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High Glucose Stimulates Angiotensinogen Gene Expression via Reactive Oxygen Species Generation in Rat Kidney Proximal Tubular Cells

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The present studies investigated whether the effect of high glucose levels on angiotensinogen (ANG) gene expression in kidney proximal tubular cells is mediated via reactive oxygen species (ROS) generation and p38 MAPK activation. Rat immortalized renal proximal tubular cells (IRPTCs) were cultured in monolayer. Cellular ROS generation and p38 MAPK phosphorylation were assessed by lucigenin assay and Western blot analysis, respectively. The levels of immunoreactive rat ANG secreted into the media and cellular ANG mRNA were determined by a specific RIA and RT-PCR, respectively. High glucose (25 mM) evoked ROS generation and p38 MAPK phosphorylation as well as stimulated immunoreactive rat ANG secretion and ANG mRNA expression in IRPTCs. These effects of high glucose were blocked by antioxidants (taurine and tiron), inhibitors of mitochondrial electron transport chain complex I (rotenone) and II (thenoyltrifluoroacetone), an inhibitor of glycolysis-derived pyruvate transport into mitochondria (α-cyano-4-hydroxycinnamic acid), an uncoupler of oxidative phosphorylation (carbonyl cyanide *m*-chlorophenylhydrazone), a manganese superoxide dismutase mimetic, catalase, and a specific inhibitor of p38 MAPK (SB 203580), but were not affected by an inhibitor of the malate-aspartate shuttle (aminooxyacetate acid). Hydrogen peroxide ($\geq 10^{-5}$ M) also stimulated p38 MAPK phosphorylation, ANG secretion, and ANG mRNA gene expression, but its stimulatory effect was blocked by catalase and SB 203580. These studies demonstrate that the stimulatory action of high glucose on ANG gene expression in IRPTCs is mediated at least in part via ROS generation and subsequent p38 MAPK activation. (*Endocrinology* 143: 2975–2985, 2002)

THE PATHOGENESIS of diabetic nephropathy involves complex structural changes, including glomerular and tubular hypertrophy, with progressive accumulation of extracellular matrix components in the glomerular mesangium and tubulointerstitium (1–3). One of the early abnormalities detected after the onset of diabetes is altered proximal tubular cell growth (4). A growing body of evidence indicates that high levels of glucose and/or angiotensin II (Ang II) may directly or indirectly be responsible for renal proximal tubular cell (RPTC) hypertrophy and tubulofibrosis in diabetes (5–10). For example, *in vitro* studies have shown that murine proximal tubular cells cultured in a high glucose medium (≥25 mM) containing Ang II ($\geq 10^{-8}$ M) exhibit increased expression of TGFβ1, cellular hypertrophy, and extracellular matrix protein accumulation (5–10). These findings are supported by clinical observations that administration of angiotensin-converting enzyme (ACE) inhibitors or Ang II (AT_1 subtype) receptor antagonists reduces proteinuria and slows the progression of nephropathy in diabetic patients (11–13).

The presence of a local intrarenal renin-angiotensin system (RAS) has been well documented. The mRNA components of the RAS, including angiotensinogen (ANG), renin, ACE, and Ang II receptors (AT_1 and AT_2 subtypes), are expressed in rodent (mouse and rat) immortalized proximal tubular cell lines (14–18). We have previously shown that high glucose (25 mM) stimulates ANG gene expression in rat immortalized RPTCs (IRPTCs) (19, 20). This stimulatory effect of high glucose can be suppressed by inhibitors of aldose reductase, protein kinase C (PKC) and p38 MAPK (19, 20). Furthermore, we have reported that ACE inhibitors (perindopril and captopril) and an Ang II (AT_1) receptor antagonist (losartan) blocked ANG gene expression and prevented the induction of hypertrophy in IRPTCs stimulated by high glucose levels (21). These data support the hypothesis that intrarenal RAS activation may play an important role in the development of renal hypertrophy in diabetes.

Recent studies have shown that hyperglycemia may induce cellular oxidative stress by increasing superoxide or reactive oxygen species (ROS) generation (22). Overproduction of ROS and/or impaired antioxidant defense in poorly controlled diabetes could contribute to endothelial and vascular dysfunction (23, 24). Studies have also shown that elevated glucose levels *per se* enhance PKC activation, cause oxidative stress, and augment membrane lipid peroxidation

Abbreviations: ACE, Angiotensin-converting enzyme; ANG, angiotensinogen; Ang II, angiotensin II; AOAC, aminooxyacetate acid; ATF-2, activating transcription factor-2; CAT, catalase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CREB, cAMP-responsive element binding protein; dFBS, depleted FBS; FBS, fetal bovine serum; IRPTC, immortalized renal proximal tubular cell; IR-rANG, immunoreactive rat angiotensinogen; MnTBAP, manganese (III) tetrakis (4-benzoic acid) porphorin chloride; 4-OHCA, α-cyano-4-hydroxycinnamic acid; PKC, protein kinase C; RAS, renin-angiotensin system; ROS, reactive oxygen species; RPTC, renal proximal tubular cell; SAPK, stress-activated protein kinase; SOD, superoxide dismutase; TTFA, thenoyltrifluoroacetone.

in glomeruli (25), suggesting that high glucose might play a role in the induction of glomerular lipid peroxidation. Although high glucose may induce oxidative stress (*i.e.* ROS generation) in the diabetic kidney (26, 27), the underlying molecular mechanism(s) remains incompletely understood. Brownlee's group (28, 29) demonstrated recently that hyperglycemia-induced mitochondrial superoxide formation might be the unifying pathway that activates PKC activity, increases intracellular sorbitol and advanced glycated end-product formation, and subsequently induces endothelial cell dysfunction.

In the present studies we investigated whether high glucose levels could evoke cellular ROS generation and modulate ANG gene expression in IRPTCs. Our studies showed that high glucose levels stimulate cellular ROS generation, p38 MAPK phosphorylation, and ANG gene expression in IRPTCs. This high glucose effect on ROS generation and ANG gene expression was blocked by antioxidants, inhibitors of mitochondrial electron transport chain complexes I and II, an inhibitor of glycolysis-derived pyruvate transport into mitochondria, an uncoupler of oxidative phosphorylation, a manganese superoxide dismutase (SOD) mimetic, a catalase (CAT), and a specific inhibitor of p38 MAPK (SB 203580), but not by an inhibitor of the malate-aspartate shuttle. Hydrogen peroxide (H_2O_2) also stimulated p38 MAPK phosphorylation and ANG gene expression in IRPTCs. Its stimulatory effect was blocked in the presence of CAT and SB 203580. These results demonstrate that high glucose stimulates ANG gene expression in IRPTCs at least in part via the generation of cellular ROS and subsequent activation of the p38 MAPK signal transduction pathway.

Materials and Methods

D(+)-Glucose, L-glucose, taurine (an antioxidant), tiron (a superoxide scavenger), rotenone (an inhibitor of mitochondrial electron transport chain complex I), thenoyltrifluoroacetone (TTFA; an inhibitor of mitochondrial electron transport chain complex II), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; an uncoupler of oxidative phosphorylation), α-cyano-4-hydroxycinnamic acid (4-OHCA; an inhibitor of glycolysis-derived pyruvate transport into mitochondria), aminooxyacetate acid (AOAC; an inhibitor of the malate-aspartate shuttle), CAT, and H_2O_2 were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Canada). SB 203580 (an inhibitor of p38 MAPK kinase) and manganese (III) tetrakis (4-benzoic acid) porphorin chloride (MnTBAP) (a cell-permeable

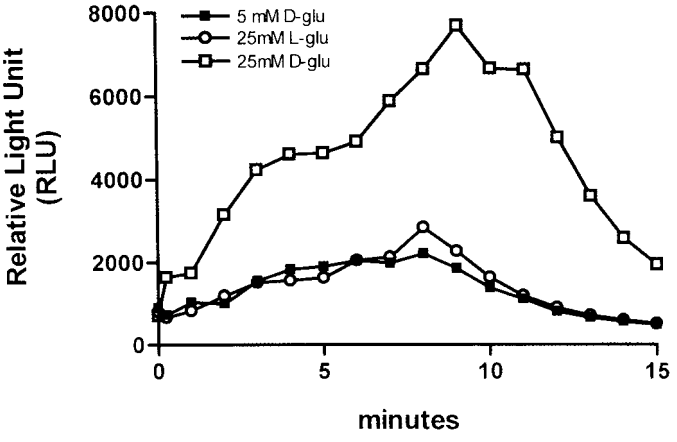

FIG. 1. Effect of D-glucose on superoxide (ROS) generation in rat IRPTCs. Measurement of ROS generation was started in the luminometer after the addition of lucigenin at time zero. Emission of relative light units (RLU) was corrected for nonspecific luminescence in the absence of cells. Representative tracings for ROS measurement using the lucigenin method are shown for cells incubated in 5 or 25 mM D-glucose or 25 mM L-glucose medium for 15 min. ■, 5 mM D-glucose; □, 25 mM D-glucose; ○, 25 mM L-glucose.

