High glucose-induced oxidative stress inhibits Na+/glucose cotransporter activity in renal proximal tubule cells

Ho Jae Han,1 Yun Jung Lee,1 Su Hyung Park,1 Jang Hern Lee,2 and Mary Taub3

1Department of Veterinary Physiology, College of Veterinary Medicine, Chonnam National University, Gwangju; 2College of Veterinary Medicine, Seoul National University, Seoul, Korea; and 3Department of Biochemistry, State University of New York at Buffalo, Buffalo, New York

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ALTERATIONS HAVE BEEN OBSERVED in renal tubular function during the early stages of the hyperglycemic state. Indeed, the early changes that occur within the tubules in hyperglycemia play a major role in determining the ultimate renal outcome. However, the underlying mechanisms responsible for the development of tissue damage in type I diabetes are not understood. Recent observations indicate that hyperglycemia triggers the generation of free radicals and oxidant stress in both mesangial and renal tubular epithelial cells (5). Previous studies with cultured neuronal cells similarly indicate that elevated glucose levels induce dysfunction and apoptosis, possibly through the formation of reactive oxygen species (ROS) (19). ROS are considered to be important mediators of several biological responses, including cell proliferation and extracellular matrix deposition. Glucose autooxidation, nonenzymatic glycation, the formation of advanced glycation end products (AGEs), and the overproduction of ROS by mitochondria are potential sources of hyperglycemia-induced oxidative stress (28, 8, 14).

AGEs interact with specific receptors and binding proteins to influence the expression of growth factors and cytokines, including transforming growth factor (TGF)-β, and connective tissue growth factor, thereby regulating the growth and proliferation of the various renal cell types (6). The observation that hyperglycemia does indeed cause an upregulation of the expression of fibronectin, TGF-β1, and basic fibroblast growth factor in human peritoneal mesothelial cells further suggests that hyperglycemia may indeed induce these effects by causing oxidative stress (18, 29). The inappropriate expression of these growth factors would have deleterious effects on cellular functions and thus may contribute to the pathogenesis of various diabetic complications.

Included among other changes that occur during chronic hyperglycemia is the activation of NADPH-dependent aldose reductase, which affects the polyol pathway and diminishes the quantity of NADPH available for the reduction of oxidized glutathione (GSH) by GSH reductase (4). In addition, the oxidative stress that occurs during hyperglycemia may trigger the NF-κB pathway (9). The deleterious effects of the increased levels of ROS that are observed in hyperglycemia are very likely further amplified by the decreased capacity of the cellular antioxidant defense system in this condition.

Alterations in transporter function may occur as a consequence of hyperglycemia, because proteins and lipids are also subject to oxidant injury (10–12). Thus oxidative stress may induce dysfunction of apical transporters in renal proximal tubule cells. Therefore, we investigated the mechanisms un-
derlying the ROS generation that occurs in response to incubations of renal cells in the presence of high glucose levels and its the consequent effects on the activity of the Na\(^+\)/glucose cotransport system in primary cultures of renal proximal tubule cells (PTCs).

The primary rabbit PTC culture system that is utilized in this study retains in vitro the differentiated phenotype typical of the renal proximal tubule, including a polarized morphology and distinct renal proximal tubule transport systems (2, 31). Previous reports have demonstrated that the Na\(^+\)-dependent and phlorizin-sensitive Na\(^+\)/glucose cotransport system, a distinctive function of renal proximal tubule cells, is present in the PTCs (11, 12, 30). Therefore, PTCs in hormonally defined, serum-free culture conditions are an appropriate tool to study the effect of high glucose on the Na\(^+\)/glucose cotransporter present in the renal proximal tubule. The present study was designed to examine the effects of high glucose on the generation of ROS and on the activity of the Na\(^+\)/glucose cotransport system in the primary PTCs.

