Cinnamaldehyde and Nitric Oxide Attenuate Advanced Glycation End Products–Induced the JAK/STAT Signaling in Human Renal Tubular Cells

Jau-Shyang Huang,[1] Ying-Ho Lee,[1,2] Lea-Yea Chuang,[3] Jinn-Yuh Guh,[4] and Jean-Yu Hwang[5]*

[1]*Department of Biological Science and Technology, Chung Hwa University of Medical Technology, Tainan, Taiwan*

[2]*Department of Pharmacy, Kaohsiung Veterans General Hospital Tainan Branch, Tainan, Taiwan*

[3]*Department of Biochemistry, Kaohsiung Medical University, Kaohsiung, Taiwan*

[4]*Department of Internal Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*

[5]*Department of Food Science and Technology, Chung Hwa University of Medical Technology, Tainan, Taiwan*

ABSTRACT

Cinnamaldehyde is a major and a bioactive compound isolated from the leaves of *Cinnamomum osmophloeum* kaneh. It possesses anti-diabetic properties in vitro and in vivo and has anti-inflammatory and anti-cancer effects. To explore whether cinnamaldehyde was linked to altered advanced glycation end products (AGE)-mediated diabetic nephropathy, the molecular mechanisms of cinnamaldehyde responsible for inhibition of AGE-reduced nitric oxide (NO) bioactivity in human renal proximal tubular cells were examined. We found that raising the ambient AGE concentration causes a dose-dependent decrease in NO generation. Cinnamaldehyde significantly reverses AGE-inhibited NO generation and induces high levels of cGMP synthesis and PKG activation. Treatments with cinnamaldehyde, the NO donor S-nitroso-N-acetylpenicillamine, and the JAK2 inhibitor AG490 markedly attenuated AGE-inhibited NOS protein levels and NO generation. Moreover, AGE-induced the JAK2-STAT1/STAT3 activation, RAGE/p27^{Kip1}/collagen IV protein levels, and cellular hypertrophy were reversed by cinnamaldehyde. The ability of cinnamaldehyde to suppress STAT activation was also verified by the observation that it significantly increased SCOS-3 protein level. These findings indicate for the first time that in the presence of cinnamaldehyde, the suppression of AGE-induced biological responses is probably mediated by inactivating the JAK2-STAT1/STAT3 cascade or activating the NO pathway. J. Cell. Biochem. 116: 1028–1038, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: ADVANCED GLYCATION END PRODUCTS; CINNAMALDEHYDE; NITRIC OXIDE; JANUS KINASE 2; DIABETIC NEPHROPATHY; HUMAN RENAL PROXIMAL TUBULAR CELLS

Advanced glycation end products (AGE) are believed to have a critical role in the development of diabetic nephropathy (DN) [Sato et al., 2006; Tan et al., 2007]. AGE arise from glucose-derived Amadori (1-deoxy-d-fructosyl derivatives) products and act to increase production of reactive oxygen species (ROS), elevate vascular permeability, enhance protein and lipoprotein deposition, promote extracellular matrix (ECM) protein synthesis and tubulointerstitial hyperplasia and hypertrophy, 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 oxidative stress and generalized cellular dysfunction [Bohlender et al., 2005; Coughlan et al., 2008].

Accumulation of AGE in human diabetic kidney begins in arterial walls and deposition in nodular and exudative lesions in glomeruli and tubulointerstitium [Friedman, 1999]. In situ hybridization studies demonstrated that AGE detected in diabetic mesangium, glomerular basement membranes, tubular basement membranes, and vessel walls [Tanji et al., 2000; Suzuki et al., 2006]. In diabetic nephropathy, AGE was preferentially located in interstitial collagen and was less consistently observed in vessel walls, mesangium, and tubular basement membranes [Daroux et al., 2010]. Furthermore, RAGE was expressed on glomeruli and tubulointerstitium and was upregulated in diabetic nephropathy [Tanji et al., 2000; Suzuki et al., 2006].

