Antioxidants attenuate high glucose-induced hypertrophic growth in renal tubular epithelial cells

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Although the etiology of the tubulointerstitial pathology in DN is not fully understood, a great deal of attention has focused on the role of high glucose (HG) per se (11, 12, 17, 25, 37). Hyperglycemia sharply increases the production of reactive oxygen species (ROS), which play a key role in the tubulointerstitial hyperplasia and hypertrophy in DN (2, 40). Recently, several mechanisms have been proposed for the oxidative damage during chronic hyperglycemia including glucose autoxidation, synthesis of advanced glycation end-products (AGEs), and mitochondrial ROS overproduction (2, 29, 40). Hyperglycemia and ROS may induce changes in cellular function by common intracellular signaling pathways. Excess generation of mitochondrial ROS due to hyperglycemia initiates a vicious circle by activating stress-sensitive pathways such as JAK/STAT, NF-κB, PKC, and p38 MAPK, polyl (sorbitol) and hexosamine pathways, and AGEs (2, 37, 39, 40).

Numerous studies demonstrated that tubular cells are a primary target of hyperglycemia and that chronic exposure to elevated blood glucose levels contributes to the tubulointerstitial changes seen in overt DN (11, 12, 28, 29, 37). Overproduction of AGEs, sorbitol, and proinflammatory cytokines exerts a positive feedback on ROS synthesis and potentiates MAPK-mediated tubular dysfunction by altering gene expression as well as tubular function and structure (2, 11, 37, 39, 40). Thus it became clear that ameliorating oxidative stress through treatment with antioxidants might be an effective strategy for reducing tubulointerstitial injury.

Protective effects of exogenously administered antioxidants have been extensively studied in animal models within recent years, thus providing some insight into the relationship between ROS and DN (6, 19, 31, 36). ROS is a key mediator of renal tubular hypertrophy in diabetic nephropathy (DN). The molecular mechanisms of antioxidants responsible for inhibition of renal tubular hypertrophy in DN are incompletely characterized. We now aim at verifying the effects of N-acetylcysteine (NAC) and taurine on cellular hypertrophy in renal tubular epithelial cells under high ambient glucose. We found that NAC and taurine treatments significantly attenuated high glucose (HG)-induced cellular growth and HG-induced hypertrophy. HG-induced Raf-1, p42/p44 mitogen-activated protein kinase (MAPK), Janus kinase 2 (JAK2), and signal transducer and activator of transcription 1 (STAT1) and STAT3 (but not STAT5) activation was markedly blocked by NAC and taurine. Moreover, NAC and taurine increased cyclin D1/cdk4 activation and suppressed p21Waf1/Cip1 and p27Kip1 expression in HG-treated cells. It seems that apoptosis was not observed in these treatments. There were no changes in bcl-2 and poly(ADP-ribose) polymerase expression, and mitochondrial cytochrome c release. However, NAC or taurine markedly inhibited the stimulation by HG of fibronectin and type IV collagen protein levels. It is concluded that both NAC and taurine significantly attenuated HG-induced activation of the Raf-1/MAPK and the JAK2-STAT1/STAT3 signaling pathways and hypertrophic growth in renal tubular epithelial cells.

N-acetylcysteine; taurine; high glucose; hypertrophy; renal tubular epithelial cells; antioxidants

THE TUBULOINTERSTITIUM COMPRISES ~90% of kidney volume and undergoes major pathological changes in diabetes (8, 29). Nephromegaly in the early stages of diabetes and the correlation of tubulointerstitial pathology with declining renal function in diabetic nephropathy (DN) suggest the involvement of the tubulointerstitium (8, 29, 37). Although the glomerulus and, in particular, the mesangium have hitherto been the focus of intense investigation in diabetes, recent studies in experimental animals have shown that the tubulointerstitium is also a major site of growth factor synthesis and matrix expression in the diabetic kidney (8, 17, 25, 28, 29). Indeed, the extent of tubulointerstitial fibrosis is strongly associated with mesangial expansion, falling glomerular filtration rate, and increasing proteinuria in human DN (11, 12, 17). Moreover, tubulointerstitial injury is an important predictor of both renal dysfunction and its response to therapeutic interventions (11, 17, 25).