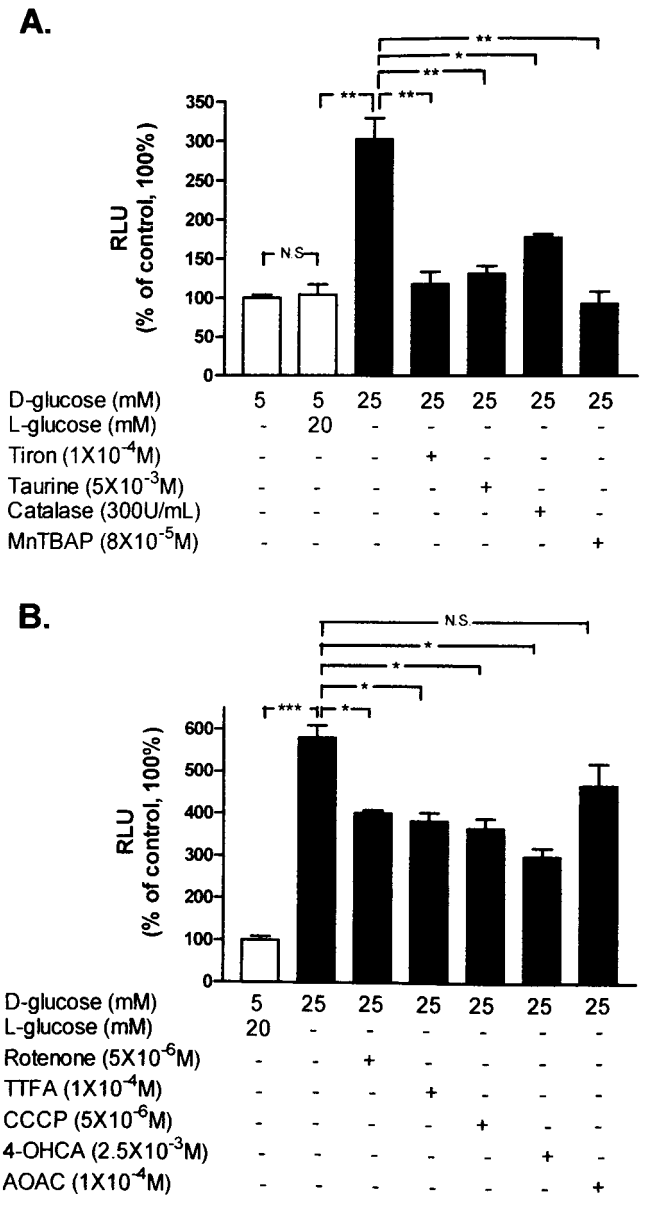

FIG. 2. Effects of antioxidants and inhibitors of mitochondrial metabolism on ROS production in IRPTCs. A, Cells were preincubated in 5 mM D-glucose DMEM containing 1% depleted FBS (dFBS) for 4 h in the absence or presence of tiron (1×10^{-4} M), taurine (5×10^{-3} M), catalase (300 U/ml), or MnTBAP (8×10^{-5} M). B, Cells were preincubated in 5 mM D-glucose DMEM containing 1% dFBS for 4 h in the absence or presence of rotenone (5×10^{-6} M), TTFA (1×10^{-4} M), CCCP (5×10^{-6} M), 4-OHCA (2.5×10^{-3} M), or AOAC (1×10^{-4} M). The cells were then trypsinized and incubated in 5 or 25 mM D-glucose for the assessment of ROS generation. The RLU measurements were compared after 10 min of incubation. Cells incubated in 5 mM D-glucose plus 20 mM L-glucose medium were considered controls (100%). Each *point* represents the mean ± SD of five and four independent experiments in A and B, respectively. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