**MATERIALS AND METHODS**

**Materials.** Adult male New Zealand White rabbits (1.5–2.0 kg) were purchased from Dai Han Experimental Animal (Chungjiu, Korea). DMEM, Ham’s nutrient mixture F-12 (DMEM/F-12), class IV collagenase, and soybean trypsin inhibitor were purchased from Life Technologies (Grand Island, NY). Aminoguanidine, pyridoxamine, and N-acetyl-L-cysteine were purchased from Dae Han Experimental Animal (Chungju, Korea). Rabbit anti-α-SGLT-1 was purchased from Chemicon International (Temecula, CA), and rabbit anti-sGLT-2 from Alpha Diagnostic International (San Antonio, TX). Goat anti-rabbit-IgG was purchased from Jackson ImmunoResearch (West Grove, PA). Liquisint was obtained from National Diagnostics (Parsippany, NJ). All other reagents were of the highest purity commercially available.

**Preparation of primary cell cultures.** Primary rabbit renal PTC cultures were prepared by the method of Chung et al. (2). The PTCs were grown in a 50:50 mixture of DMEM and Ham’s F12 containing 15 mM HEPES and 20 mM sodium bicarbonate (pH 7.4; DMEM/F-12 medium). Immediately before use of the medium, three growth supplements (5 μg/ml insulin, 5 μg/ml transferrin, and 5 × 10\(^{-8}\) M hydrocortisone) were added. The kidneys of each rabbit utilized for culture studies were perfused via the renal artery, first with PBS and subsequently with DMEM/F-12 containing 0.5% iron oxide (wt/vol) until the kidney turned gray black in color. Renal cortical slices prepared from the kidney were homogenized with four strokes (wt/vol) until the kidney turned gray black in color. Renal cortical slices prepared from the kidney were homogenized with four strokes (wt/vol) until the kidney turned gray black in color. The dissociated tubules were washed by centrifugation, resuspended in DMEM/F-12, and transferred into 35-mm tissue culture dishes. The cultures were then maintained at 37°C in a 5% CO\(_2\)-95% humidified air environment. The medium was changed 1 day after plating and every 3 days thereafter.

**α-MG uptake.** α-MG uptake experiments were conducted as described by Sakhrani et al. (26). To study α-MG uptake, the culture medium was removed by aspiration. Monolayers were gently washed twice with the uptake buffer (136 mM NaCl, 5.4 mM KCl, 0.41 mM MgSO\(_4\), 1.3 mM CaCl\(_2\), 0.44 mM Na\(_2\)HPO\(_4\), 0.44 mM KH\(_2\)PO\(_4\), 5 mM HEPES, 2 mM glutamine, and 0.5 μg/ml BSA, pH 7.4). After washing, the monolayers were incubated at 37°C for 30 min in an uptake buffer containing 0.5 mM α-MG and \(^{14}\)C-labeled α-MG (0.5 μCi/ml). At the end of the incubation period, the monolayers were again washed three times with ice-cold uptake buffer, and the cells in each culture dish were solubilized in 1 ml 0.1% SDS. To determine the [α-\(^{14}\)C]MG incorporated intracellularly, 900 μl of each sample were removed and counted in a liquid scintillation counter (LS 6500, Beckman Instruments, Fullerton, CA). The remainder of each sample was used for protein determination (1). The radioactive counts in each sample were normalized with respect to protein and corrected for zero time uptake per milligram protein. All uptake measurements were made in triplicate.

**Quantitative assay for hydrogen peroxide.** The level of hydrogen peroxide (H\(_2\)O\(_2\)) in experimental samples was determined by a modification of the method of Zhou et al. (32). The cells were washed twice with ice-cold PBS, harvested by microcentrifugation, and suspended in a modified Krebs-Ringer solution (KRPG; 145 mM NaCl, 5.5 mM sodium phosphate, 4.86 mM KCl, 0.54 mM CaCl\(_2\), 1.22 mM MgSO\(_4\), 5.5 mM glucose, pH 7.35). One hundred microliters of reaction mixture (50 μM Amplex red reagent containing 0.1 U/ml horseradish peroxidase in KRPG) were added to each microplate well, followed by a 10-min preincubation at 37°C. The reaction was started by adding resuspended cells in 20 μl of KRPG. Fluorescence readings were stable within 30 min of the beginning of the reaction. The fluorescence intensities of the reaction mixtures were measured at 30 min, using a fluorescence microplate reader (Multiskan, Thermo Labsystems, Franklin, MA) equipped for absorbance at ~560 nm.