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NAC and taurine have been successfully used in diabetes to attenuate cardiac damage and peripheral neuropathy in experimental models (21, 23, 27, 33). They have also been demonstrated to reduce the expression of extracellular matrix (ECM) and transforming growth factor-β (TGF-β) in glomeruli from streptozotocin-induced diabetic rats (14, 20). Moreover, HG-induced overproduction of oxidative stress in renal tubular epithelial cells has been previously documented (6, 19, 31, 36). Nevertheless, the effects of antioxidants on the HG-induced hypertrophy in experimental DN have not yet been well elucidated. Therefore, the objective of the present study was to verify whether NAC and taurine 1) reduced HG-induced renal tubular hypertrophy; 2) attenuated HG-enhanced activation of the JAK/STAT and the Raf-1/MAPK pathways; and 3) affected HG-mediated cell cycle progression, ECM synthesis, and apoptosis in renal tubular epithelial cells.

MATERIALS AND METHODS

Reagents. Fetal bovine serum (FBS), DMEM, antibiotics, d-glucose, d-mannitol, molecular weight standards, trypsin-EDTA, trypsinization buffer, and all medium additives were obtained from Life Technologies (Gaithersburg, MD). Anti-JAK2, -STAT1, -STAT3, -STAT5, -Raf-1, -phospho-Raf-1 (Ser 338), -p42/p44 MAPK, -bcl-2, -poly(ADP-ribose) polymerase (PARP), -cytochrome c, -collagen IV, -c-myc, -cyclin D1, -cdk4, -p27kip1, and -p21Waf1/Cip1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-p42/p44 MAPK antibody was purchased from New England Biolabs (Beverly, MA). Anti-phospho-JAK2 antibody was obtained from Cell Signaling Technologies (Gaithersburg, MD). Anti-JAK2, -STAT1, -STAT3, -STAT5, -Raf-1, and -stat-phospho-Raf-1 (0.75 μg/ml) antibodies were obtained from Upstate Biotechnology (Charlottesville, VA). Protein A/G-coupled agarose beads, AG-490, GW5074, PD98059, N3-mono-methyl-l-arginine, and anti-protein kinase G-I antibody were purchased from Calbiochem (La Jolla, CA). Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody, [γ-32P]ATP, and the enhanced chemiluminescence system as described above. Reagents for cell cycle analysis were obtained from BD Biosciences (San Jose, CA). Protein A/G-coupled agarose beads, AG-490, GW5074, PD98059, N3-mono-methyl-l-arginine, and anti-protein kinase G-I antibody were purchased from Calbiochem (La Jolla, CA). Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody, [γ-32P]ATP, and the enhanced chemiluminescence system as described above. Reagents for cell cycle analysis were obtained from BD Biosciences (San Jose, CA).

Cellular hypertrophy analysis. Cells were made quiescent for 2 days in serum-free DMEM containing 100 μg/dl d-glucose. The cultures were then treated with HG and various agents, after which the cells were trypsinized, washed twice with PBS, and counted using a hemocytometer. Equal numbers of cells were lysed in RIPA buffer [0.1% (wt/vol) SDS, 0.5% (wt/vol) sodium deoxycholate, and 1.0% (wt/vol) Nonidet P-40 in PBS]. The total protein content was measured using a Bio-Rad protein assay kit. Total protein was expressed as micrograms of protein per 10^6 cells.

Western blot analysis. For protein analysis, 1.0 × 10^7 serum-deprived cells were treated with agents or HG as described above. Total cell lysates were harvested, resolved by 10% SDS-PAGE, and then transferred to Protran membranes (0.45 μm, Schleicher & Schuell, Keene, NH). The membranes were blocked in blocking solution and subsequently probed with primary antibodies. The membrane was incubated in 4,000× diluted horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody. The protein bands were detected using the enhanced chemiluminescence system, and the percentage of the phosphorylated form of protein was determined using a scanning densitometer. For JAK/STAT and Raf-1/MAPK activation assays, proteins were resolved by SDS-PAGE and transferred to Protran membranes. The membranes were probed with anti-phospho-JAK2 (0.75 μg/ml), anti-phospho-STAT1 (1 μg/ml), anti-phospho-STAT3 (1 μg/ml), anti-phospho-STAT5 (1 μg/ml), anti-phospho-Raf-1 (0.75 μg/ml), anti-phospho-p42/p44 MAPK (0.75 μg/ml), anti-JAK2 (0.75 μg/ml), anti-STAT1 (1 μg/ml), anti-STAT3 (1 μg/ml), anti-STAT5 (1 μg/ml), anti-Raf-1 (0.75 μg/ml), and anti-p42/p44 MAPK (0.75 μg/ml) antibodies. The anti-phospho-STAT5a polyclonal antibody detects the tyrosine-phosphorylated form of both STAT5a and STAT5b. Immunoreactive proteins were detected with the enhanced chemiluminescence system as described above.