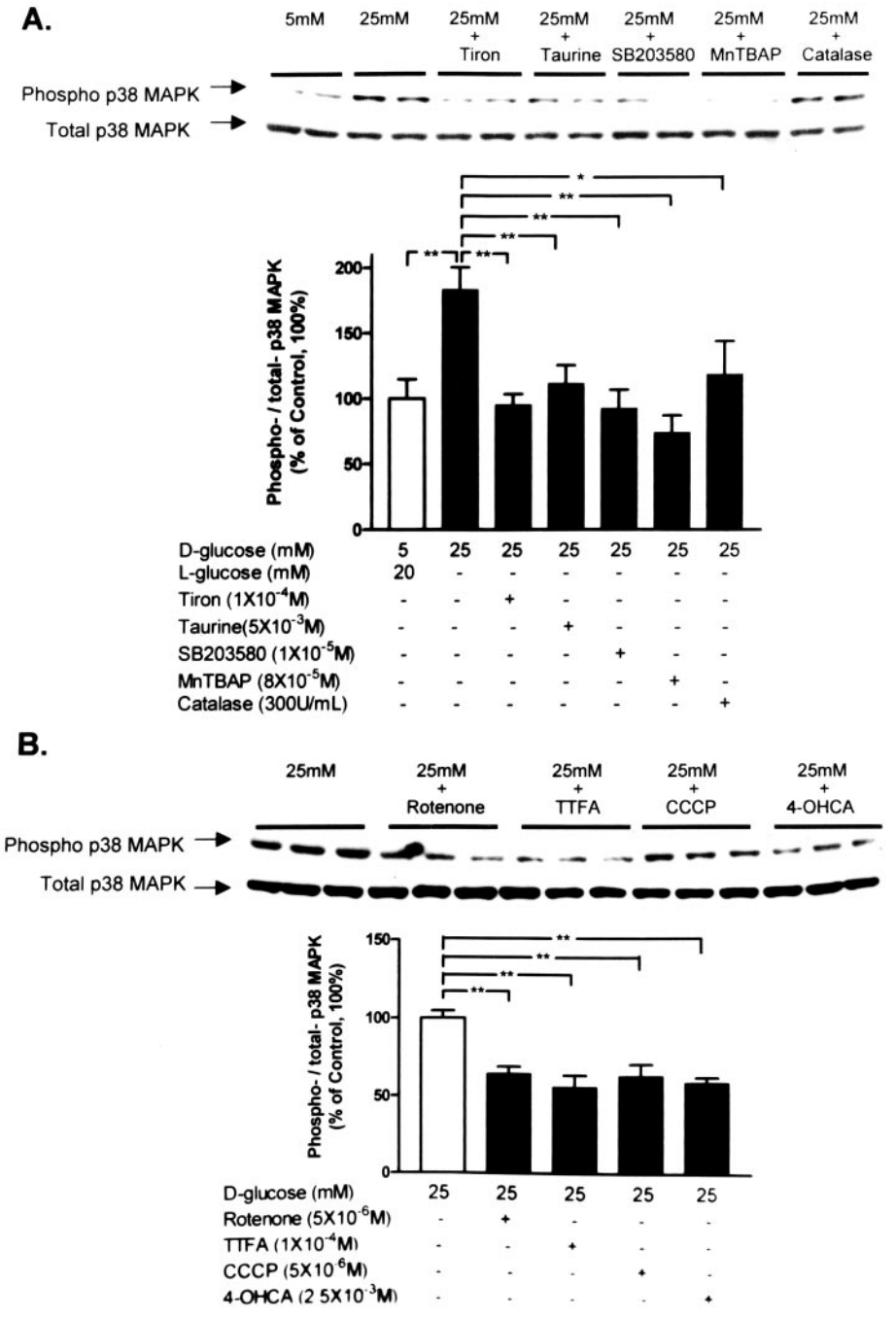

FIG. 3. Effects of antioxidants and inhibitors of mitochondrial metabolism on p38 MAPK phosphorylation in IRPTCs. A, Cells were preincubated in 5 mM D-glucose DMEM containing 1% dFBS for 4 h in the absence or presence of tiron (1×10^{-4} M), taurine (5×10^{-3} M), SB 203580 (1×10^{-5} M), MnTBAP (8×10^{-5} M), or catalase (300 U/ml). B, Cells were preincubated in 5 mM D-glucose DMEM containing 1% dFBS for 4 h in the absence or presence of rotenone (5×10^{-6} M), TTFA (1×10^{-4} M), CCCP (5×10^{-6} M), 4-OHCA (2.5×10^{-3} M), or AOAC (1×10^{-4} M). Cells then were incubated in 5 or 25 mM D-glucose for 30 min and subsequently lysed and assayed for phosphorylated p38 MAPK and total p38 MAPK. The relative densities of phosphorylated p38 MAPK were compared with total p38 MAPK. The ratio of phosphorylated p38 MAPK to total p38 MAPK in cells cultured in 5 mM glucose (A) was considered the control (100%). Each point represents the mean ± SD of six independent experiments. Similarly, the ratio of phosphorylated p38 MAPK to total p38 MAPK in cells cultured in 25 mM glucose (B) was considered the control (100%). The inhibitory effect of various agents is compared with cells incubated in 25 mM glucose (without the presence of any agents). The results are expressed as the percentage of controls (mean ± SD; n = 4 independent experiments). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

synthetic manganese SOD mimetic) were purchased from Calbiochem (La Jolla, CA). Normal glucose (5 mM) DMEM (catalog no. 12320) and 100× penicillin/streptomycin were purchased from Life Technologies, Inc. (Burlington, Canada). $Na^{125}I$ was obtained from Amersham-Pharmacia Biotech (Baie d'Urfé, Canada).

The Phospho Plus p38 MAPK antibody kit was purchased from New England Biolabs, Inc. (Mississauga, Canada). This kit was used for the rapid analysis of p38 MAPK (Thr^{180}/Tyr^{182}) phosphorylation status that functions in the stress-activated protein kinase cascade.

Cell culture

IRPTCs at passages 13–18 were used in the present study. Their characteristics have been described previously (30). These cells express the mRNA and protein of ANG, renin, ACE, and Ang II receptors (30). IRPTCs were grown in 100-mm plastic petri dishes (Life Technologies, Inc.) in normal glucose (*i.e.* 5 mM glucose DMEM, pH 7.45) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc.), 100 U/ml penicillin, and 100 μg/ml of streptomycin until used. The cells were grown in a humidified atmosphere in 95% air/5% CO_2 at 37 C and passaged twice a week by harvesting with trypsin/EDTA. Cells were synchronized for 24 h in serum-free 5 mM DMEM at 80–90% confluence before the experiments.

Study design

To determine whether the stimulatory effect of high glucose on ANG gene expression is mediated via ROS generation, cells were preincubated in 5 mM glucose DMEM containing 1% dFBS for 4 h in the absence or presence of tiron (1×10^{-4} M), taurine (5×10^{-3} M), rotenone ($5 \times$

10^{-6} M), TTFA (1×10^{-4} M), CCCP (5×10^{-6} M), AOAC (1×10^{-4} M), 4-OHCA (2.5×10^{-3} M), MnTBAP (8×10^{-5} M), or CAT (300 U/ml). Then, 20 mM L-glucose or 20 mM D(+)-glucose were added into the culture medium, and the cells were cultured for an additional 30 min or 24 h. At the end of the incubation period, the media were collected and analyzed by RIA for rat ANG (rANG). The cells were harvested for p38 MAPK phosphorylation assay or ANG mRNA quantification. To assess the role of H_2O_2 on p38 MAPK phosphorylation and ANG gene expression, the cells were incubated with different concentrations of H_2O_2 (*i.e.* 10^{-7}–10^{-4} M) in 5 or 25 mM glucose DMEM containing 1% dFBS for 30 min or 24 h. At the end of the incubation period, the media were collected and assayed by RIA for rANG. The cells were harvested for p38 MAPK phosphorylation assay or ANG mRNA quantification.

Measurement of superoxide or ROS generation in IRPTCs

ROS production was determined by the lucigenin method (31) with minor modifications. Briefly, the cells were preincubated in 5 mM glucose DMEM containing 1% dFBS for 4 h in the absence or presence of tiron (1×10^{-4} M), taurine (5×10^{-3} M), rotenone (5×10^{-6} M), TTFA (1×10^{-4} M), CCCP (5×10^{-6} M), AOAC (1×10^{-4} M), 4-OHCA (2.5×10^{-3} M), MnTBAP (8×10^{-5} M), or CAT (300 U/ml). Then, cells were trypsinized and collected by centrifugation, and the pellet was washed in modified Krebs buffer containing NaCl (130 mM), KCl (5 mM), $MgCl_2$ (1 mM), $CaCl_2$ (1.5 mM), K_2HPO_4 (1 mM), and HEPES (20 mM), pH 7.4. After washing, the cells were resuspended in Krebs buffer with 1 mg/ml BSA, and the cell concentration was adjusted to 1×10^7 in 900 μl buffer. To measure ROS production, the cell suspension was transferred into plastic tubes and assessed in a luminometer (LB 9507, Berthold, Wildbad, Germany). Measurement was started by an injection of 100 μl lucigenin (final concentration, 5×10^{-4} M) and 25 μl 1 M L-glucose or D-glucose (final concentration, 25 mM) with or without inhibitors, as indicated. Photon emission was counted every 1 min for up to 20 min. Modified Krebs buffer was used as a control (blank). Solutions containing glucose or inhibitors in the absence of cells did not display any significant interference in the lucigenin assay.

Phosphorylation of p38 MAPK in IRPTCs

The activation of p38 MAPK signaling transduction pathways in IRPTCs was evaluated by p38 MAPK phosphorylation with the Phospho Plus p38 MAPK antibody kit (New England Biolabs, Inc., Beverly, MA), as described previously (20). Briefly, 1×10^7 cells were plated in 100-mm petri dishes in 5 mM glucose DMEM containing 10% FBS until 80–90% confluence, then synchronized in 5 mM glucose medium for 24 h. Subsequently, cells were incubated in high glucose in the presence or absence of inhibitors of ROS generation, or H_2O_2 for 30 min. Then cells were lysed in 700 μl lysis buffer [62.5 mM Tris-HCl, pH 6.8, containing 2% (wt/vol) sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, and 0.1% (wt/vol) bromophenol blue] and transferred into Eppendorf tubes (Ultident Scientific, St. Laurent, Québec, Canada). The cell lysates were sonicated for 15 sec, heated at 95 C for 5 min, and centrifuged at 12,000 $\times$ *g* for 5 min. Thirty-five microliters of the supernatants were subjected to SDS-10% PAGE, then transferred onto a polyvinylidene difluoride membrane (Hybond-P, Amersham-Pharmacia Biotech). The membrane was initially blotted for phosphorylated p38 MAPK and then reblotted for total p38 MAPK according to the instructions of the supplier (New England Biolabs, Inc.).

RIA for rANG

The RIA for rANG, developed in our laboratory (J.S.D.C.), has been described previously in detail (32). Purified plasma rANG (*i.e.* ≥90% pure, as analyzed by SDS-PAGE) and iodinated rANG were used as hormone standard and tracer, respectively. This RIA is specific for intact rat (62–65 kDa) ANG and has no cross-reactivity with pituitary hormone preparations or other rat plasma proteins (32). The lower limit of detection for the RIA is approximately 2 ng rANG. The intra- and interassay coefficients of variation were 9% (n = 10) and 14% (n = 10), respectively.