The Janus kinase family (JAK)-mediated tyrosine phosphorylation of signal transducers and activators of transcription (STATs) enables translocation of these transcription factors to the nucleus and lead to an augmentation of gene transcription [Kiu and Nicholson, 2012; Trengove and Ward, 2013]. Furthermore, the JAK-STAT signaling pathway is negatively regulated by the suppressor of cytokine signaling (SOCS) proteins [Linossi et al., 2013; Trengove and Ward, 2013]. Many cytokines and pathogenic mediators induce expression of SOCS, which act in a negative feedback loop to inhibit further signal transduction [Linossi et al., 2013]. Recent studies have shown that the JAK-STAT pathway is activated in a variety of renal diseases and has been implicated in the pathogenesis of DN [Marrero et al., 2006; Matsui and Meldrum, 2012]. However, the change in SOCS expression levels and the correlation between the JAK-STAT signaling in renal cellular hypertrophy is incompletely understood.

NO is synthesised by NO synthase (NOS), three distinct isoforms of which have been identified: inducible (iNOS), endothelial (eNOS), and neuronal (nNOS) [Kone, 2004]. Normal kidney specimens showed weak immunoreactivity for iNOS and eNOS in the proximal tubules and a lack of immunoreactivity for nNOS [Chertin et al., 2002; Kone, 2004]. It has been reported that iNOS and eNOS modulate immunologic injury and accumulation of extracellular matrix in the glomerulus and tubulointerstitial space [Chertin et al., 2002; Ito et al., 2004]. NO modulates a large variety of physiological functions and initiates diverse cellular signaling cascades which comprise nitrosylation of proteins or stimulation of soluble guanylyl cyclases which catalyze intracellular guanosine $3',5'$-cyclic monophosphate (cGMP) synthesis [Cerra and Pellegrino, 2007]. It is well known that the cGMP activates cGMP-dependent protein kinases (PKG) which mediate localized and global signaling. Considerable evidence suggests that advanced DN leading to severe proteinuria, immune injuries, declining renal function, and hypertension is associated with a state of progressive NO deficiency [Niedowicz and Daleke, 2005; Cerra and Pellegrino, 2007].

Cinnamaldehyde is a major and a bioactive compound isolated from the leaves of *Cinnamomum osmophloeum* kaneh [Chang et al., 2001; Cheng et al., 2004]. There are disparate outcomes from cinnamaldehyde treatments. In general, it has anti-tumorigenic effects [Ka et al., 2003], immunomodulatory [Chao et al., 2005; Chao et al., 2008; Tung et al., 2008], and anti-diabetic effects [Subash et al., 2007; Zhang et al., 2010]. The consequence of treatment with cinnamaldehyde may be cell and tissue specific. Recent studies report that cinnamaldehyde decreases glycosylated-hemoglobin but increases plasma insulin in STZ-induced diabetic rats [Subash et al., 2007]. It also enhances the antioxidant defense against ROS produced under hyperglycemic conditions and thus protects pancreatic β-cells against their loss and exhibits anti-diabetic properties [Subash et al., 2014]. However, the outcome of cinnamaldehyde treatment on diabetes-induced renal tubulointerstitial fibrosis, which is known to involve NO bioavailability and oxidative stress, has yet to be elucidated. Therefore, in this study, we examined the molecular mechanisms of cinnamaldehyde responsible for inhibition of AGE-reduced NO production in human renal proximal tubular cells. Our objective was to verify: (1) the effects of AGE and cinnamaldehyde on NO generation, cGMP production, PKG activation, and NOS protein levels; (2) the role of cinnamaldehyde in AGE-induced JAK/STATs signaling and SOCS protein levels; and (3) whether cinnamaldehyde and the NO donor affected AGE-mediated NOS, $p27^{Kip1}$ and collagen IV protein levels and cellular hypertrophy.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

DMEM/F12, trypsin-EDTA, trypan blue stain, antibiotics, molecular weight standards, FBS, and all medium additives were obtained from Life Technologies (Gaithersburg, MD). Anti-iNOS, -eNOS, -JAK2, -STAT1, -STAT3, -STAT5, -$p27^{Kip1}$, -collagen IV, -RAGE, -SOCS-1, and -SOCS-3 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-JAK2, -STAT1, -STAT3, and -STAT5 antibodies were obtained from Cell Signaling Technology (Beverly, MA). Lactate dehydrogenase (LDH)-cytotoxicity assay kit was purchased from BioVision (Mountain View, CA). NO colorimetric assay kit, SNAP, anti-PKG-I antibody, and AG490 were purchased from Calbiochem (La Jolla, CA). HRP-conjugated goat anti-rabbit or anti-mouse secondary antibody, streptavidin-peroxidase, and the enhanced chemiluminescence kit were obtained from Amersham Corp. (Arlington Heights, IL). N,N'-methylenebisacrylamide, acrylamide, SDS, ammonium persulfate, Temed, and Tween 20 were purchased from Bio-Rad Laboratories (Hercules, CA). BSA, DMSO, Nω-nitro-L-arginine-methyl ester (L-NAME), anti-β-actin antibody, and all other chemicals were purchased from Sigma-Aldrich Chemical (St. Louis, MO).

