Effects of Nitric Oxide and Antioxidants on Advanced Glycation End Products-Induced Hypertrophic Growth in Human Renal Tubular Cells

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The accumulation of advanced glycation end products (AGE) is a key mediator of renal tubular hypertrophy in diabetic nephropathy (DN). Reactive oxygen species and nitric oxide (NO) were involved in the progression of DN. In this study, the molecular mechanisms of NO and antioxidants responsible for inhibition of AGE-induced renal tubular hypertrophy were examined. We found that AGE (but not nonglycated bovine serum albumin) significantly suppressed the NO/cGMP/PKG signaling in human renal proximal tubular cells. NO donors S-nitroso-N-acetylpenicillamine (SNAP)/sodium nitroprusside (SNP) and antioxidants N-acetylcysteine (NAC)/taurine treatments significantly attenuated AGE-inhibited NO production, cGMP synthesis, and inducible NO synthase/cGMP-dependent protein kinase (PKG) activation. Moreover, AGE-induced extracellular signal-regulated kinase (ERK)/p38 mitogen-activated protein kinase activation was markedly blocked by antireceptor for AGE (RAGE), SNAP, SNP, NAC, and taurine. The abilities of NO and antioxidants to inhibit AGE/RAGE–induced hypertrophic growth were verified by the observation that SNAP, SNP, NAC, and taurine inhibited fibronectin, p21Waf1/Cip1, and RAGE expression. Therefore, antioxidants significantly attenuated AGE/RAGE-enhanced cellular hypertrophy partly through induction of the NO/cGMP/PKG signaling.

Key Words: nitric oxide; antioxidant; advanced glycation end products; hypertrophy; renal proximal tubular cells.

Diabetic nephropathy (DN) and hypertension frequently coexist, leading to additive increases in the risk of life-threatening end-stage renal disease in the world (Giunti et al., 2006; Schutta, 2007). Two key mediators implicated in the development of DN include high glucose and advanced glycation end products (AGE) (Fukami et al., 2007; Sato et al., 2006; Tan et al., 2007). AGE arise from glucose-derived Amadori (1-deoxy-d-fructosyl derivatives) products and act to increase oxidative stress, elevate vascular permeability, enhance protein and lipoprotein deposition, promote extracellular matrix (ECM) protein synthesis, and tubulointerstitial fibrosis, and exert a number of toxic effects of renal cells (Bohlender et al., 2005; Sato et al., 2006; Tan et al., 2007). AGE can mediate their effects via specific receptors, such as the receptor for AGE (RAGE), activating diverse signal transduction cascades, and downstream pathways, including generation of generalized cellular dysfunction and induction of reactive oxygen species (ROS) (Bohlender et al., 2005; Sato et al., 2006; Tan et al., 2007).

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 (Da Ros et al., 2004; Niedowicz and Daleke, 2005). N-acetylcysteine (NAC), a thiol-containing radical scavenger and glutathione precursor, has been found to protect β-cells in culture and in vivo from “glucose toxicity,” preserving insulin synthesis and secretion (Haber et al., 2003). AGE-RAGE–mediated ROS generation induces mesangial cell hypertrophy, and fibronectin synthesis was inhibited by NAC (Fukami et al., 2004). The antioxidant properties of taurine, an amino acid abundant in human tissues, are ascribed to its scavenging activity on hypochlorous acid a highly toxic molecule (Verzola et al., 2002). Taurine might also act as an antiglycative compound, providing free amino groups that may compete for the reducing sugars. Furthermore, a high concentration of taurine regulates many cellular functions, including cell growth, homeostasis, protein stabilization, apoptosis, calcium mobilization, and immunity (Da Ros et al., 2004; Haber et al., 2003; Niedowicz and Daleke, 2005; Verzola et al., 2002).