RT-PCR

Total RNA, isolated with TRIzol reagent (Life Technologies, Inc.) according to the protocol of the supplier and quantified by its absorbance at 260 nm, was used in RT-PCR to quantify the amount of ANG mRNA expressed in IRPTCs. Briefly, 2 μg total RNA were used to synthesize first strand cDNAs by employing the SuperScript preamplification system, following the protocol described by the supplier (Life Technologies, Inc.). Then, the first strand cDNA was diluted with water to a ratio of 1:4, and aliquots were used to amplify the rat ANG and β-actin cDNA fragments with the PCR core kit according to the supplier's instructions

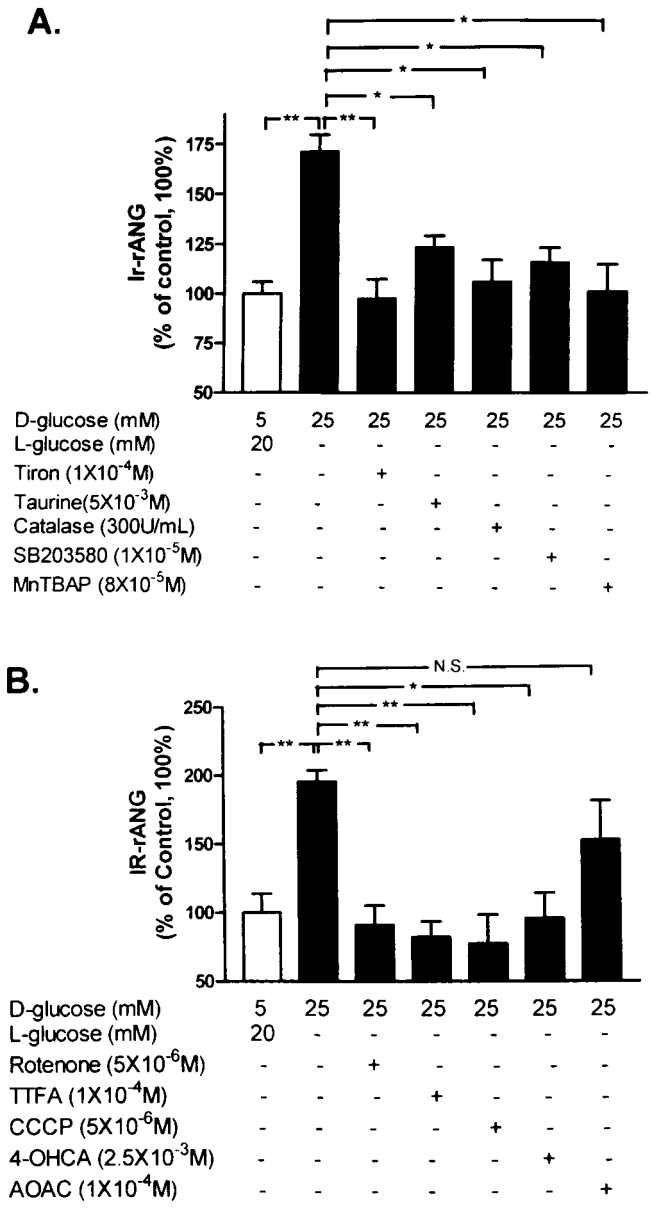

FIG. 4. Effects of antioxidants and inhibitors of mitochondrial metabolism on ANG secretion in IRPTCs. A, Cells were incubated in 5 or 25 mM D-glucose DMEM containing 1% dFBS for 24 h in the absence or presence of tiron (1×10^{-4} M), taurine (5×10^{-3} M), catalase (300 U/ml), SB 203580 (1×10^{-5} M), or MnTBAP (8×10^{-5} M). B, Cells were incubated in 5 or 25 mM D-glucose DMEM containing 1% dFBS for 24 h in the presence or absence of rotenone (5×10^{-6} M), TTFA (1×10^{-4} M), CCCP (5×10^{-6} M), 4-OHCA (2.5×10^{-3} M), or AOAC (1×10^{-4} M). The media were then harvested and assayed for IR-rANG. The IR-rANG levels in 5 mM glucose medium are expressed as 100%. The inhibitory effect of various agents is compared with cells incubated in 25 mM glucose (without the presence of any agents). The results are expressed as the percentage of controls (mean ± SD; n = 7 independent experiments in A or B). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

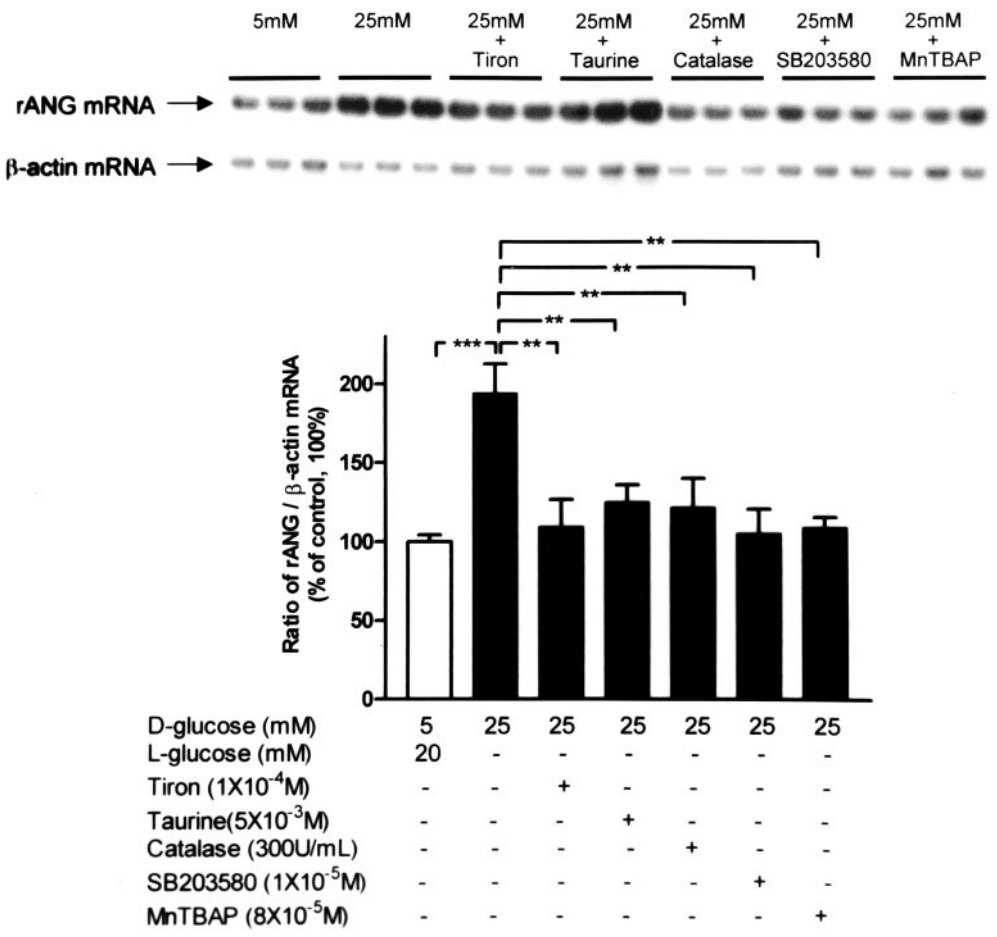

FIG. 5. Effects of antioxidants on ANG mRNA expression in IRPTCs. After a 24-h incubation with 5 or 25 mM D-glucose DMEM in the absence or presence of tiron (1×10^{-4} M), taurine (5×10^{-3} M), catalase (300 U/ml), SB 203580 (1×10^{-5} M), or MnTBAP (8×10^{-5} M), cells were harvested and assayed for ANG mRNA levels by RT-PCR. DNA fragments of the RT-PCR mixture were separated on 1.5% agarose gel, then transferred onto a nylon membrane. Subsequently, the membrane was blotted with a digoxigenin-labeled oligonucleotide corresponding to nucleotides +775 to +798 of rat ANG and +9 to +35 of exon 4 of rat β-actin, respectively. The relative densities of the PCR band of ANG were compared with the β-actin control. The rANG mRNA level in cells normalized in 5 mM glucose was considered the control (100%). Each point represents the mean ± SD of four independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

(La Roche Biochemicals, Inc., Laval, Canada). First strand cDNA (5 μl) and primers of rat ANG (800 nM) and rat β-actin (100 nM) were added in a final volume of 50 μl PCR mixture (final concentrations, 1× PCR buffer, 0.2 mM deoxy-NTP, 2 mM $MgCl_2$, and 2.0 U *Taq* DNA polymerase; Life Technologies, Inc.). The PCR mixture was amplified in a Perkin Elmer Cetus (Norwalk, CT) 2400 thermocycler. After denaturation at 94 C for 3 min, rANG and β-actin cDNA were coamplified in the same tube under the following conditions: 94 C for 1 min, 60 C for 1 min, and 72 C for 1 min and 30 sec. After 30 cycles of amplification, PCRs were further extended at 72 C for 7 min.