MTT assay. MTT assays were performed to evaluate the proliferation of renal tubular epithelial cells. Cells (5 × 10^5 cells/dl) were plated and incubated for 24 h in wells of a 96-well plate. Then, various concentrations of each drug were added to the wells. After 24-h incubation, 10 μl of sterile MTT dye were added, and the cells were incubated for 6 h at 37°C. Then, 100 μl of acidic isopropyl (0.04 M HCl in isopropanol) were added and thoroughly mixed. Spectrophotometric absorbance at 595 nm (for formazan dye) was measured with the absorbance at 655 nm for reference.

Flow-cytometric analysis. For flow-cytometric experiments to determine relative cell size, DNA content, and the cell cycle profile, cells were plated at a density of 1 × 10^6 to 1.2 × 10^6 cells per T-25 flask. At various time points, cells were harvested and fixed with ice-cold 100% ethanol with vortexing at low speed, cells were then placed at −20°C for overnight. After fixation, cells were centrifuged and washed once with PBS containing 1% bovine serum albumin. For staining with DNA dye, cells were resuspended in 0.5–1 ml of propidium iodide (PI) solution containing RNase and incubated at 37°C for 30 min, followed by overnight incubation at 4°C. Cell viability (assessed by PI staining) was >90% in each experiment. Forward-side scatter was expressed as arbitrary units, and the histogram of the mean average cell size was shown. Cell cycle profiles were obtained with a FACScan flow cytometer (Becton Dickinson, San Jose, CA), and the data were analyzed with ModFit software (Verity Software House) for cell cycle analysis.

Cdk4 kinase assay. For immunoprecipitation of cdk4, cells were washed twice with ice-cold PBS, harvested in cdk4 lysis buffer [50 mM HEPES (pH 7.5), 10% glycerol, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, and 0.1% Tween 20, supplemented with the phosphatase and protease inhibitors 5 mM NaF, 0.1 mM sodium orthovanadate, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 50 μg/ml phenylmethylsulfonyl fluoride, and 5 μg/ml pepstatin A] and lysed by repeated passages through a 25-gauge needle. Cellular debris was removed from soluble extracts by centrifugation at 16,000 g for 10 min at 4°C. Following normalization of protein content, lysates were preclarified by incubation with protein A/G-agarose beads and preimmune rabbit serum for 30 min at 4°C. Endogenous cdk4-containing complexes were immunoprecipitated for 3 h at 4°C, using a rabbit polyclonal anti-human cdk4 antibody. Immunoprecipitates were washed twice with cdk4 lysis buffer and four times with glutathione S-transferase-RB (GST-RB) kinase buffer [50 mM HEPES (pH 7.5), 10 mM MgCl2, 1 mM dithiothreitol, 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM orthovanadate, 1 mM NaF] and then resuspended in 50 μl of GST-RB kinase buffer. The kinase activity associated with anti-cdk4 immunocomplexes was assessed in...
50 μl of GST-RB kinase buffer containing 2 μg of GST-RB substrate, and in each case supplemented with 2 mM EGTA and 2 μCi of [γ-32P]ATP. Reactions were carried out for 30 min at room temperature, and cold ATP (final concentration, 30 μM) was then added to each reaction mixture to reduce background signal. Reactions were stopped by addition of Laemmli sample buffer, and the reaction products were electrophoresed in 12% SDS-PAGE, whereupon the gels were dried, visualized by autoradiography, and quantitated with a scanning densitometer.

Preparation of nuclear extracts. Nuclear extracts were prepared as in our previous study (16). Briefly, cells were maintained in 5% FBS medium for 1 day. After fasting (0.1% FBS) the cells for 48 h, fresh DMEM with different concentrations of HG or antioxidants was added. After stimulation, cells were harvested and vigorously vortexed. Then, cell lysates were centrifuged and nuclear pellets were resuspended in nuclear extraction buffer. Nuclear proteins were measured for protein by using a Bio-Rad protein assay kit. The extracts were stored at −70°C for further use.

Synthesis of oligodeoxynucleotides. The sequences of phosphorothioate double-stranded oligodeoxynucleotides (ODNs) used in this study were synthesized using a DNA/RNA synthesizer (Applied Biosystems Division, PerkinElmer). STAT1, STAT3, and STAT5 ODNs corresponded to the high-affinity Ly-6E interferon-β site (GAS), the acute-phase response element (APRE) in the rat α2-macroglobulin gene, and the GAS-like element from the β-casein promoter, respectively: STAT1 ODN, 5′-ATATTCCTGTAAAGT-3′, 3′-TATAAGGACATTCA-5′; STAT3 ODN, 5′-GATCCTTGAGATTCCTAGAC-3′, 3′-CTGAGAGGCTTAAATAGTC-5′; STAT5 ODN, 5′-TGCTTCTGGAAATTT-3′, 3′-ACGAAGAACCTTAA-5′ (16). The single-stranded ODNs were annealed for 2 h while the temperature descended from 80 to 25°C.