The gaseous molecule nitric oxide (NO) modulates a large variety of physiological functions and initiates diverse cellular signaling cascades which comprise nitrosylation of proteins, adenosine 5′-diphosphate (ADP)-ribosylation, or stimulation of soluble guanylyl cyclases which catalyze intracellular guanosine 3′,5′-cyclic monophosphate (cGMP) synthesis (Cerra and Pellegrino, 2007). cGMP activates cGMP-dependent protein kinases (PKG) which mediate localized and global signaling. Considerable evidence suggests that...
advanced DN leading to severe proteinuria, declining renal function, and hypertension is associated with a state of progressive NO deficiency (Cerra and Pellegrino, 2007; Nakagawa, 2007). Several factors including AGE, hyperglycemia, ROS, as well as activation of janus kinase/signal transducers and activators of transcription and transforming growth factor-β1 (TGF-β1) contribute to decrease NO production and/or availability (Freire-de-Lima et al., 2006; Huang et al., 2005; Nakagawa, 2007; Wilcox, 2005). Interestingly, NO may exert destructive effects (hyperperfusion, peroxynitrite-mediated tissue injury) as well as exhibit certain protective properties (reduced TGF-β expression and ECM expansion) (Levine, 2006; Nakagawa, 2007; Wilcox, 2005).

Therefore, in this study, we examined the molecular mechanisms of antioxidants and NO/PGK activation responsible for inhibition of AGE-induced cellular hypertrophy. Our objective was to verify the following: (1) the effects of nonglycated bovine serum albumin (BSA) and AGE on generation of NO and cGMP and PKG activation in human renal proximal tubular cells; (2) whether NO donors and antioxidants affected AGE-mediated the inducible NO synthase (iNOS)/NO/cGMP/PGK signaling; (3) whether NO donors and antioxidants attenuated AGE/RAGE-enhanced activation of the extracellular signal-regulated kinase (ERK)/c-Jun N-terminal kinase (JNK)/p38 mitogen-activated protein kinase (MAPK) pathway; and (4) the roles of NO and antioxidants in AGE-induced RAGE/ECM/p21Waf1/Cip1 synthesis and hypertrophic growth.

MATERIALS AND METHODS

Materials. Fetal bovine serum (FBS), Dulbecco’s Modified Eagle’s Medium (DMEM)/F12, molecular weight standards, antibiotics, trypsin-EDTA, trypan blue stain, and all medium additives were obtained from Life Technologies (Gaithersburg, MD). Anti-iNOS, JNK, -p38, -RAGE, -p42/p44 MAPK, and -p21Waf1/Cip1 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-JNK, -phospho-p38, and -p42/p44 MAPK antibodies were obtained from Upstate Biotechnology, Inc. (Charlottesville, VA). SNAP, SNP, KT5823, 8-pCPT-cGMPs, and anti-PKG-I antibody were purchased from Calbiochem (La Jolla, CA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse secondary antibody, streptavidin-peroxidase, and the enhanced chemiluminescence kit (ECL) system were purchased from Bio-Rad Laboratories (Hercules, CA). BSA, NAC, taurine, d-glucose, dimethyl sulfoxide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay kit, anti-β-actin antibody, anti-fibronectin antibody, and all other chemicals were purchased from Sigma-Aldrich Chemical (St Louis, MO).

Culture conditions. Human renal proximal tubular cells (HK-2) were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in culture flasks (Nunclo, Denmark) and maintained in DMEM/F12 (5.5mM D-glucose) supplemented with 100 i.u./ml penicillin, 100 μg/ml streptomycin, and 5% FBS in a humidified 5% CO2 incubator at 37°C. Cell viability was assessed by the trypan blue exclusion test and was routinely more than 95%. For cell number analysis, cells were cultured in six-well culture plates (Nunclo) and grown in the added test agents. Cells were harvested and counted with a hemocytometer. Each experimental data point represents the mean of duplicate wells from three independent experiments.