COLLECTION OF ESSENTIAL OIL FROM *CINNAMOMUM OSMOPHLOEUM* LEAF

The leaves of 13-year-old *C. osmophloeum* were collected from the Da-Pin-Ting of the Taiwan Sugar Farm located in Nantou County in central Taiwan. The fresh leaf oils of *C. osmophloeum* were obtained by using water distillation in a Clevenger-type apparatus for 6 h and their constituents determined by GC-MS [Chao et al., 2008]. The sampling of essential oil was performed by a mass spectrometer, which was equipped with a PoLaris Q mass selective

detector in the electron impact (EI) ionization mode (70 eV), and using a RTx-5 capillary column (30 m × 0.25 mm; film thickness 0.25 μm). The oven temperature was held at 80°C for 1 min to 200°C held for 5 min at a rate of 4°C/min. The injection temperature was 250°C, detector temperature was 280°C, and helium was used as a carrier gas at a split ratio of 10:1. Identification of the major components of *C. osmophloeum* leaf oil was confirmed by comparison with standards, by spiking, and on the basis of their mass spectral fragmentation using the Wiley GC-MS library. The quantity of compounds was obtained by integrating the peak area of the spectrograms.

PURIFICATION OF CINNAMALDEHYDE

Leaf oils (1.5 g) of *C. osmophloeum* were purified by semi-preparative HPLC on a model L-7150 instrument (Hitachi, Japan) with a 250 × 10 mm i.d., 5 μm Luna Silica (absorption wavelength $\lambda = 254$ nm) (Phenomenex, American) column, ethyl acetate/hexane (15:85) mobile phase, 4 mL/min flow rate, and Hitachi L-7490 RI detector [Chao et al., 2008]. The pure cinnamaldehyde compound (yield % = 85% and purity > 98%) was obtained ($t_R = 9.04$ min).

CELL CULTURE

Human renal proximal tubular cells (HK-2) were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in culture flasks (Nunclon, Denmark) and maintained in DMEM/F12 (5.5 mM D-glucose) supplemented with 100 i.u./ml penicillin, 100 μg/ml streptomycin and 5% FBS in a humidified 5% CO_2 incubator at 37°C. In this study, cells were exposed to serum-free (0.1% FBS) DMEM/F12 supplemented with cinnamaldehyde or the JAK2 kinase inhibitor AG490 for 4 h prior to timed exposure to DMEM/F12 containing 5% FBS. Because the pure cinnamaldehyde compound was dissolved in DMSO, the vehicle DMSO alone was added as solvent control. Each experimental data point represents the mean of duplicate wells from three independent experiments.

PREPARATION OF AGE

AGE-BSA was prepared according to our previous study [Huang et al., 2009]. 20% (wt/vol) fatty acid-free BSA was incubated at 37°C under sterile phosphate-buffered saline (PBS), pH 7.2, supplemented with 250 mM 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 phosphate-buffered saline. Control non-glycated BSA was incubated in the same conditions with the exception of the absence of glucose-6-phosphate. The dialyzed preparations were sterile-filtered through 0.2 μm nylon filters. Preparations were tested for endotoxin using Endospecy ES-20S system where no endotoxin was detectable. The AGE content of the preparations was determined spectrofluorometrically 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 level was determined by using the Griess reaction [Huang et al., 2009]. Briefly, cells (1.0×10^4 cells per well) 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 kD ultrafilters, 20 μl of the medium was diluted with 120 μl assay buffer and mixed with 5 μl cofactor and 5 μl nitrate reductase. 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 $NaNO_3$ to nitrite.