**Measurement of lipid peroxides.** The levels of lipid peroxides (LPOs) in monolayer cultures were determined by measuring malondialdehyde content, as described by Ohkawa et al. (23). Monolayers were washed twice with PBS. The cells were removed from the dish by scraping with a policeman and sonicated. One hundred microliters of sonicated cells were mixed with 8% SDS, 0.8% 2-thiobarbituric acid, and 20% acetic acid. The mixture was heated to 95°C for 60 min, followed by cooling in ice-cold water. To extract nonspecific red pigment, 1 ml of an n-butanol-pyridine mixture (15:1) was added to the solution containing the sonicated cells. This mixture was shaken vigorously and centrifuged at 1,550 g for 10 min. The upper organic layer was measured by spectrophotometry with an emission wave length of 553 nm and an excitation wavelength of 515 nm. 1,1,3,3-Tetraethoxypropane was used as a standard. The LPO value obtained for each sample was expressed as nanomoles per milligram protein. NAC was added to cell mixtures to prevent any initiation of membrane lipid peroxidation during the assay. The effect of NAC addition to the standard, 1,1,3,3-tetraethoxypropane, or to the sample was examined, and no affect of NAC on color development with thiobarbituric acid was observed (data not shown). Values are the means ± SE of five independent experiments with triplicate dishes.

**Catalase assay.** The levels of catalase were determined at pH 7.4 after the breakdown of H\(_2\)O\(_2\) using the spectrophotometric method by a modification of Mueller et al. (20). The cells were removed from the culture dish by scraping with a policeman and collected by centrifugation at 200 g for 10 min at 4°C. Each reaction sample contained the indicated amounts of catalase and 20 μM H\(_2\)O\(_2\) in 0.1 M Tris-HCl and was incubated for 30 min. The final reaction containing 50 μM Amplex red reagent and 0.2 U/ml horseradish peroxidase was added and was incubated at 37°C for 30 min. Catalase activity in each sample was determined after a 30-min incubation in the dark at 25°C and measuring absorbance at 563 nm. Enzyme activity was expressed as units per milligram protein. Values are means ± SE of five independent experiments with triplicate dishes.

**Measurement of intracellular GSH.** Cells (5 × 10\(^5\)) were treated for various times and with various concentrations of d-glucose. The cells were removed from the culture dish by scraping with a policeman and collected by centrifugation at 200 g for 10 min at 4°C. Eighty
microliters of 10 mM HCl were added, and the cells were lysed by freezing and thawing. After addition of 5% sulfoaspiric acid, the cell lysate was centrifuged at 8,000 g for 10 min. The intracellular GSH (in the supernatant) was measured using a total GSH quantification kit (Dojindo Molecular Technologies, Gaithersburg, MD). The reactions were conducted on microtiter plates, and the absorbance in each of the wells was measured at 415 nm using a microplate reader. Values are the means ± SE of five independent experiments with triplicate dishes.

Preparation of cytosolic and total membrane fractions. After the PTCs had become confluent, the culture medium (DMEM/F-12) was changed 48 h before the use of the cultures for the preparation of cytosolic and total membrane fractions. At the time of the preparation, the medium was removed, and the cells were washed twice with ice-cold PBS. The monolayers were removed by scraping with a policeman and then subjected to microcentrifugation. The cells were then resuspended in buffer A [137 mM NaCl, 8.1 mM Na2HPO4, 2.7 mM KCl, 1.5 mM KH2PO4, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 10 μg/ml leupeptin (pH 7.5)] and then mechanically lysed at 4°C by trituration with a 21.1-gauge needle. The lysates were centrifuged at 1,000 g for 10 min at 4°C. The supernatants were then removed and centrifuged at 100,000 g for 1 h at 4°C to obtain cytosolic and membrane fractions. The cytosolic fraction (the supernatant) was then precipitated by the addition of 5 vol of acetone, followed by a 5-min incubation at 4°C. The precipitate was collected by centrifugation at 20,000 g for 20 min at 4°C. The resultant pellet was resuspended in buffer A containing 1% (vol/vol) Triton X-100. The membrane fraction (the 100,000-g pellet) was washed twice and resuspended in buffer A containing 1% (vol/vol) Triton X-100. The protein content of each fraction was quantified by the Bradford method (1976).