Preparation of AGE. AGE-BSA was prepared according to standard procedures. Twenty percent (wt/vol) fatty acid-free BSA was incubated at 37°C under sterile phosphate-buffered saline (PBS), pH 7.2, supplemented with 250mM glucose-6-phosphate, 100 μg/ml gentamycin, and 10 μg/ml ceftriaxone for 8 weeks. Unincorporated glucose-6-phosphate and antibiotics were then removed by dialysis against PBS. Control nonglycated BSA was incubated in the same conditions with the exception of the absence of glucose-6-phosphate. The diazylated preparations were sterile filtered through 0.2-μm nylon filters. Preparations were tested for endotoxin using Endospecy ES-20S system (Seikagaku Co., Tokyo, Japan) where no endotoxin was detectable. The AGE content of the preparations was determined spectrophotometrically with excitation set at 390 nm and emission set at 450 nm, and expressed as the percentage of relative fluorescence compared with nonincubated aliquots of the same batch of BSA in glucose-6-phosphate solution, stored frozen immediately after preparation.

NO analysis. NO synthesis was determined by measuring the accumulation of nitrite (NO2-), a stable metabolite of NO, in culture supernatants using the Griess reaction. Briefly, cells were plated in each well of a 96-well plate in DMEM/F12 medium with 5% FBS and medium were deproteinized prior to assay. After being passed through 25-kDa ultrafilters, 20 μl of the medium was diluted with 120 μl of assay buffer and mixed with 5 μl of cofactor and 5 μl of nitrate reductase (NO colorimetric assay kit, Calbiochem). After the medium had been kept at room temperature for 2 h to convert nitrate to nitrite, total nitrite was measured at 540 nm absorbance by reaction with Griess reagent (sulfanilamide and naphthalene-ethylene diamine dihydrochloride). Amounts of nitrite in the medium were estimated by a standard curve obtained from enzymatic conversion of NaNO2 to nitrite. The detection limit of the method is 0.2μM and the assay was reliable and reproducible with interassay and intra-assay variation coefficients of 3.5 and 4.2%, respectively.

Cellular hypertrophy analysis. Cells were grown in six-well plates until approximately 50% confluent and then made quiescent for 2 days in serum-free DMEM/F12 medium. The cultures were then treated with BSA, AGE, or AGE plus various agents, after which the cells were trypsinized, washed twice with phosphate-buffered saline (PBS) and counted using a hemocytometer. Equal numbers of cells were lysed in buffer (0.1% [wt/vol] SDS, 0.5% [wt/vol] sodium deoxycholate, 1.0% [wt/vol] sodium dodecyl sulfate, 40 μM PMSF, and 1 mg/ml protease inhibitors, pH 8.0 in PBS). The total protein content was measured using the Bio-Rad protein assay kit. Total protein was expressed as micrograms of protein per 106 cells.

Western blot analysis. For protein analysis, serum-deprived cells were treated with indicated agents or AGE as described above. Total cell lysates were harvested, resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to Protran membranes (0.45 μm, Schiecher & Schuell, Keene, NH). The membranes were blocked in blocking solution and subsequently probed with primary antibodies. The membrane was incubated in 4000× diluted HRP-conjugated goat anti-rabbit or anti-mouse secondary antibody. The protein bands were detected using the enhanced chemiluminescence (ECL) system, and the percentage of phosphorylated form of protein was determined using a scanning densitometer. For the ERK/JNK/p38 MAPK activation assay, proteins were resolved by SDS-PAGE and transferred to Protran membranes. The membranes were probed with anti-phospho-ERK, anti-phospho-JNK, anti-phospho-p38 MAPK, anti-ERK, anti-JNK, and anti-p38 MAPK antibodies. Immunoreactive proteins were detected with the ECL system as described above.

Measurement of cGMP production. Cells were grown in 24-well plates until approximately 70% confluent. Sixteen hours prior to stimulation, culture media were replaced by serum-free DMEM/F12 supplemented with the specific inhibitors or other drugs. Basal cGMP level was checked in the wells to which a corresponding volume of incubation medium without activators was added. Reaction was terminated by adding 10% (vol/vol) ice-cold lysis buffer from the
direct cGMP ELISA kit (Assay Designs, Ann Arbor, MI) according to the manufacturer’s instructions. Briefly, cells were lysed using 1 ml of 0.1M HCl for 10–15 min after removal of the medium. The acetylated version was conducted to detect low levels of cGMP. Measurements were performed in triplicate.