The sense and antisense rat ANG primers were 5′-CCT CGC TCT CTG GAC TTA TC-3′ and 5′-CAG ACA CTG AGG TGC TGT TG-3′, corresponding to the nucleotide sequences of +676 to +695 and +882 to +901 of rANG cDNA (33), respectively. The sense and antisense rat β-actin primers were 5′-ATG CCA TCC TGC GTC TGG ACC TGG C-3′ and 5′-AGC ATT TGC GGT GCA CGA TGG AGG G-3′, corresponding to the nucleotide sequences of +155 to +139 of exon 3, and nucleotide sequences of +115 to +139 of exon 5 of the rat β-actin gene (34), respectively.

To identify rANG and β-actin cDNA fragments, 10 μl of the PCR product were electrophoresed on 1.2% agarose gels and transferred onto a Hybond XL nylon membrane (Amersham Pharmacia Biotech). Digoxigenin-labeled oligonucleotide 5′-GAG GGG GTC AGC ACG GAC AGC ACC-3′, corresponding to nucleotide +775 to +798 of rANG cDNA (33) prepared with a digoxigenin oligonucleotide 3′-end labeling kit (La Roche Biochemicals, Inc.), was used to hybridize the PCR products on the membrane. After stringent washing, the membrane was detected with a digoxigenin luminescent detection kit (La Roche Biochemicals, Inc.) and exposed to Kodak BMR film (Eastman Kodak Co., Rochester, NY). After rANG mRNA analysis, the same membrane was stripped and rehybridized with a β-actin oligonucleotide probe (sequence: 5′-TCC TGT GGC ATC CAT GAA ACT ACA TTC-3′, corresponding to nucleotides +9 to +35 of exon 4 of the rat β-actin gene) (34). ANG mRNA levels were normalized by corresponding β-actin mRNA levels.

Statistical analysis

Four to seven separate experiments were performed per protocol, and each treatment group was assayed in triplicate. The data were analyzed with *t* test or ANOVA. $P \leq 0.05$ was regarded as statistically significant.

Results

Effects of high glucose on ROS generation in IRPTCs

The lucigenin assay revealed that ROS generation was significantly higher (*i.e.* 2- to 4-fold increase) in IRPTCs cultured in high glucose medium (*i.e.* 25 mM D-glucose) compared with those in normal glucose medium (*i.e.* 5 mM D-glucose) or 25 mM L-glucose medium (Fig. 1). The addition of tiron (1×10^{-4} M), taurine (5×10^{-3} M), CAT (300 U/ml), or MnTBAP (8×10^{-5} M) to the culture medium abolished high glucose (25 mM)-stimulated ROS generation (Fig. 2A). Likewise, the addition of rotenone (5×10^{-6} M), TTFA (1×10^{-4} M), CCCP (5×10^{-6} M), or 4-OHCA (2.5×10^{-3} M) abolished the stimulatory effect of high glucose, but not the addition of AOAC (1×10^{-4} M; Fig. 2B). These results indicate that high glucose induces ROS generation at least in part via mitochondrial membrane-bound electron transport chain complex I/II, oxidative phosphorylation, and glycolysis-derived pyruvate transport into mitochondria, but not via the malate-aspartate shuttle.

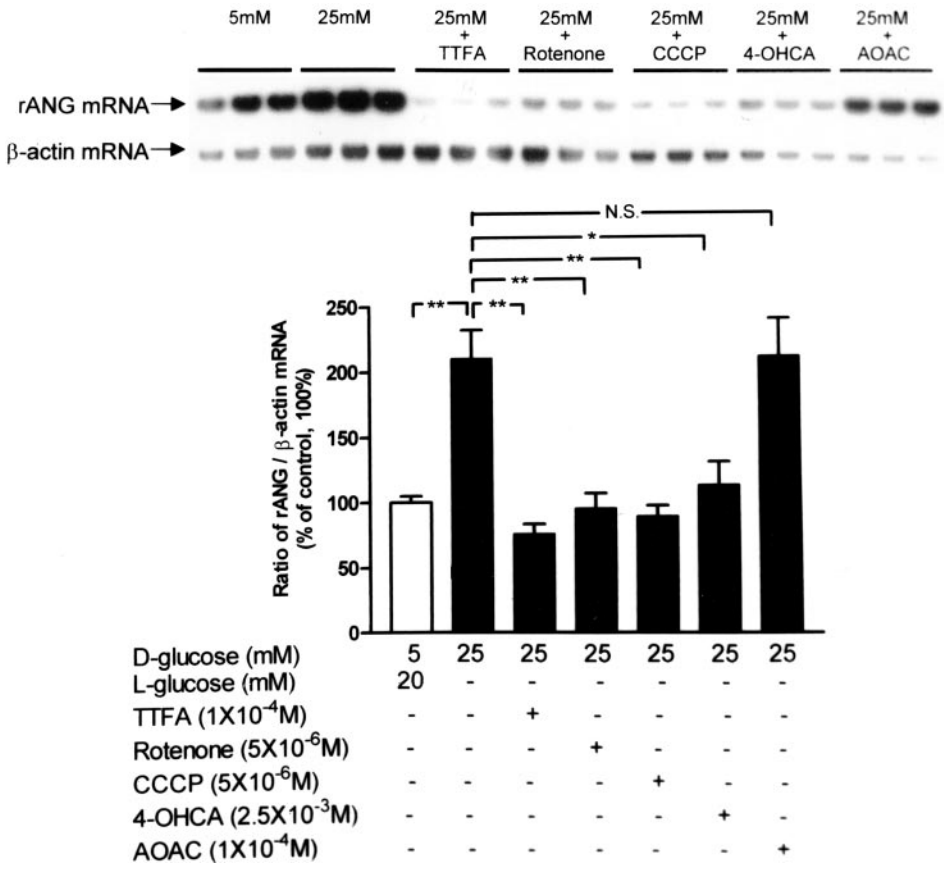

FIG. 6. Effects of inhibitors of mitochondrial metabolism on ANG mRNA expression in IRPTCs. After 24-h incubation with 5 or 25 mM D-glucose DMEM in the absence or presence of TTFA (1×10^{-4} M), rotenone (5×10^{-6} M), CCCP (5×10^{-6} M), 4-OHCA (2.5×10^{-3} M), or AOAC (1×10^{-4} M), the cells were then harvested and assayed for ANG mRNA levels by RT-PCR according to the method described in Fig. 5. The relative densities of the PCR band of ANG were compared with the β-actin control. The rANG mRNA level in cells normalized in 5 mM glucose was considered the control (100%). Each *point* represents the mean ± SD of four independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

Effect of high glucose and antioxidants on p38 MAPK phosphorylation in IRPTCs

Figure 3A shows that 25 mM glucose stimulated p38 MAPK phosphorylation in IRPTCs compared with IRPTCs in 5 mM glucose medium. The stimulatory effect of high glucose (25 mM) was blocked in the presence of tiron (1×10^{-4} M), taurine (5×10^{-3} M), MnTBAP (8×10^{-5} M), or SB 203580 (1×10^{-7} M). The addition of rotenone (5×10^{-6} M), TTFA (1×10^{-4} M), CCCP (5×10^{-6} M), or 4-OHCA (2.5×10^{-3} M) also inhibited p38 MAPK phosphorylation in IRPTCs incubated in 25 mM (Fig. 3B). In contrast, the addition of AOAC did not block the stimulatory effect of high glucose on p38 MAPK phosphorylation (data not shown). These results demonstrate that the effect of high glucose on p38 MAPK phosphorylation is mediated at least in part via mitochondrial ROS generation.