Western blot analysis. Cell homogenates (containing 20 μg of protein) were separated by electrophoresis through 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blots were then washed with H2O, blocked for 1 h with 5% skim milk powder in TBST [10 mM Tris·HCl (pH 7.6), 150 mM NaCl, 0.05% Tween 20], and incubated with the appropriate primary antibody at the dilutions recommended by the supplier. The nitrocellulose membrane was washed, and primary antibodies were detected with either goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase. The bands were visualized by means of enhanced chemiluminescence (Amersham Pharmacia Biotech).

EMSA. EMSA was performed by the method of Jeon et al. (16) with modifications. PTCs were lysed with hypotonic buffer (10 mM HEPES, 1.5 mM MgCl2, 1% NP-40, pH 7.5), and nuclei were pelleted by centrifugation at 3,000 g for 5 min. Nuclei were lysed using a hypertonic buffer (30 mM HEPES, 1.5 mM MgCl2, 450 mM KCl, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μg/ml of aprotinin, and 1 μg/ml of leupeptin) and then centrifuged at 14,500 g for 15 min. The supernatant was retained for use in DNA binding assays. A synthetic double-stranded oligonucleotide homologous to the consensus sequence recognized by NF-κB/Rel, 5'-GAT-CTC-AGA-GAG-TCA-GAC-ACA-3' (24), was end-labeled with [γ-32P]ATP using polynucleotide kinase. Nuclear extracts (5 μg) were incubated for 10 min in binding buffer (100 mM KCl, 30 mM HEPES, 1.5 mM MgCl2, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μg/ml of aprotinin, and 1 μg/ml of leupeptin) containing poly (dl-dC) and the γ-32P-labeled DNA probe. The nuclear protein/DNA complexes that formed during the binding reaction were separated from the free 32P-labeled probe by electrophoresis through a 4.8% polyacrylamide gel in 0.5 x TBE buffer. After electrophoresis, the gel was dried and subjected to autoradiography.

Statistical analysis. Results were expressed as means ± SE. The difference between two mean values was analyzed by the nonparametric Wilcoxon sign test or ANOVA. The difference was considered statistically significant when P < 0.05.

RESULTS

Involvement of oxidative stress in high glucose-induced inhibition of α-MG uptake. To determine whether the activity of the Na+/glucose cotransport system was effected by hyperglycemia, initially 14C-labeled α-MG uptake studies were conducted with the primary PTCs. Both the effect of the incubation time with 25 mM D-glucose (0–48 h) and the effect of increasing the D-glucose concentration (5–50 mM) were studied. As shown in Fig. 1A, a significant decrease in the α-MG uptake rate was first observed after 12 h of incubation with 25 mM D-glucose (12.6% decrease vs. control; P < 0.05). The inhibition was maximal between 48 and 72 h (a 36.5% de-

![Fig. 1. Time- and dose-dependent effect of glucose on 14C-labeled α-methyl-D-glucose (α-MG) uptake.](http://ajprenal.physiology.org/)
crease at 72 h. vs. control; \( P < 0.05 \). As shown in Fig. 1B, at glucose concentrations above 15 mM \( \alpha \)-MG uptake decreased significantly after a 48-h incubation.

To determine whether oxidative stress was involved in mediating the effect of glucose, the effect of either 1) aminoguanidine or pyridoxamine (inhibitors of the accumulation of AGEs, as well as antioxidants); 2) rotenone (an inhibitor of NADH-UQ reductase, a component of complex I of the mitochondrial electron transfer chain); or 3) apocynin or DPI (inhibitors of NADPH oxidase) on the high glucose-induced inhibition of the Na\(^+\)/glucose cotransporter was examined. Figure 2 shows that 1 \( \mu \)M aminoguanidine, 1 \( \mu \)M pyridoxamine, 100 nM rotenone, 1 \( \mu \)M apocynin, and 100 nM DPI all prevented the inhibitory effects of 25 mM \( \delta \)-glucose on \( \alpha \)-MG uptake. Incubation with either mannitol or \( \lambda \)-glucose at the same concentration as the added \( \delta \)-glucose (25 mM) failed to inhibit \( \alpha \)-MG uptake (data not shown).