**Expression of PKG and assay of PKG activity.** Cells were treated with AGE or agents as described above. After rinsing the monolayer with PBS, cells were harvested and homogenized with cold buffer consisting of 20mM sodium phosphate, pH 6.8, 2mM EDTA, 150mM NaCl, 2mM benzamidine, 0.1mM phenylmethylsulfonyl fluoride, 10 µg/ml pepstatin A, and 10 µg/ml leupeptin. The suspension was centrifuged for 10 min at 18,000 g to obtain cell extract. Aliquots of extract were analyzed for PKG activity and also for Western blotting with an affinity-purified polyclonal rabbit anti-PKG-I antibody. The anti-PKG-I polyclonal antibody detects the activation forms of both PKG-Iα and PKG-Iβ. To measure the PKG activity, cells were homogenized in a buffer containing 50mM Tris-HCl (pH 7.4), 10mM EDTA, 2mM dithiotreitol, 1mM isobutylmethylxanthine, 100µM nitro-L-arginine, and 10µM indomethacin. The homogenate was sonicated and centrifuged at 13,000 x g for 10 min at 4°C. In brief, PKG activity was assayed with a peptide substrate (RKISASEFDRPL) selective for PKG. The difference in the phosphorylation of substrate in the presence and absence of cGMP was taken as PKG activity.

**MTT assay.** MTT assays were performed to evaluate the proliferation of renal proximal tubular cells. Cells were grown in 96-well plates until approximately 50% confluent and made quiescent for 2 days in serum-free DMEM/F12 medium. Then various concentrations of each drug were added to the wells. After treatments, 10 µl of sterile MTT dye was added to each well, and the cells were incubated for 6 h at 37°C. Then 100 µl of acidic isopropanol (0.04M HCl in isopropanol) was added and thoroughly mixed. Spectrometric absorbance at 595 nm (for formazan dye) was measured with the absorbance at 655 nm for reference.

**Flow-cytometric analysis.** Relative cell size and DNA content were assessed by flow-cytometric analysis. 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% BSA. 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 greater than 90% in each experiment. Forward side scatter was expressed as arbitrary units, and the histogram of the mean average cell size is shown.

**Statistical analysis.** Analysis and graphing of data were performed with Prism 3.0 (GraphPad Software, San Diego, CA). Data are expressed as means ± SEM. Statistical analysis was performed by ANOVA for multiple group comparison and by Student’s t-test for direct comparison of two groups. p values < 0.05 were considered significant.

**RESULTS**

**Effects of NO Donors and Antioxidants on AGE-modulated NO Generation and iNOS Expression**

To investigate if AGE could affect NO generation in renal proximal tubular cells, we first treated these cells with AGE and BSA and analyzed endogenously NO generation by Griess method. We found that AGE significantly decreased nitrite production compared with nonglycated BSA or control when the incubation period was for 12 h (Fig. 1A). Raising the ambient AGE concentration causes a dose-dependent decrease in nitrite production. NAC and taurine were used to determine whether AGE-suppressed NO generation could be prevented by antioxidants. Interestingly, we found that enhancing SNAP, SNP, NAC, and taurine concentration caused dose-dependent increase in NO generation. Moreover, we found that SNAP (10µM), SNP (10µM), NAC (100µM), and taurine (100µM) markedly reversed AGE (300 µg/ml) inhibited iNOS protein expression (Fig. 1B) at 12 h. We also examined whether HK-2 cells express other NOS isoenzymes and found that the amount of endothelial NOS (eNOS) or neuronal NOS (nNOS) in the cells is very little. AGE (300 µg/ml) slightly inhibited the expression of eNOS or nNOS when compared with BSA (data not shown). In addition, treatment of cells with SNAP, SNP, NAC, or taurine alone did not alter NO generation and iNOS expression regardless of time course (data not shown). Together, these results suggest that antioxidants inhibit AGE-mediated effects maybe involve the iNOS/NO-dependent mechanism.