Effect of high glucose and antioxidants on ANG secretion and mRNA expression in IRPTCs

Immunoreactive (IR)-rANG secretion by IRPTCs into the culture medium was increased (*i.e.* 150%) in the presence of a high glucose level (*i.e.* 25 mM) compared with that under normal glucose conditions (*i.e.* 5 mM; $P \leq 0.05$) after a 24-h incubation period (Fig. 4). The stimulatory effect of high glucose (25 mM) was blocked by taurine (5×10^{-3} M), tiron (1×10^{-4} M), CAT (300 U/ml), MnTBAP (8×10^{-5} M), or SB 203580 (1×10^{-5} M; Fig. 4A) as well as by rotenone (5×10^{-6} M), TTFA (1×10^{-4} M), CCCP (5×10^{-6} M), or 4-OHCA (2.5×10^{-3} M; Fig. 4B). In contrast, the addition of AOAC had no such effect. These studies demonstrate that the hyperglycemia-stimulated secretion of IR-rANG in IRPTCs is mediated at least in part via ROS generation and p38 MAPK activation.

Figure 5 shows that 25 mM glucose enhanced ANG mRNA accumulation to levels 2.5-fold higher than those found in control cells cultured in medium containing 5 mM glucose ($P \leq 0.01$). The addition of taurine, tiron, catalase, MnTBAP, or SB 203580 inhibited ANG mRNA expression stimulated by high glucose. Similarly, the addition of rotenone, TTFA, CCCP, or 4-OHCA also inhibited ANG mRNA expression in IRPTCs incubated in 25 mM glucose medium (Fig. 6). AOAC, however, did not block the stimulatory effect of high glucose on ANG mRNA expression (Fig. 6). These data demonstrate that the stimulatory effect of high glucose levels on cellular ANG mRNA expression is mediated at least in part via ROS generation and p38 MAPK signal transduction pathways.

Effect of H_2O_2 on p38 MAPK phosphorylation, ANG secretion, and ANG mRNA expression in IRPTCs in high glucose

Figure 7A shows that H_2O_2 (*i.e.* 10^{-7}–10^{-4} M) stimulated p38 MAPK phosphorylation in IRPTCs incubated in 5 or 25 mM glucose in a dose-dependent manner with a maximal impact observed at 10^{-4} M. This dose of H_2O_2 (10^{-4} M) was then used routinely in all subsequent experiments. Its stimulatory effect on p38 MAPK phosphorylation in IRPTCs was prevented by CAT and SB 203580 (Fig. 7B). These studies

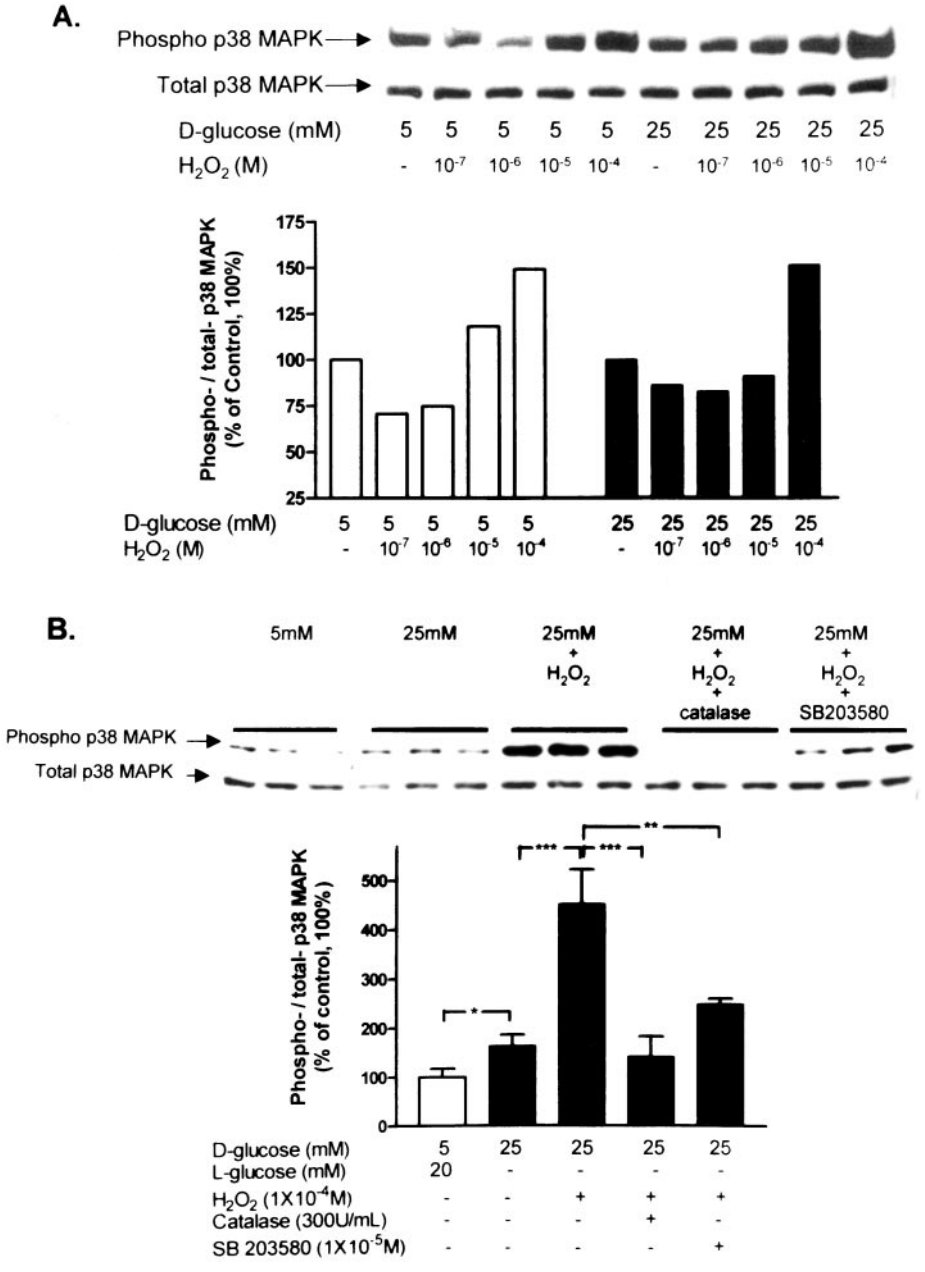

FIG. 7. Effect of H_2O_2 on p38 MAPK phosphorylation in IRPTCs. A, After a 24-h incubation period in 5 mM glucose, the cells were incubated in 5 or 25 mM glucose medium for 30 min in the presence or absence of various concentrations of H_2O_2. B, Cells were incubated in 25 mM glucose plus H_2O_2 in the absence or presence of catalase or SB 203580. Then, cells were harvested and assayed for p38 MAPK phosphorylation with the Phospho Plus p38 MAPK antibody kit. The same membrane was reblotted for total p38 MAPK. The *upper panel* shows the raw data, and the *lower panel* shows the relative densities for phosphorylated p38 MAPK to total p38 MAPK. Cells incubated in 5 mM glucose medium were considered controls (100%). Each *point* represents the mean ± SD of four independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

demonstrate that H_2O_2 could directly stimulate p38 MAPK phosphorylation in IRPTCs.