**Effect of high glucose on \( \text{H}_2\text{O}_2 \) generation.** The ability of high glucose to increase the generation of LPOs in PTCs was examined as a function of time (Fig. 3A) and glucose concentration (Fig. 3B). Figure 3A shows that an increase in the LPO level (30.2%) was first observed after a 4-h incubation with 25 mM \( \delta \)-glucose and that this increase in the LPO level was maintained over a 48-h incubation. When the effect of \( \delta \)-glucose concentration was examined (Fig. 3B), a significant increase in LPO level was observed at 15 mM \( \delta \)-glucose, and maximal effects were observed at both 25 and 50 mM \( \delta \)-glucose. The effect of high glucose on the \( \text{H}_2\text{O}_2 \) level in particular was examined. Figure 4A shows that unlike the case with LPOs in general, the \( \text{H}_2\text{O}_2 \) level showed a statistically significant increase after a 2-h incubation with 25 mM glucose, and moreover the increase observed at 2 h was the maximal observed over the 48-h incubation period. However, the \( \text{H}_2\text{O}_2 \) level was observed to depend on \( \delta \)-glucose concentration in a manner similar to that observed with LPOs in general (Fig. 4B).

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![Figure 2](http://ajprenal.physiology.org/)

**Figure 2.** Effect of aminoguanidine, pyridoxamine, rotenone, apocynin, and diphenylene iodonium (DPI) on high glucose-induced inhibition of \( \alpha \)-MG uptake. PTCs were incubated with aminoguanidine, pyridoxamine (1 \( \mu \)M), rotenone (100 nM), apocynin (1 \( \mu \)M), and DPI (100 nM) for 30 min before treatment with 25 mM glucose for 48 h. Values are means \( \pm \) SE of 5 independent experiments. \({}^* P < 0.05\) vs. 5 mM glucose alone (open bars). \({}^{**} P < 0.05\) vs. 25 mM glucose alone (filled bars).

![Figure 3](http://ajprenal.physiology.org/)

**Figure 3.** Time- and dose-dependent effect of glucose on lipid peroxidase (LPO) formation. PTCs were treated with 25 mM glucose for different time periods (0–48 h; A) and different glucose concentrations (5–50 mM) for 4 h (B). Values are means \( \pm \) SE of 5 independent experiments with triplicate dishes. \( \bigcirc \), Control; \( \blacksquare \), 25 mM glucose. \( \alpha P < 0.05\) vs. 0 h or 5 mM glucose. \( \alpha{}^{**} P < 0.01\) vs. SmM glucose.

Figure 5 shows that both the high glucose-induced increase in LPO formation and the high glucose-induced increase in \( \text{H}_2\text{O}_2 \) content were significantly inhibited by the individual addition of 1) either aminoguanidine or pyridoxamine (which act as AGE inhibitors, as well as antioxidants); 2) rotenone (an NADH-UQ reductase inhibitor); as well as 3) either apocynin or DPI (which are NADPH oxidase inhibitors). However, when added individually to PTC cell culture maintained at the control \( \delta \)-glucose level, aminoguanidine, pyridoxamine, rotenone, apocynin, and DPI did not have a significant effect on either LPO formation or the cellular \( \text{H}_2\text{O}_2 \) content.