**Effects of NO Donors and Antioxidants on AGE-mediated cGMP Synthesis**

It is well established that NO stimulates the synthesis of the second messenger cGMP, which in turn regulates various cellular functions by activating downstream targets including PKG (Cerra and Pellegrino, 2007; Levine, 2006). We then examined whether AGE could prevent cGMP synthesis. First, AGE significantly decreased cGMP synthesis when compared with nonglycated BSA or control (Fig. 2). Next, we found that SNAP, SNP, NAC, and taurine markedly reversed AGE-inhibited cGMP synthesis. However, treatment of cells with SNAP, SNP, NAC, or taurine alone did not alter cGMP expression regardless of time course (data not shown).

**Effects of NO Donors and Antioxidants on AGE-Modulated PKG Activation**

Recent literature suggests that cytokine-induced increase in iNOS expression causes the subsequent production of pathophysiological high levels of NO and PKG (Cerra and Pellegrino, 2007; Levine, 2006; Wilcox, 2005). Thus, the effects of antioxidants on AGE-modulated PKG activation were examined. We found that SNAP, SNP, NAC, and taurine markedly reversed AGE (300 µg/ml)–inhibited PKG protein expression (Fig. 3A) and activity (Fig. 3B) of PKG at 4 h. Figure 3 also illustrates that the PKG inhibitor KT5823 (10 µM) slightly enhanced AGE-inhibited PKG activation. The PKG activator 8-pCPT-cGMPS (10µM) markedly reversed AGE-inhibited PKG activation. Treatment of cells with SNAP, SNP, NAC, or taurine alone did not alter PKG expression and PKG activation regardless of time course (data not shown). Taken together, these results suggest that sustained NO generation was regulated by persistent activation of PKG and antioxidants inhibited AGE-mediated effects that may involve the NO/cGMP/PKG-dependent mechanism.
Effects of Anti-RAGE, NO Donors, and Antioxidants on AGE-Mediated ERK/JNK/p38 MAPK Activation

To determine if the NO-PKG pathway and antioxidative activity played roles in AGE-mediated signaling cascades, NO donors and antioxidants were used to treat AGE-cultured cells for ERK/JNK/p38 MAPK activity assay. After exposure of cultured cells to AGE and nonglycated BSA, we found that AGE (but not BSA) markedly induced phosphorylation of ERK, JNK, and p38 at 4 h (Fig. 4). Interestingly, anti-RAGE, SNAP, SNP, NAC, and taurine significantly reduced phospho-ERK, phospho-JNK, and phospho-p38 without obviously affecting ERK, JNK, and p38 protein levels in AGE-treated cells. These observations demonstrate that the ERK/JNK/p38 MAPK may play important signal mediators in the AGE/RAGE–induced biological responses. Stimulating the NO/PKG pathway and antioxidative activity may inhibit AGE/RAGE-enhanced the ERK/JNK/p38 MAPK pathway.

Effects of Anti-RAGE, NO Donors, and Antioxidants on AGE-Mediated Protein Production of Fibronectin, p21Waf1/Cip1, and RAGE

Several lines of evidence indicate that ECM (e.g., fibronectin, collagen) and cyclin-dependent kinase inhibitors (e.g., p21Waf1/Cip1) are required for the development of growth factors/cytokines–mediated hypertrophy, but interference with the expression of one of these proteins may attenuate hypertrophy. AGE and its signal transducing receptor (RAGE) are implicated in the pathogenesis of diabetic vascular complications. To annex a better understanding of mechanisms involved in AGE/RAGE–induced renal proximal tubular hypertrophy, we further examined the effects of anti-RAGE, NO donors, and antioxidants on AGE-mediated fibronectin, p21Waf1/Cip1, and RAGE protein synthesis. As depicted in Figure 5, the de novo protein syntheses of fibronectin, p21Waf1/Cip1, and RAGE were significantly increased in AGE-cultured renal proximal tubular cells as compared with control
Results were expressed as the mean assayed for cGMP synthesis as described under "Materials and Methods." Serum-deprived cells were treated with 5% FBS (control), BSA (300 μg/ml), AGE (300 μg/ml), and SNAP (10μM), SNP (10μM), NAC (100μM), and taurine (100μM) in the presence of AGE for 0, 1, 2, 4, 8, and 12 h, and then assayed for cGMP synthesis as described under "Materials and Methods." Results were expressed as the mean ± SEM (n = 4). *p < 0.05 versus control; #p < 0.05 versus AGE.