H_2O_2 (*i.e.* 10^{-7}–10^{-4} M) had no effect on IR-rANG secretion in IRPTCs incubated in 5 mM glucose (Fig. 8A), whereas it stimulated IR-rANG secretion in IRPTCs incubated in 25 mM glucose medium in a dose-dependent manner with a maximal effect observed at 10^{-5}–10^{-4} M (Fig. 8B). Its stimulatory effect was inhibited in the presence of CAT and SB 203580 (Fig. 8C). H_2O_2 (*i.e.* 10^{-4} M) also enhanced ANG mRNA expression in IRPTCs in high glucose medium (Fig. 9). This stimulatory effect on ANG mRNA expression in IRPTCs was blocked in the presence of CAT and SB 203580 (Fig. 9). These studies demonstrate that H_2O_2 stimulates IR-rANG secretion and ANG mRNA expression in IRPTCs via p38 MAPK activation in high glucose medium.

Discussion

Here we report a novel mechanism by which high glucose induces ANG gene expression in rat RPTCs via ROS generation and subsequent activation of the p38 MAPK phosphorylation.

p38 MAPK is a member of the MAPK superfamily, *i.e.* the extracellular signal-regulated kinase (p42/p44 MAPKs), p38 MAPK, and stress-activated protein kinases(s) (SAPK)/Jun-N terminal kinase (35). These three kinases represent distinct, but related, signal transduction pathways by which kinases are activated by extracellular signals and then translocated into the nucleus to stimulate specific gene expression (36). Studies have shown that p42/p44 MAPKs are activated by a variety of growth and neurotropic factors and are linked to

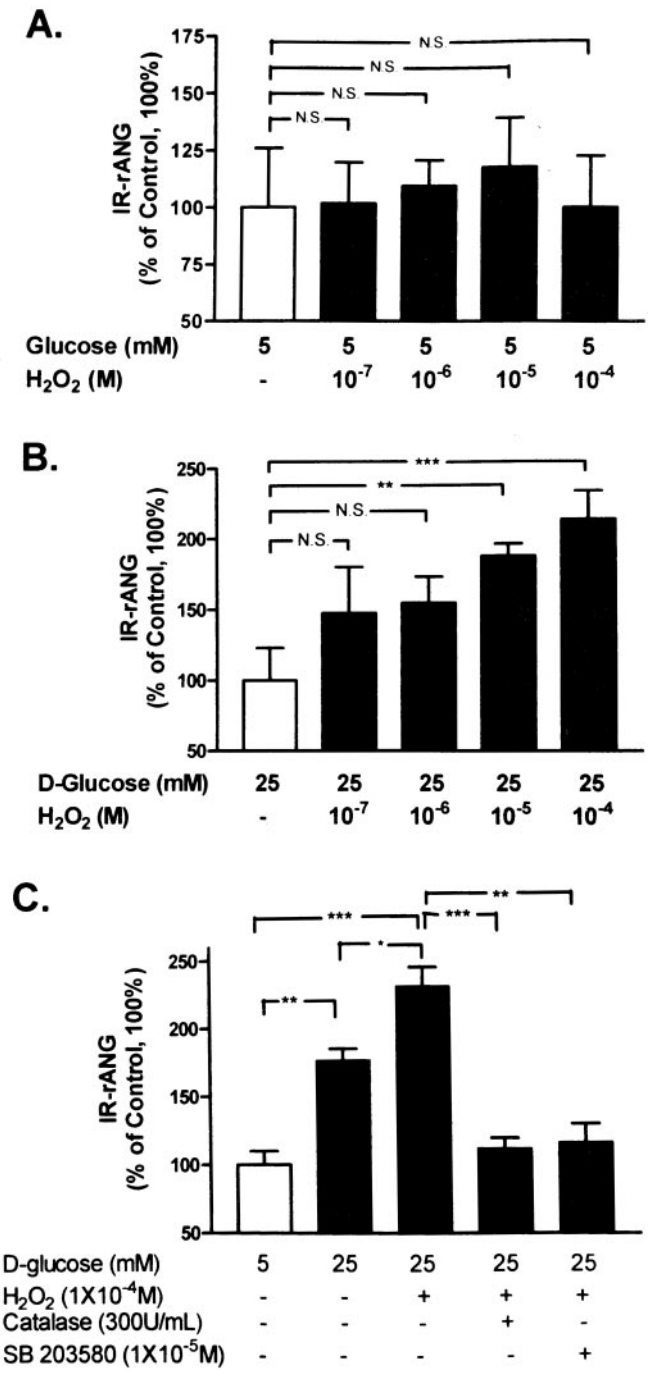

FIG. 8. Effect of exogenous H_2O_2 on ANG secretion in IRPTCs. A, Cells were incubated in 5 mM glucose DMEM with various concentrations of H_2O_2 for 24 h. B, Cells were incubated in 25 mM glucose DMEM with various concentrations of H_2O_2 for 24 h. C, Cells were incubated in 25 mM glucose DMEM plus 10^{-4} M H_2O_2 in the absence or presence of catalase (300 U/ml) or SB 203580 (10^{-5} M) for 24 h. Then the media were collected and assayed for IR-rANG. The IR-rANG levels in medium containing 5 mM glucose in A, B, or C were considered controls (100%). The inhibitory effect of catalase or SB 203580 is compared with cells incubated in 25 mM glucose and 10^{-4} M H_2O_2 (in the absence of catalase or SB 203580). Each *point* represents the mean ± SD of seven independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

cell growth and differentiation (37). In contrast, the p38 MAPK and SAPK/Jun-N terminal kinase pathways have been implicated in the cellular response to environmental stress, such as high extracellular osmolarity, UV irradiation, heat shock, and inflammatory cytokines (38–40). We have previously demonstrated that high glucose levels (*i.e.* 25 mM) stimulate ANG gene expression and p38 MAPK phosphorylation in IRPTCs in a time-dependent manner that can be prevented by SB 203580 (20). These studies suggest that the stimulatory effect of high glucose (25 mM) on ANG gene expression may be mediated at least in part via the p38 MAPK signal transduction pathway. High glucose was reported to stimulate p38 MAPK phosphorylation consistently in rat mesangial cells and vascular smooth muscle cells (41, 42). The molecular mechanism(s) of high glucose action on p38 MAPK activation in IRPTCs, however, remain(s) undefined.

To elucidate the molecular mechanism(s) by which high glucose activates p38 MAPK and induces ANG gene expression in IRPTCs, we investigated the possibility that high glucose may stimulate superoxide generation and subsequently activate p38 MAPK signaling. The present studies showed that, indeed, 25 mM D-glucose stimulates ROS generation in a time-dependent manner compared with 5 mM glucose, whereas 25 mM L-glucose has no effect (Fig. 1). Moreover, the addition of antioxidants (tiron, taurine, SOD mimetic, and CAT) and inhibitors of mitochondrial oxidation (rotenone, TTFA, CCCP, and 4-OHCA) prevented ROS generation stimulated by high glucose (Fig. 2, A and B). These results are in agreement with previous studies reporting that antioxidants, superoxide scavengers, and inhibitors of mitochondrial oxidation prevent ROS formation in various cell lines, *i.e.* vascular smooth muscle cells, endothelial cells, mesangial cells, and murine proximal tubular cells (23, 24, 27–29, 43). The addition of AOAC did not block the stimulatory effect of high glucose (25 mM) on ROS generation in IRPTCs, indicating that the role of high glucose on ROS generation is not mediated via the malate-aspartate shuttle. These results are in agreement with the findings of Brownlee's group (29) that AOAC had no influence on mitochondrial ROS generation in bovine endothelial cells. Furthermore, the effective doses of the inhibitors used here were similar to those of reported by Brownlee's group (29). On the other hand, the inhibitory effect of retenone on ROS generation in IRPTCs is in disagreement with the results of Brownlee's group (29). The exact reasons for these discrepancies are not known at present. One possible explanation might be that the response to rotenone is specific to the cell type involved (endothelial compared with epithelial cells).