The effect of an incubation with 25 mM \( \delta \)-glucose on catalase activity and GSH level of PTCs was then examined. Figure 6 shows that 25 mM \( \delta \)-glucose had an inhibitory effect on both catalase activity (22.6% decrease vs. control; \( P < 0.05 \)) and the level of GSH (19.8% decrease vs. control; \( P < 0.05 \)). However, Fig. 6 also shows that both the high glucose-induced decrease in catalase activity and the high glucose-induced decrease in the cellular GSH level were blocked by individually adding to the culture medium 1) either aminoguanidine or pyridoxamine (which act as AGE inhibitors, as well as antioxidants); 2) rotenone (an NADH-UQ reductase inhibitor); or 3) either apocynin or DPI (NADPH oxidase inhibitors).
The possible involvement of TGF-β1 in mediating the high glucose-induced inhibition of α-MG uptake was examined. Primary PTCs were incubated for 4 h with D-glucose at concentrations ranging from 5 to 50 mM. As shown in Fig. 7A, TGF-β1 secretion increased when the D-glucose level was increased to either 25 or 50 mM. Aminoguanidine, pyridoxamine, rotenone, apocynin, and DPI all individually blocked the high glucose-induced stimulation of TGF-β1 secretion.

To summarize, all of the observed effects of high glucose on the primary PTCs, including the decreased activity of the Na+/H+ cotransport system, the increase in lipid peroxidase levels, the increase in H2O2 release, the increase in catalase activity, the decrease in cellular GSH levels, and the increase in TGF-β1 secretion, were all prevented by agents that acted either 1) as inhibitors of AGEs and/or as antioxidants; 2) as inhibitors of NADH-UQ reductase; or 3) as inhibitors of NADPH oxidase.

**Involvement of NF-κB on high glucose-induced inhibition of α-MG uptake.** NF-κB is a ubiquitous transcription factor that is regulated by ROS. The possibility that NF-κB was involved in mediating the effects of high glucose in the primary PTCs was examined. The effect of incubation with 25 mM D-glucose on the level of nuclear NF-κB p65 and cellular IkBα was examined by means of Western blot analysis (Fig. 8A). In addition, EMSAs were conducted (Fig. 8B and C). Figure 8A shows that following an increase in the D-glucose level to 25 mM, the level of NF-κB p65 increased 1.8-fold, whereas the cytosolic level of IkBα protein decreased 1.5-fold. Figure 8A also shows that these effects of high glucose were prevented by the individual addition of either pyridoxamine, rotenone, or DPI.

The effect of an increase in the D-glucose level on the ability of nuclear proteins to bind to the consensus NF-κB binding site was examined by EMSAs. Nuclear extracts derived from PTCs treated with 25 mM D-glucose as well as from control cultures were incubated with a 32P-labeled deoxyoligonucleotide homologous to the consensus NF-κB binding site. Figure 8B shows the presence of retarded bands, indicative of the nuclear binding of NF-κB.

**Fig. 4.** Time- and dose-dependent effect of glucose on H2O2 release. PTCs were treated with 25 mM glucose for different time periods (0–48 h; A) and different glucose concentrations (5–50 mM) for 4 h (B). Values are means ± SE of 5 independent experiments with triplicate dishes. *P < 0.05 vs. 0 h or 5 mM glucose (A). **P < 0.01 vs. 0 h or 5 mM glucose (B).

**Fig. 5.** Effect of aminoguanidine, pyridoxamine, rotenone, apocynin, and DPI on high glucose-induced alteration of LPO formation (A) and H2O2 release (B). PTCs were pretreated with aminoguanidine, pyridoxamine, rotenone, apocynin, and DPI for 30 min before treatment with 25 mM glucose and H2O2 (100 μM) alone for 4 h. Values are means ± SE of 5 independent experiments with triplicate dishes. *P < 0.05 vs. 5 mM glucose alone (open bars). **P < 0.05 vs. 25 mM glucose alone (filled bars).
protein/DNA complex formation that occurred during the binding reaction. The intensity of two of the retarded bands increased 2.6-fold when nuclear extracts derived from cells that had been incubated with high glucose were used. Figure 8B also shows that the intensity of these two bands was reduced to the control level when nuclear extracts derived from cells treated with either aminoguanidine, rotenone, or apocynin in addition to 25 mM glucose were used. The specificity of the binding reaction was confirmed by the ability of excess unlabelled (cold) NF-κB oligodeoxynucleotide (cold κB) to inhibit binding (Fig. 8C), indicating the specificity of the binding reaction.