EMSA. EMSA was performed as in our previous study (15, 16). Briefly, 32P labeling of STAT ODNs was carried out using T4-polyynucleotide kinase and [γ-32P]ATP (3,000 Ci/mmol). Labeled DNA was separated from the unincorporated radioactivity. Binding reactions were carried out by adding 5 μg of nuclear protein to 20 μl of binding buffer and [γ-32P]ATP-labeled STAT ODN probes. Where indicated, cold competitive oligonucleotides were included during the preincubation periods. Samples were incubated at room temperature for 25 min and fractionated by electrophoresis. Following electrophoresis, gels were transferred to 3 MM paper, dried, and exposed to X-ray Hyperfilm-MP at −70°C using an intensifying screen. The results were quantified by a scanning densitometer.

Determination of cytochrome c release from mitochondria. Renal tubular epithelial cells were treated with normal glucose (NG), high mannitol (HM), HG, the antioxidants, and H2O2 for 12 h and harvested from culture. After a washing with cold PBS, cells were incubated in the buffer containing 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 20 μM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Following incubation for 10 min in an ice bath, the cells were homogenized with the homogenizer for 30 strokes, and a buffer containing 200 mM mannitol, 70 mM sucrose, 5 mM EDTA, 5 mM Tris·HCl, pH 7.5, was added to the homogenates. After centrifugation at 1,000 g for 10 min at 4°C, the supernatants were centrifuged at 15,000 g at 1°C for 30 min. The supernatants were collected and used as the cytosol fraction. The cytochrome c level in the cytosol fraction was detected by Western blot analysis.

Statistical analysis. Analysis and graphing of data were performed with Prism 3.0 (GraphPad Software, San Diego, CA). Data are expressed as means ± SE. Statistical analysis was performed by ANOVA for multiple group comparison and by Student’s t-test for direct comparison of two groups. P values <0.01 were considered significant.

RESULTS

HG inhibited cell growth in renal tubular epithelial cells. To investigate whether HG has an effect on cell growth, renal tubular epithelial cells were seeded in culture plates in equal numbers and left to grow to 30–40% confluence in medium containing 5% FBS. Cells were then washed twice with PBS buffer and maintained in medium containing 0.1% FBS. After 48 h, the growth-arrested cells were washed and incubated in serum-free medium for 2 h before the addition of NG, HM, HG, or FBS (5%), after which they were incubated for the indicated time. The MTT assay and cell number analysis showed that HG significantly inhibited cellular mitogenesis compared with NG (100 mg/dl) when the incubation period was 5 days (Fig. 1). Raising the ambient d-glucose concentration causes a dose-dependent decrease in cellular mitogenesis. The inhibitory effect was not mediated by an increase in osmolarity, since raising the osmolarity by the addition of d-mannitol did not significantly decrease cellular mitogenesis.

Effects of NAC and taurine on HG-induced cellular hypertrophy. It is well established that oxidative stress contributes to the pathogenesis of hyperglycemia-induced renal hypertrophy (2, 31, 36, 39, 40). Addition of two different antioxidants, NAC and taurine, with HG was tested to determine whether hyper-
glycemia-induced renal hypertrophy could be prevented. At a cellular level, we further measured quantitatively by assessing the ratio of cell protein to cell number, as hypertrophy is characterized by larger cells with a higher protein content than nonhypertrophic cells. We found that HG significantly increased cellular hypertrophy compared with NG or HM when the incubation time was 5 days (Fig. 2A). Raising NAC and taurine concentration caused a dose-dependent decrease in the hypertrophy index, but the low concentration of NAC (10 μM) did not induce a significant decrease in HG-enhanced cellular hypertrophy. The MTT assay and cell number analysis showed that NAC (100 μM) and taurine (100 μM) suppressed HG-inhibited cell proliferation when the incubation time was 5 days (Fig. 2, B and C). However, these antioxidants were without effects in NG-cultured cells. Additionally, hypertrophy is the result of a greater increase in cell protein than DNA and represents a phenomenon of increasing cell size (17, 25, 29). Thus we measured cell size using forward cell scatter and found that NAC and taurine significantly decreased cell size in HG-cultured cells (Fig. 2D). These results show that the renal tubular hypertrophy induced by HG was probably abolished by both antioxidants.