High glucose stimulated p38 MAPK phosphorylation in IRPTCs. This stimulatory effect was suppressed in the presence of antioxidants (*i.e.* tiron, taurine, CAT, and SOD mimetic), inhibitors of mitochondrial metabolism (*i.e.* rotenone, TTFA, CCCP, and 4-OHCA), and an inhibitor of p38 MAPK (*i.e.* SB 203580). These results demonstrate that the stimulatory action of high glucose levels on p38 MAPK phosphorylation is mediated at least in part via mitochondrial ROS generation. Our data are in agreement with previous studies showing that ROS-dependent p38 MAPK activation in various cell types (44–47). Taken together, these findings sup-

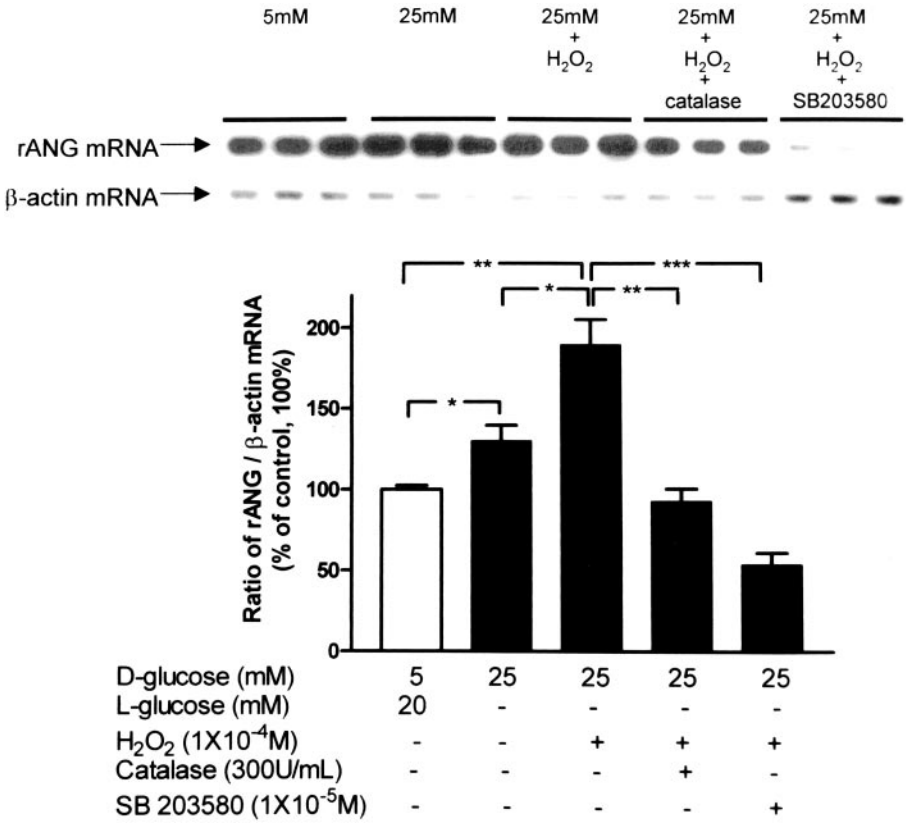

FIG. 9. Effect of exogenous H_2O_2 on the expression of rat ANG mRNA in IRPTCs. After a 24-h incubation period in 5, 25, or 25 mM glucose DMEM plus H_2O_2 (10^{-4} M) in the absence or presence of catalase (300 U/ml) or SB 203580 (10^{-5} M), cells were harvested and assayed for ANG mRNA levels by RT-PCR according to the method described in Fig. 5. The relative densities of the PCR band of ANG were compared with those of the β-actin control. The rANG mRNA level in cells cultured in 5 mM glucose was considered the control (100%). Each *point* represents the mean ± SD of four independent experiments. *, $P \leq 0.05$.

port the idea that the high glucose (*i.e.* 25 mM) effect on p38 MAPK phosphorylation is mediated at least in part via mitochondrial ROS generation.

The addition of antioxidants, inhibitors of mitochondrial electron transport complex I/II, inhibitors of glycolysis-derived pyruvate transport into mitochondria, uncoupler of oxidative phosphorylation, and SB 203580 blocked IR-rANG secretion and ANG mRNA expression in IRPTCs stimulated by high glucose. These studies provide evidence that the effect of high glucose levels on ANG gene expression in IRPTCs may be mediated at least in part via ROS generation and p38 MAPK signal transduction pathways. At present it is not known whether high glucose levels and/or p38 MAPK increase the transcription or affect the stability of ANG mRNA in IRPTCs. Experiments are underway in our laboratory to investigate these possibilities.

Oxidative defense is provided by several key enzymes, such as SOD and CAT, and by chain-breaking scavengers, such as vitamin E, vitamin C, and glutathione (48). SOD is the key antioxidant enzyme involved in the detoxification of superoxide radicals. It is a metalloprotein and its manganese form (MnSOD) is present in mitochondria. Two other forms, containing copper and zinc, have cytoplasmic or extracellular location (49). Our studies revealed that cell-permeable MnSOD mimetic, *i.e.* MnTBAP, and CAT prevented the stimulatory effect of high glucose on ROS generation, p38 MAPK activation, and ANG mRNA expression in IRPTCs, suggesting that the expression of endogenous MnSOD and CAT is effective in preventing cellular ROS accumulation and subsequently cellular injury.

Recent evidence suggests that certain forms of ROS, such as H_2O_2, may act as signal molecules. Indeed, studies have shown that H_2O_2 significantly increases extracellular matrix proteins (collagen types I, III, and IV and fibronectin) and TGFβ1 mRNA by approximately 2-fold in human mesangial cells (50). This increase in extracellular matrix mRNA may be mediated via TGFβ (50). Our present studies showed that H_2O_2 stimulates p38 MAPK phosphorylation, IR-rANG secretion, and ANG mRNA expression in IRPTCs. This stimulatory effect of H_2O_2 is inhibited in the presence of CAT and SB 203580. These studies suggest that the stimulatory impact of ROS on p38 MAPK phosphorylation and ANG gene expression could be mediated at least in part via H_2O_2 generation in IRPTCs. The molecular mechanism(s) of H_2O_2 action on p38 MAPK phosphorylation is not clear at present. Investigations are ongoing along this line in our laboratory.

At present we do not know the exact molecular mechanism(s) underlying the stimulatory effect of high glucose (that is, the downstream pathway after p38 MAPK activation) on rat ANG gene expression in IRPTCs. One possibility might be that high glucose induces the phosphorylation of nuclear activating transcription factor-2 (ATF-2) via the p38 MAPK signal transduction pathway. Indeed, studies have shown that p38 MAPK phosphorylates ATF-2 (51, 52). The phosphorylated ATF-2 then forms the heterodimer complex with phosphorylated cAMP-responsive element binding protein (CREB). CREB phosphorylation is induced by high glucose levels via the PKC signal transduction pathway, as demonstrated by Kreisberg *et al.* (53). The phosphorylated ATF-2/CREB heterodimer then binds to the cAMP-respon-

sive element in the 5′-flanking region of the rANG gene (54, 55) and subsequently enhances gene expression. This possibility is supported by our recent studies which showed that high glucose levels stimulated the phosphorylation of 43-kDa CREB and ATF-2 in IRPTCs (56). Indeed, experiments are ongoing in our laboratory to explore the downstream molecular mechanism(s) of the stimulatory effect of high glucose on ANG gene expression in IRPTCs.

In summary, the present studies showed that high glucose levels directly stimulate ROS generation, p38 MAPK phosphorylation, and ANG gene expression in IRPTCs. The addition of antioxidants, inhibitors of mitochondrial oxidation, and SB 203580 blocked the stimulatory effect of high glucose, implicating mitochondrial ROS and the p38 MAPK signal transduction pathway in up-regulation of renal ANG gene expression under hyperglycemic conditions. These results suggest that blockade of ROS generation may represent a novel therapeutic approach to prevent or attenuate glucose-induced ANG gene expression and, consequently, the development of diabetic nephropathy. However, it remains to be seen whether long-term blockade of ROS generation may indeed be beneficial in the treatment of diabetic nephropathy. This interesting possibility warrants further investigation.

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