To further evaluate the relationship between oxidative stress and the high glucose-induced inhibition of α-MG uptake, the effect of incubation with 25 mM D-glucose on the level of the SGLT proteins was examined by means of Western blot analysis. Figure 9 shows that the increase in the glucose level was associated with a decrease in the level of both SGLT1 and SGLT2. The decrease in the level of SGLT1 and SGLT2 was prevented by incubation with either aminoguanidine, rotenone, or apocynin.

**DISCUSSION**

The present study has demonstrated that the induced production of H$_2$O$_2$ and LPOs that occurs when PTCs are cultured in the presence of elevated D-glucose levels is dependent on mitochondrial ROS and NADPH oxidase. Previously, we showed that the high glucose-induced generation of H$_2$O$_2$ in PTCs is effectively blocked both by inhibitors of PKC (13) as
well as by the downregulation of PKC, which occurs during prolonged incubations with phorbol 12-myristate 13-acetate. PKC has been shown to play an important role in the activation of both phagocytic and nonphagocytic NADPH oxidase (15). Thus our previously reported results suggest that the increased H$_2$O$_2$ generation in PTCs treated with high levels of D-glucose is dependent on PKC, as previously reported for aortic smooth muscle and endothelial cells (15).

NADPH oxidase has been found to be a critical determinant in the receptor-stimulated formation of superoxide anion, which subsequently results in the generation of H$_2$O$_2$ by superoxide dismutase in both phagocytic and nonphagocytic cells (3). Nishikawa et al. (21) have demonstrated that incubation of bovine aortic endothelial cells with high glucose does indeed result in the generation of the superoxide anion, suggesting that the ROS produced by NADPH oxidase do indeed play a critical role in generating the diabetic vascular complications that are induced by hyperglycemia.

We have observed that both apocynin and DPI, NADPH oxidase inhibitors, and rotenone, an inhibitor of complex I of the mitochondrial electron transport chain, effectively block the high glucose-induced generation of H$_2$O$_2$ in PTCs. ROS production was abolished by DPI as well as rotenone, indicating that a nonmitochondrial flavoprotein such as NADPH oxidase is a source of ROS in proximal tubule cells (25).

High glucose may also induce the generation of H$_2$O$_2$ indirectly through the synthesis of AGEs as well as through the synthesis of cytokines such as TGF-$eta_1$ (29). Indeed, AGEs have been observed to stimulate the generation of intracellular ROS in neonatal mesangial and proximal tubular epithelial cells (27). In human umbilical vein endothelial cells, the AGE/receptor for the AGE-mediated generation of ROS is
NADPH oxidase dependent (17). However, in the studies presented here, it is unclear that AGEs are actually involved in mediating the effects of high glucose, due to the relatively rapid time course of the observed effects in our studies. However, oxidative stress that occurs in diabetes may result from a variety of other abnormalities that can emerge with a more rapid time course, including decreased activities of antioxidant enzymes and increased H$_2$O$_2$ production. Indeed, our previously published data indicate that metabolites that may emerge, such as oxalate, stimulate H$_2$O$_2$ production in primary cultured renal proximal tubule cells after only a 30-min incubation, and this stimulatory effect was maintained for 4 h (3).

Both aminoguanidine and pyridoxime, which were utilized in this report, also act as antioxidants, while being inhibitors of AGE formation (1, 2). Some reports have demonstrated that the antioxidant activity of aminoguanidine and pyridoxime is due, at least in part, to their ability to scavenge ROS (3, 4). Both aminoguanidine and pyridoxime have been observed to inhibit the oxidation of ascorbic acid in a concentration-dependent manner (6). However, when examining the effects of aminoguanidine on post-Amadori AGE formation, Booth et al. (7) found that aminoguanidine had only a minor inhibitory effect, whereas pyridoxime was identified as an effective post-Amadori AGE inhibitor. This discrepancy may be due to the difference of experimental model (in vivo vs. in vitro), species (rat vs. rabbit), or cell specificity.