**Effects of NAC and taurine on HG-mediated JAK/STAT and Raf-1/MAPK activation.** Activation of JAK/STAT and Raf-1/MAPK pathways has been implicated in diverse cellular pro-
cesses, including cell growth, proliferation, hypertrophy, differentiation, and survival (3, 4, 24, 38). To determine whether the antioxidative pathway played a role in HG-mediated signaling cascades, NAC and taurine were used to pretreat renal tubular epithelial cells in JAK/STAT and Raf-1/MAPK activities. We found that HG markedly induced tyrosine phosphorylation of JAK2, STAT1, and STAT3 at 20 min (Figs. 3 and 4). NAC, taurine, and AG-490 (a specific JAK2 inhibitor) significantly reduced phospho-JAK2, phospho-STAT1, and phospho-STAT3 without affecting JAK2, STAT1, and STAT3 protein levels in HG-treated cells. Surprisingly, HG, the antioxidants, and the JAK2 inhibitor did not affect tyrosine phosphorylation.

Fig. 4. Laser densitometry of the gels showed in Fig. 3 and 2 additional phosphorylation experiments. Results are shown for JAK2 (A), STAT1 (B), STAT3 (C), and STAT5 (D), respectively. It is evident that NAC, taurine, and AG-490 significantly decreased HG-induced phosphorylation of JAK2, STAT1, and STAT3 at 20 min. Results are expressed as arbitrary units plotted against time (means ± SE; n = 3). ##p < 0.01 vs. NG. *p < 0.01 vs. HG.

Fig. 5. Effects NAC, taurine, and AG-490 on HG-induced STAT protein-DNA binding activities. Serum-deprived cells were treated with NAC (100 μM), taurine (100 μM), and AG-490 (5 μM) in the presence of HG (500 mg/dl D-glucose) for 20 min. The nuclear extracts were prepared and assayed by EMSA, as described in MATERIALS AND METHODS. A: it was obvious that 20-fold molar excess of unlabeled STAT probes (Comp.) markedly abolished the DNA-protein complexes. Consistent with the results from the phosphorylation assays, treatment with NAC, taurine, and AG-490 inhibited HG-enhanced STAT1 and STAT3 (but not STAT5) protein-DNA binding activities. The concentrations were 100 and 400 mg/dl in NG and HM, respectively. B: laser densitometry of the gels shown in A and 2 additional phosphorylation experiments. Shown is a representative experiment independently performed 3 times. ##p < 0.01 vs. NG. *p < 0.01 vs. HG.
of STAT5. To test whether the inhibition seen at the tyrosine phosphorylation of STAT1 and STAT3 were also observed on protein-DNA binding activities, EMSA was performed. As shown in Fig. 5A, the binding protein complexes were characterized by incubation with a 20-fold molar excess of unlabeled consensus ODNs (Fig. 5A, Comp.). Consistent with the results from the phosphorylation assays, treatment with NAC-, taurine-, and AG-490-inhibiting HG enhanced STAT1 and STAT3 (but not STAT5) protein-DNA binding activities. Moreover, we tested whether the antioxidants regulate Raf-1/ MAPK activity in renal tubular epithelial cells treated with HG. We found that HG markedly increased Raf-1 and p42/p44 MAPK phosphorylation at 30 min compared with NG or HM (Fig. 6). However, HG-enhanced Raf-1 and p42/p44 MAPK phosphorylation were suppressed by NAC, taurine, GW5074 (a specific Raf-1 inhibitor), and PD98059 (a specific MAPK inhibitor). These observations demonstrate that both the JAK2-STAT1/STAT3 and Raf-1/MAPK pathways may play important signal mediators in the HG-mediated biological responses and treatment with antioxidants might be an effective strategy for reducing HG-enhanced activation of the JAK2-STAT1/STAT3 and Raf-1/MAPK signaling cascades.