(5% FBS) or nonglycated BSA when the incubation period was for 3 days. Anti-RAGE, SNAP, SNP, NAC, taurine markedly reversed AGE-induced these effects. Altogether, these results suggest that NO and antioxidants inhibit AGE/RAGE–induced cellular hypertrophy through similar mechanisms, partly by decreasing ERK/JNK/p38 MAPK activities and fibronectin/p21Waf1/Cip1/RAGE synthesis. Thus, these findings indicate that the first time that antioxidants inhibit AGE/RAGE–induced hypertrophic effect at least partly by increasing NO/cGMP synthesis and iNOS/PKG activation in renal tubular cells. NO donors and antioxidants can potentially act as antihypertrophy agents.

DISCUSSION

We demonstrated in the present studies that inhibition of the NO/cGMP synthesis and the iNOS/PKG activity by AGE-induced renal cellular hypertrophy was associated with induction of the ERK/JNK/p38 MAPK pathway. NO donors (SNAP and SNP) and antioxidant (NAC and taurine) treatments significantly attenuated AGE-inhibited NO/cGMP/PKG signaling and AGE/RAGE–enhanced the ERK/JNK/p38 MAPK activation. Furthermore, the abilities of NO and antioxidants to inhibit AGE/RAGE–induced renal cellular hypertrophy was verified by the observation that SNAP, SNP, NAC, and taurine inhibited hypertrophic growth and fibronectin/p21Waf1/Cip1/RAGE synthesis. Thus, these findings indicate the first time that antioxidants inhibit AGE/RAGE–induced hypertrophic effect at least partly by increasing NO/cGMP synthesis and iNOS/PKG activation in renal tubular cells. NO donors and antioxidants can potentially act as antihypertrophy agents.

DN is increasing dramatically worldwide and is now the most common cause of end-stage renal failure requiring renal replacement therapy (Fukami et al., 2007; Schutta, 2007; Verzola et al., 2002). Its molecular pathogenesis is thus becoming a target of active research. There is a growing body of evidence to show that the formation and accumulation of AGE are involved in the development and progression of glomerulosclerosis and tubulointerstitial fibrosis in patients with diabetes (Bohlender et al., 2005; Sato et al., 2006; Tan et al., 2007). Recent studies have been indicated on the critical roles of AGE, that is, AGE alter the function and structure of tissue matrix proteins, and AGE-modified proteins trigger a variety of cellular responses through a specific cell surface receptor, resulting in the expression and activation of pathogenic mediators, for example, oxidative stress, ECM, and TGF-β1, implicated in the development and induction of DN (Bohlender et al., 2005; Cameron et al., 2005; Ha and Lee, 2005; Sato et al., 2006). NO may serve an important role in reducing renal fibrosis by modulating hypertrophic interactions between renal tubular cells, fibroblasts, and mesangial cells (Levine, 2006; Nakagawa, 2007; Wilcox, 2005). AGE-modified albumin rapidly decreases NOS activity in human umbilical vein endothelial cells and giving rise to a decrease in serine phosphorylation of eNOS (Xu et al., 2005). AGE-RAGE
interaction also elicits angiogenesis via the transcriptional activation of the VEGF gene that can be prevented by aldose reductase inhibitors (Bandello et al., 2003; Yamamoto et al., 2007). However, this study demonstrated that AGE-decreased NO generation and NOS activity may not mediate by the TGF-β1–dependent pathway. On the other hand, hyperglycemia is associated with a decrease in NO production and the loss of NO-mediated repression of aldose reductase is a significant factor in the activation of the polyol pathway and the development of several diabetic complications (Forbes et al., 2007; Schrijvers et al., 2004). Moreover, increase in NO generation may be a compensatory phenomenon in response to enhanced oxidative stress induced by hyperglycemia because inhibiting NOS, scavenging superoxide, or supplementing the NOS cofactor tetrahydrobiopterin blocked the formation of ROS (Cai et al., 2005; Miyazaki-Akita et al., 2007; Prabhakar, 2001).

