carboxy-H2DFFDA. Under cell-free conditions, 10 μM carboxy-H2DFFDA was also incubated with the indicated concentrations of H2O2 for 24 min, and then the fluorescence was measured.

Statistical analysis
All values are presented as mean ± standard deviation. One-way analysis of variance followed Tukey’s test or student’s t-test was performed for statistical comparisons; p-values of less than 0.05 were considered significant.

Results
Effects of insulin, high glucose, AGEs, or H2O2 on SGLT2 expression
We first examined whether insulin, high glucose, AGEs, or H2O2 stimulated SGLT2 expression in cultured proximal tubular cells. High glucose up to 33 mM or 100 μg/ml
AGEs, comparable levels with plasma concentrations under diabetic situations [18], did not affect SGLT2 expression in tubular cells (data not shown). However, as shown in Figs. 1a and b, insulin significantly increased tubular SGLT2 expression level in a dose-dependent manner; whereas bell-shaped dose-response curves were observed for H$_2$O$_2$-treated cells. Since maximum response was obtained in 50 ng/ml insulin-treated cells (Fig. 1a), we chose the condition of 50 ng/ml insulin for the following experiments.

**Effects of NAC on insulin-induced SGLT2 expression and glucose uptake by tubular cells**

We next studied the effects of an anti-oxidant NAC on insulin-induced SGLT2 expression and glucose uptake by tubular cells. As shown in Fig. 2a, 1 mM NAC completely blocked the insulin-induced up-regulation of SGLT2 level in tubular cells. Furthermore, 50 ng/ml insulin significantly increased glucose entry into tubular cells, which was also completely prevented by the treatment with 1 mM NAC (Fig. 2b).

**Effects of insulin on ROS generation in tubular cells**

We investigated the effects of insulin on ROS generation in tubular cells. As shown in Fig. 3, insulin dose-
Indeed, insulin-induced ROS generation significantly increased SGLT2 expression in the kidney of type 2 diabetes. These observations suggest that insulin might stimulate SGLT-2-mediated glucose entry into cultured proximal tubular cells via oxidative stress generation.

There is some controversy about the expression level of SGLT2 in the diabetic kidney of type 1 diabetic animals; SGLT2 were decreased, unchanged or increased in streptozotocin-induced diabetic rats [19–22]. However, in contrast to type 1 diabetic animals, increased SGLT2 expression was consistently observed in the kidney of type 2 diabetic animals [23–25] and in tubular cells harvested from the urine of type 2 diabetic subjects [17], the latter of which was correlated with glucose reabsorption capacity in these patients. Therefore, insulin resistance and resultant hyperinsulinemia may contribute to SGLT2 overexpression in the kidney of type 2 diabetes. The ability of insulin to stimulate sodium reabsorption in proximal tubules is preserved in insulin-resistant subjects despite resistance to insulin metabolic effects [26]. So, the selective insulin resistance might be involved in SGLT2 induction under hyperinsulinemic conditions.

In the present study, although high glucose or AGEs have been reported to stimulate ROS generation in tubular cells [12, 13, 17], neither of them increased tubular SGLT2 expression (data not shown). High glucose or AGEs has been reported to induce apoptotic cell death of cultured proximal tubular cells [12, 13, 17], whereas insulin has anti-apoptotic properties in tubular cells and stimulates proliferation of this cell type [27, 28]. Furthermore, while high level of ROS is toxic to various types of cells, including proximal tubular cells [29–31], relatively low level of intracellular ROS could function as a second messenger in signaling cascades involved in gene expression [30–32]. Indeed, insulin-induced ROS generation has been coupled with its action in insulin-sensitive cells [30]. Therefore, the action of ROS on SGLT2 expression in tubular cells might also depend on its concentration. ROS generation evoked by high glucose or AGEs may be higher than 300 μM H2O2 and toxic to cells, which might partly explain the reason why these two agents cannot induce tubular SGLT2 expression. In this study, (1) SGLT2 level induced by 500 μM H2O2 was less than that by 300 μM H2O2 (Fig. 1b), and (2) total protein amounts obtained from 500 μM H2O2-exposed tubular cells were decreased to about 70 % of those of non-treated controls or 300 μM H2O2-exposed cells, thus supporting the concept that toxic level of ROS could affect SGLT2 expression in tubular cells.

We have previously shown that SGLT2-mediated glucose overload in tubular cells could augment the cells’ susceptibility toward pro-apoptotic effects of AGEs via overexpression of receptor for AGEs (RAGE) [13]. Furthermore, we, along with others, have recently found that empagliflozin, an inhibitor of SGLT2, suppresses oxidative, inflammatory and fibrotic reactions in the kidney and aorta of diabetic rats partly via suppression of the AGEs-RAGE axis [21, 33]. Apoptosis of proximal tubular cells plays a central role in tubular atrophy and atubular glomeruli of diabetic nephropathy [34, 35], which are most closely correlated with declining creatinine clearance in patients with diabetes [4, 5]. Given the pathological role of the AGEs-RAGE axis in tubular cell apoptosis [12, 13, 17], blockade of insulin-induced SGLT2 overexpression may not just improve hyperglycemia by promoting urinary glucose excretion, but could also directly inhibit glucotoxicity to proximal tubular cells, thus protecting against tubulointerstitial damage in diabetic nephropathy.

**Limitations**

We did experiments of Figs. 1a, b and 2a separately. So the 50 ng/ml column in Figs. 1a and 2a was not the
same data. Since exposure time of an enhanced chemiluminescence detection system in each experiment differed, we were not able to show the data with the same units. This was a reason why we showed the data of non-treated cells in each experiment as a control. The physiological concentration of insulin in humans is 0.5-5 ng/ml. Oral administration of NAC for the treatment of acetaminophen poisoning obtained a plasma level of NAC at 10 μM [36]. Therefore, the concentration of insulin (50 ng/ml) and NAC (1 mM) having biological effects on tubular cells used in the present experiments may be in the superphysiologic range. This study was only analyzed in cell culture, not investigated about animal models and human. Therefore, further study is needed to clarify whether hyperinsulinemia may contribute to SGLT2 overexpression in animal model or human diabetic kidneys.

Conclusions
Our present study demonstrated that insulin could stimulate SGLT2-mediated glucose entry into cultured proximal tubular cells via oxidative stress generation. Suppression of the insulin-induced overexpression of SGLT2 in tubular cells might be a novel therapeutic strategy for the treatment of diabetic nephropathy.

Abbreviations
Abs: Antibodies; AGEs: Advanced glycation end products; BSA: Bovine serum albumin; DM5O: Dimethyl sulfoxide; NAC: N-acetylcyesteine; 2-NBDG: 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose; ROS: Reactive oxygen species; SGLT2: Sodium-glucose cotransporter 2; RAGE: Receptor for AGEs.

Competing interests
The authors declare that they have no competing interest.

Authors' contributions
NN, TM, and YI acquired, analyzed, and interpreted data. SY mainly contributed to the present study, conceptualized and designed the study, acquired, analyzed, and interpreted data, and drafted the manuscript, and took responsibility for the integrity of the data and the accuracy of the data analysis. All authors read and approved the final manuscript.

Acknowledgments
This work was supported in part by Grants-in-Aid for Scientific Research (B) 22390111 (SY) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Received: 7 April 2015 Accepted: 20 May 2015
Published online: 24 May 2015

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