NAC and taurine blocked effect of HG on cell cycle progression. To gain further insight into the mechanism exerted by antioxidants, we next wished to determine whether the antioxidative effect of NAC/taurine is responsible for inhibition of HG-mediated cell cycle progression. HG-incubated cells were treated for the indicated times with NAC, taurine, AG-490, GW5074, and PD98059 and then examined by flow-cytometric analysis. We found that a significant proportion of the cells remained arrested in the G1 phase after HG treatment for 12 h, whereas control (NG) cells largely entered the S phase (Table 1). Clearly, the antioxidants and specific kinase inhibitors may also have the ability to induce cell cycle progression from HG-treated cells. To investigate the possible role of the antioxidants in cell cycle regulation, we examined the effects of NAC, taurine, AG-490, GW5074, and PD98059 on HG-mediated expression of cell cycle-regulatory molecules and cdk4 kinase activity. Western blot analysis revealed that HG, the antioxidants, and specific kinase inhibitors cannot significantly affect protein expression of c-myc, cyclin D1, and cdk4 (Fig. 7A). However, cdk4 kinase analysis showed that NAC, taurine, and the specific kinase inhibitors markedly reduced HG-inhibited cdk4 kinase activity (Fig. 7B). Interestingly, NAC, taurine, and the specific kinase inhibitors significantly reversed HG-induced expression of p27Kip1 and p21Waf1/Cip1 (Fig. 7A). These results indicated that suppression of cdk4 activation and induction of p27Kip1 and p21Waf1/Cip1 are the underlying mechanisms by HG to promote inhibition of cell cycle progression. Furthermore, the antioxidants negatively regulated HG-retarded cell growth by accelerating G1/S phase transition.

Effects of NAC and taurine on HG-mediated protein production of fibronectin and type IV collagen. The effects of NAC and taurine administration on the accumulation of the ECM proteins fibronectin and type IV collagen were determined by Western blot analysis. As depicted in Fig. 8, A and B, protein expressions of fibronectin and type IV collagen were significantly increased in HG-cultured cells compared with NG. HM treatment had no effect on these ECM protein expressions. The increased expressions of fibronectin and type IV collagen were markedly reduced in the HG-cultured cells treated with NAC, taurine, and the specific kinase inhibitors.

Fig. 6. Effects of NAC, taurine, GW5074, and PD98059 on HG-induced Raf-1 and p42/p44 MAPK phosphorylation. A: total cell lysates from cells treated with NAC (100 μM), taurine (100 μM), GW5074 (10 μM), and PD98059 (10 μM) in the presence of HG (500 mg/dl d-glucose) for 30 min. Proteins were separated by polyacrylamide gels and immunoblotted with anti-phospho-Raf-1 (Ser 338) and anti-phospho-p42/p44 MAPK antibodies (top) or antibodies corresponding to the above antibodies (bottom). B: laser densitometry of the gels shown in A and 2 additional phosphorylation experiments. The concentrations were 100 and 400 mg/dl in NG and HM, respectively. Shown are representative experiments independently performed 3 times. #P < 0.01 vs. NG. *P < 0.01 vs. HG.
These observations were confirmed by enzyme-linked immunosorbent assay of fibronectin and type IV collagen protein expressions (data not shown). Thus we suggest that the JAK-STAT and Raf-1/MAPK pathways may play important roles in the HG-stimulated fibronectin and type IV collagen protein expressions and the antioxidant treatments did alter the HG-enhanced ECM protein productions in renal tubular epithelial cells.

Effect of HG or antioxidants on apoptosis in renal tubular epithelial cells.

To assess whether HG inhibited cell growth through an apoptotic mechanism, we analyzed in detail changes in expression of antiapoptotic and apoptogenic proteins. Table 1 shows the effects of NAC, taurine, AG-490, GW5074, and PD98059 on HG-mediated cell cycle progression in renal tubular epithelial cells.

Table 1. Effects of NAC, taurine, AG-490, GW5074, and PD98059 on HG-mediated cell cycle progression in renal tubular epithelial cells

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<th>Time/Phase</th>
<th>NG</th>
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<th>HG</th>
<th>NAC</th>
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<td>G1</td>
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Values are means ± SE of 3 independent experiments. NG, normal glucose; HM, high mannitol; HG, high glucose; NAC, N-acetylcysteine. Serum-deprived cells were treated with NAC (100 μM), taurine (100 μM), AG-490 (5 μM), GW5074 (10 μM), and PD98059 (10 μM) in the presence of HG (500 mg/dl D-glucose) for the indicated time, and the cell cycle distribution in G1, S, or G2/M phase was analyzed by flow cytometry. NG (100 mg/dl D-glucose) and HM (400 mg/dl D-mannitol) represent cells grown in the absence of added test agents.