The increased expression and activity of NADPH oxidase and increased production of superoxide that occur in diabetes may synergize with the subsequent AGE-related changes (5). The relative contributions of PKC, NADPH oxidase, and mitochondrial metabolism to the high glucose-induced generation of ROS have not yet been clearly defined. However, the hypothesis that aminoguanidine and pyridoxime act through their antioxidant effects in the studies presented here is consistent with the mechanisms inferred from similar results obtained in experiments with DPI (an NADPH oxidase inhibitor) and rotenone (an inhibitor of mitochondrial oxidative metabolism).

GSH is a direct free radical scavenger, whose concentration has been observed to decrease in the kidneys of chemically induced diabetic animals (22). However, there is a contradictory report regarding increased GSH levels in diabetic rat kidneys and lens, which suggests that the observed changes in GSH status are also dependent on other aspects of the physiological state (22). In hyperglycemia, as much as 30% of the glucose is channeled into the polyol pathway, resulting in a substantial depletion of NADPH and a significant decrease in the GSH level. Catalase, located in peroxisomes, metabolizes H$_2$O$_2$ to water and molecular oxygen. Catalase activity has been consistently found to be elevated in the heart as well as in the brain of diabetic rats (7), unlike in the liver and kidneys where catalase activity is decreased. In the present study, the alterations in the GSH level and in catalase activity due to high glucose were prevented by treatment with 1) aminoguanidine and pyridoxamine, which act as inhibitors of the formation of AGEs as well as antioxidants; 2) rotenone, which inhibits NADH-UQ reductase, a component of complex I of the electron transport chain; as well as 3) apocynin and DPI, which inhibit NADPH reductase.

In addition, we observed a high glucose-induced increase in the level of NF-κB. Evidence for increased binding of nuclear proteins to the NF-κB consensus deoxyoligonucleotide was obtained. These effects were prevented by the same inhibitors that blocked the effects of high glucose on catalase activity and GSH levels. Previously, we reported that the high glucose-induced activation of NF-κB in PTCs was effectively inhibited by antioxidants (13). NF-κB is a ubiquitous transcription factor that is activated by ROS, and ROS-mediated NF-κB activation may very well play an important role in the pathogenesis of diabetic nephropathy.

The generation of ROS by high glucose was associated with an inhibition of apical transporter activity of renal proximal tubule cells. Our experiments with aminoguanidine, pyridoxamine, rotenone, apocynin, and DPI indicate that the inhibition of the activity of the Na$^+$/glucose cotransporter is dependent on the formation of AGEs, mitochondrial metabolism, and NADPH oxidase. Oxidant-induced alterations in transporter function may occur at the membrane level via the oxidation of critical amino acid residues within the transporter or by alterations of the lipid environment of the plasma membrane, which result from increased levels of lipid hydroperoxides (10). Indeed, our observations indicate that the inhibition of the activity of apical Na$^+$/glucose cotransport by high glucose is the consequence of an increase in the level of LPOs, in particular H$_2$O$_2$. However, we also observed that high glucose caused a reduction in the overall level of the Na$^+$/glucose cotransport system in PTCs. To gain a better understanding of the mechanisms by which high glucose induces these effects, further studies must be conducted to identify other possible target molecules that are activated by ROS in renal cells cultured under high glucose.

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**Fig. 9.** Effects of aminoguanidine, rotenone, and apocynin on high glucose-induced alteration of SGLT protein expression. PTCs were treated with aminoguanidine, rotenone, and apocynin for 30 min before treatment with 25 mM glucose for 48 h. The amount of SGLT proteins was determined by Western blot analysis of membrane fraction, as described in MATERIALS AND METHODS. Bands represent 70–77 kDa of SGLT1, SLGT2, and 41 kDa of β-actin, respectively. The example shown is representative of 4 experiments.
REFERENCES


25. Petit AI, Wong RK, Lee V, Jennings S, Quinn PA, and Ng LL. Increased free radical production in hypertonosis due to increased expression of the NADPH oxidase subunit p22(phox) in lymphoblust cell lines. *J Hypertens* 20: 677–683, 2002.