It has been previously observed that blockade of oxidative stress in mice or rats with diabetes reduced CDK inhibitor p21Waf1/Cip1 levels and improved renal function (Da Ros et al., 2004; Haber et al., 2003; Wolf, 2000). Because p21Waf1/Cip1 induces cell cycle arrest and hypertrophic growth, which augment ECM production and tubulointerstitial fibrosis, pharmacological reduction of ROS by antioxidants may also exert its beneficial effects in DN by downregulation of renal p21Waf1/Cip1. NO and/or cGMP modulate mesangial cell function and in particular inhibit fibronectin production in response to a thromboxane analog (Studer et al., 1996). Treatment of mesangial cells with lipopolysaccharide, which activate the iNOS, inhibits synthesis of collagens and fibronectin and this effect is blocked by the NOS inhibitor N-nitro-l-arginine methyl ester (Noh et al., 2002). In this study, we found that treatment with AGE

FIG. 3. Effects of antioxidants and NO donors on AGE-mediated PKG-I protein synthesis and PKG activity. Total cell lysates from cells treated with SNAP (10μM), SNP (10μM), NAC (100μM), taurine (100μM), KT5823 (10μM), and 8-pCPT-cGMPs (10μM) in the presence of AGE (300 μg/ml) for 4 h were subjected to analysis for PKG-I protein synthesis (A) PKG activity (B) as described under “Materials and Methods.” The concentration of BSA was 300 μg/ml. These are representative experiments, each performed at least four times. *p < 0.05 versus control; #p < 0.05 versus AGE.
FIG. 4. Effects of anti-RAGE, NO donors, and antioxidants on AGE-mediated phosphorylation of ERK, JNK, and p38 MAPK. (A) Total cell lysates from cells treated with anti-RAGE (50 µg/ml), SNAP (10µM), SNP (10µM), NAC (100µM), and taurine (100µM) in the presence of AGE (300 µg/ml) for 4 h were subjected to Western blot analysis for phospho-p42/p44 MAPK, phospho-JNK, and phospho-p38 MAPK (upper panel) or proteins corresponding to the above phosphorylated proteins (lower panel). (B) Laser densitometry of the gels shown in (A) and two additional phosphorylation experiments. The concentration of BSA was 300 µg/ml. This is a representative experiment independently performed four times. *p < 0.05 versus control; #p < 0.05 versus AGE.
leads not only to iNOS/PKG inactivation but also to renal cellular hypertrophy. NO induction could effectively suppress AGE-induced the ERK/JNK/p38 MAPK pathway and fibronectin/p21Waf1/Cip1 synthesis. Thus, there is reason to believe that impaired NO production plays a role in cellular hypertrophy of diabetic kidney.

There is a growing body of evidence to show that AGE is a positive regulator of cell expression of RAGE (Ann et al., 1999; Sho-ichi et al., 2006; Thorsten et al., 2007). AGE can activate the RAGE gene through nuclear factor kappa B (NF-kB) and Sp1, causing enhanced AGE-RAGE interactions, which would lead to an exacerbation of diabetic

FIG. 5.  Effects of anti-RAGE, NO donors, and antioxidants on AGE-mediated synthesis of fibronectin, p21Waf1/Cip1, and RAGE. (A) Total cell lysates from cells treated with anti-RAGE (50 μg/ml), SNAP (10μM), SNP (10μM), NAC (100μM), and taurine (100μM) in the presence of AGE (300 μg/ml) for 3 days were subjected to Western blot analysis for fibronectin, p21Waf1/Cip1, and RAGE. (B) Laser densitometry of the gels showed in (A) and two additional experiments. The concentration of BSA was 300 μg/ml. This is a representative experiment independently performed four times. *p < 0.05 versus control; #p < 0.05 versus AGE.
microvasculopathy (Nobushige et al., 1995). In human renal tubular cells, we found that AGE markedly induced RAGE synthesis and that NO donor or antioxidant significantly reversed this AGE-induced effect. Although the molecular mechanism underlying NO inhibition of AGE-induced RAGE expression is still incompletely understood, NO is already known to inhibit NF-κB activation (De Caterina et al., 1995; Peng et al., 1995). In addition, Sp1 is also inhibited by incubation with NO donor (Berendji-Grün et al., 2001). Therefore, the inhibition of NF-κB and/or Sp1 transcription factor(s) by NO donor is implicated in the inhibition of AGE-induced RAGE upregulation.