Fig. 7. Effects of NAC, taurine, AG-490, GW5074, and PD98059 on HG-regulated expression of cell cycle-regulatory molecules and cdk4 kinase activity. A: total cell lysates from cells treated with NAC (100 μM), taurine (100 μM), GW5074 (10 μM), and PD98059 (10 μM) in the presence of HG (500 mg/dl D-glucose) for 4 h were subjected to Western blot analysis for c-myc, cyclin D1, cdk4, p27Kip1, and p21Waf1/Cip1. B: serum-deprived cells were treated with the same above test agents for 4 h and then assayed for cdk4 kinase activity as described in MATERIALS AND METHODS. The concentrations were 100 and 400 mg/dl in NG and HM, respectively. Shown are representative experiments, each performed at least 3 times. #P < 0.01 vs. NG. *P < 0.01 vs. HG.
teins, notably, bcl-2, PARP, and cytochrome c. As shown in Fig. 9, HG and the antioxidant treatments had no significant effect on bcl-2, PARP, or cytochrome c expression compared with NG or HM at 12 h. However, a pronounced increase in cytosolic cytochrome c and significant PARP cleavage were detected after H2O2 treatment. Furthermore, no differences were observed regarding the lactate dehydrogenase activity between NG and HG cultures in renal tubular epithelial cells (data not shown). Therefore, we suggest that the inhibited cell growth induced by HG suggests that HG may not promote cell death (apoptosis or necrosis) but rather decreases the proliferation rate of cells.

DISCUSSION

The present study shows that in renal tubular epithelial cells, HG acting through the JAK2-STAT1/STAT3 and Raf-1/MAPK pathways, enhances the expression of p27Kip1 and p21Waf1/Cip1 and ECM proteins (fibronectin and type IV collagen). These results support a putative role for the JAK/STAT or Raf-1/MAPK-mediated cell cycle arrest and ECM accumulation in HG-induced cellular hypertrophy. In addition, we suggested that HG-induced cellular hypertrophy was independent of osmolar forces and partly triggered by oxidant stress. Both the antioxidants NAC and taurine significantly prevented HG-induced tubular cell hypertrophy. This important predictor of renal tubular fibrosis is associated with progressive DN, which is concomitantly inhibited by reducing oxidative stress. Thus the JAK2-STAT1/STAT3 and Raf-1/MAPK activations are two parts of the redox-sensitive signaling pathways in renal tubular epithelial cells. Moreover, we probably provide the evidence that both the JAK/STAT and Raf-1/MAPK pathways contribute to HG-induced renal tubular hypertrophy.

Renal hypertrophy in early DN is mainly caused by hyperplasia and/or hypertrophy of mesangial cells and renal tubule epithelial cells (8, 11, 17, 25). The majority of studies focusing on the pathogenic mechanism of HG in promoting renal hypertrophy have highlighted the antiproliferative effects of HG on mesangial and renal tubular cell growth (11, 25, 37). In this study, we showed that inhibition of cdk4 kinase activity and induction of the cyclin-dependant kinase inhibitors p27Kip1 and p21Waf1/Cip1 are thought to play the crucial role in tubular cell cycle arrest. In another study, several factors such as TGF-β1, PDGF, or angiotensin II have been reported to induce the expression of p27Kip1 and p21Waf1/Cip1 and cause cell cycle arrest and hypertrophy in renal tubular epithelial cells (26, 32, 34, 44). Most renal cells, and particularly tubular cells, undergo growth perturbations, characterized by cell cycle arrest at the G0/G1 phase and subsequent hypertrophy (34, 44). These cellular perturbations are accompanied by a progressive accumulation of ECM (fibronectin and type IV collagen) components and contribute to tubulointerstitial fibrosis and a decline in renal function (25, 26, 29, 34, 37).

It is well known that increased oxidative stress is present in diabetes and its complications (2, 40). Diabetic subjects with complications may have a defective cellular antioxidant response against the oxidative stress generated by hyperglycemia, which can predispose the patient to organ damage (6, 11, 19, 31, 36). Several mechanisms have been proposed for the oxidative stress effects during chronic hyperglycemia including accumulation of AGEs, induction of insulin resistance, overproduction of inflammatory cytokines, ROS augmentation, promotion of programmed cell death, and renovascular hypertension (2, 31, 36, 39, 40). The excessive production of ROS induced by metabolic changes in DN, including autooxidation...
and increased advanced glycation, has been investigated for several decades (31, 36, 39). It has recently been reported that the antioxidant status is poor in both glucose intolerance and non-insulin-dependent diabetes, and it is possible that antioxidant therapy mitigates or retards the progress of glucose intolerance (6, 14, 19, 22, 36).

In addition to inducing hypertrophy, other studies in some renal cell lines have shown that HG can induce cell death by apoptosis through mechanisms related to classic apoptosis pathways (1, 5), offering a possible explanation of the mechanism for renal atrophy observed in advanced stages of DN. However, it is still unknown whether NAC or taurine can play a role as a protective agent in apoptosis induced by oxidative stress in tubular cells. It is now well known that cytochrome c release by mitochondria and caspase activation are critical events in triggering oxidative stress-mediated apoptosis (13). The nuclear DNA repair enzyme PARP is a target of caspase-3, and its cleavage can serve as a biochemical marker of apoptosis (42). Interestingly, our results demonstrated that exposure of renal tubular epithelial cells to HG cannot trigger these apoptotic signaling events compared with cells treated with H₂O₂. On the other hand, several antioxidative mechanisms are utilized by the induction of bcl-2 to interrupt transmission of death signals and inhibition of cytochrome c release from mitochondria (30, 43). Nevertheless, we found that NAC and taurine did not alter the bcl-2 expression and cytochrome c release in these cells. Thus it is possible that hyperglycemia selectively triggers apoptosis in renal tubular epithelial cells.

NAC, a glutathione precursor and thiol-containing radical scavenger, as well as several endogenous antioxidants may prevent the progression of DN by improving the impaired renal hemodynamics and function (19, 20), but its effects on renal tubulointerstitial fibrosis remain to be elucidated. In this study, we found that in renal tubular epithelial cells, HG activated JAK-STAT or Raf-1-MAPK signaling and subsequently suppressed cell cycle progression and induced cellular hypertrophy, which was completely prevented by NAC. Recent experiments using rats on a sucrose-rich diet have shown that NAC offers promising therapeutic values in prevention of the dyslipidemic profile and alleviation of hyperglycemia in high-sucrose intake conditions by improving antioxidant defenses (7). It has been shown that NAC effectively protects from hyperglycemia-induced myocyte cell death and compensatory hypertrophy through direct scavenging of ROS and replenishment of intracellular glutathione content (9). In cultured mesangial cells and in diabetic glomeruli, ROS mediate HG-induced upregulation of plasminogen activator inhibitor-1 expression (20). NAC can prevent accumulation of ECM protein in diabetic glomeruli partly by abrogating upregulation of plasminogen activator inhibitor-1 and suppression of plasmin activity. Furthermore, incubation of human aortic endothelial cells with HG in the presence of NAC prevented the drop in intracellular glutathione content and decreased both ROS generation and the number of cells committed to apoptosis (35).
In our study, treatment with the potent free radical scavenger taurine, as well as with NAC, significantly prevented HG-induced renal tubular hypertrophy. Considerable evidence shows that taurine acts as a major scavenger of endogenously generated HOCl, a highly toxic molecule (6, 19). Taurine chloride, formed from the reaction of taurine with HOCl, has been shown to act as a cellular signaling molecule that can downregulate the expression of a number of mediators involved in tubulointerstitial injury (6, 10). Recently, it has been reported that taurine significantly reduced plasma lipid peroxide, glomerular plasminogen activator inhibitor-1 expression, glomerular volume, fractional mesangial area, and proteinuria in streptozotocin-induced diabetic rats (6, 10, 14, 19, 22). In addition, taurine can attenuate hyperglycemia-induced human umbilical endothelial cell apoptosis through ROS inhibition and intracellular Ca2+ concentration stabilization, which suggests that taurine may exert a beneficial effect in preventing diabetes-associated microangiopathy (45). Moreover, in human renal tubule cells, taurine attenuates hyperglycemia-induced apoptosis via an inhibition of oxidative stress (41). In the present study, HG-induced ECM overproduction and the delay in cell cycle progression as well as the upregulation of JAK-STAT or Raf-1-MAPK signaling in renal tubular epithelial cells were found to be completely prevented by taurine. Taken together, these results appear to suggest that taurine might act as an endogenous antioxidant in renal tubular epithelial cells and could exert a beneficial effect in preventing renal tubular fibrosis in DN.

In summary, we would like to propose the possible molecular mechanism of antioxidants on HG-mediated renal tubular hypertrophy, as shown in Fig. 10. In particular, there are evidences that NAC and taurine reduce HG-inhibited cell cycle progression and HG-induced ECM accumulation, probably via the effects on the JAK2-STAT1/STAT3 and Raf-1-MAPK signaling pathways. Thus we suggest that the altered antioxidative activity plays an important role in the pathogenesis of renal hypertrophy and suggest novel means of modulating hyperglycemia-dependent renal tubular fibrosis.

GRANTS

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