Increased oxidative stress and reduced NO bioactivity are key features of diabetes mellitus that eventually result in DN (Levine, 2006; Sato et al., 2006; Tan et al., 2007; Wilcox, 2005). Several studies have suggested that AGE stimulates production of ROS, including superoxide anions, hydrogen peroxide, and hydroxyl radicals, in a variety of cell types (Bohlender et al., 2005; Da Ros et al., 2004; Niedowicz and Daleke, 2005; Sato et al., 2006; Tan et al., 2007). NAC and taurine are antioxidants that can increase the intracellular concentration of glutathione and augment cellular antioxidant capacity and can also directly scavenge ROS (Da Ros et al., 2004; Haber et al., 2003; Niedowicz and Daleke, 2005). Alternatively, NO may directly affect protein kinases and phosphatases that regulate cell cycle progression, hypertrophic growth, and apoptosis. One attractive speculation is that iNOS expression by renal tubular cells or interstitial fibroblasts would produce relatively large amounts of NO, implicating an autoprotective regulatory mechanism (Bohlender et al., 2005; Da Ros et al., 2004; Freire-de-Lima et al., 2006; Huang et al., 2005; Levine, 2006; Niedowicz and Daleke, 2005; Wilcox, 2005). Previous studies have reported that antioxidants could prevent the hyperglycemia-induced increase in oxidative stress, restore NO availability, and prevent depression of arterial blood pressure and heart rate in vivo in experimental diabetes (Xia et al., 2006). Interestingly, this study showed that antioxidants NAC and taurine significantly attenuated AGE-induced cellular hypertrophy in renal tubular cells. NAC and taurine restored reduced NO generation and attenuated reductions in iNOS and PKG activities caused by AGE. However, treatment of cells with SNAP, SNP, NAC, or taurine alone did not alter the expression of iNOS, NO, cGMP, and PKG regardless of time course.

NAC is a thiol-containing antioxidant. It may be emphasized that the role of thiol group is responsible for the stabilization and prolongation of NO half-life potentiating its renoprotective effect. In support of the suggestion that NO production was increased is the in vitro observation that NAC can induce NOS expression (Jiang et al., 1999). In addition to increasing NO production, NAC can also potentiate the hemodynamic effects of NO by binding to it and forming a more stable substance, as evident from human studies where NAC treatment significantly enhanced endothelium-dependent vasodilation (Zhou et al., 2002). Moreover, animal studies showed that NAC treatment had a protective effect in models involving increased oxidative stress such as renal failure and nephrotoxicity (Conesa, 2001;
Dimari, 1997; Salom, 1998). On the other hand, there is increasing evidence that taurine enhances NO generation. Increased plasma concentrations of chenodeoxycholic acid (free, taurine, and glycine conjugates) in patients with hepatobiliary diseases may induce endothelial NO production (Chisaki et al., 2001). Taurine effectively improves metabolism in OLETF rats by decreasing serum cholesterol and triacylglycerol, presumably via increased secretion of cholesterol into bile acid and decreased production of cholesterol because of increased NO production (Nakaya et al., 2000). Moreover, taurocholate exposure did not affect epithelial cell viability, but it increased iNOS expression, NO production, and epithelial permeability (Mustonen et al., 2008).

In summary, our studies demonstrate that NO donors and antioxidants are capable of regulating both the ERK/JNK/p38 MAPK pathway and ECM synthesis in renal tubular cells stimulated by AGE, possibly in part through increasing the activation of NO/cGMP/PKG pathway. These results may provide a cellular and molecular insight into the treatment of renal tubular hypertrophy using NO pathway modulators or antioxidant agents.

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