CELL VIABILITY ASSAY

Cell viability (MTT) assay was performed to evaluate the proliferation of renal proximal tubular cells [Huang et al., 2009]. 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 (0.04 M HCl in isopropanol) were added and thoroughly mixed. Spectrometric absorbance at 595 nm (for formazan dye) was measured with the absorbance at 655 nm for reference.

CYTOTOXICITY ASSAY

LDH content was determined by using an LDH-cytotoxicity assay kit, which is based on a coupled enzymatic reaction that results in the conversion of a tetrazolium salt into a red formazan product. The increase in the amount of formazan produced in culture supernatant directly correlates with the increase in the number of lysed cells. The formazan was quantified spectrophotometrically by measuring its absorbance at 490 nm. A group of wells were treated with 1% Triton X-100 solution for maximum LDH release. The mean of the background value was subtracted from all other values. Cytotoxicity in experimental samples expressed as a percentage of the LDH release of 5% FBS-treated cells (control).

CGMP DETERMINATION

HK-2 cells (2.5×10^4 cells/well) were grown in 24-well plates until approximately 70% confluent. 16 h 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 [Huang et al., 2009]. Reaction was terminated by adding 10% (vol/vol) ice-cold lysis buffer A from cGMP ELISA kit (Assay Designs, Ann Arbor, MI). After 30 min of extraction on ice, the supernatant was collected to the Eppendorf tubes and stored at −20°C until cGMP assay. cGMP was determined using the acetylation method in a cGMP immunoassay system as described in the manual provided by the manufacturer. Measurements were performed in triplicate.

WESTERN BLOT ANALYSIS

One day before treatment, 1.5×10^6 cells were seeded in T-75 flasks. Cell growth medium was replaced 24 h after seeding, followed by addition of test compounds after medium change. For

protein analysis, total cell lysates were harvested, resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to Protran membranes (0.45 μm, Schieicher & Schuell, Keene, NH). The membranes were blocked in blocking solution and subsequently probed with primary antibodies. The membrane was incubated in 4,000 × diluted HRP-conjugated goat anti-rabbit or anti-mouse secondary antibody. The protein bands were detected using the enhanced chemiluminescence (ECL) system. For the JAK2/STATs activation assay, proteins were resolved by SDS-PAGE and transferred to Protran membranes. The membranes were probed with antiphospho-JAK2 (1 μg/ml), antiphospho-STAT1 (1 μg/ml), antiphospho-STAT3 (1 μg/ml), antiphospho-STAT5 (1 μg/ml), anti-JAK2 (0.75 μg/ml), anti-STAT1 (1 μg/ml), anti-STAT3 (1 μg/ml), and anti-STAT5 (1 μg/ml) antibodies. Immunoreactive proteins were detected with the ECL system as described above. The intensity of Western blot bands was quantified by densitometric analysis. Results were expressed as the ratio of intensity of the protein of interest to that of β-actin or the indicated protein from the same sample.

EXPRESSION OF PKG AND ASSAY OF PKG ACTIVITY

Cells (1.5×10^6 cells) were harvested and homogenized with cold buffer consisting of 20 mM sodium phosphate, pH 6.8, 2 mM EDTA, 15 mM 2-mercaptoethanol, 150 mM NaCl, 2 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. The suspension was centrifuged for 10 min at 14,000 rpm 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 (1.5 μg/ml). The anti-PKG-I polyclonal antibody detects the activation forms of both PKG-Iα and PKG-Iβ. PKG activity was assayed as described in Suhasini and Soh [Demple, 2002; Pilz and Casteel, 2003] 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.

CELLULAR HYPERTROPHY ANALYSIS

Cells were grown in 6-well plates until approximately 50% confluent and then made quiescent for 2 days in DMEM/F12 medium containing 0.1% FBS. The cultures were then treated with BSA (500 μg/ml), AGE (500 μg/ml) or AGE (500 μg/ml) plus various agents for 5 days, after which the cells were trypsinized, washed twice with PBS and counted using a haemocytometer. Equal numbers of cells were lysed in buffer (0.1% [wt/vol] SDS, 0.5% (wt/vol) sodium deoxycholate, 1.0% (wt/vol) Nonidet P-40, in PBS). The total protein content measured using the Bio-Rad protein assay kit. Total protein was expressed as micrograms of protein per 10^4 cells.

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 Dunnett's test for individual differences between groups. P-values < 0.05 were considered significant.

RESULTS

EFFECTS OF BSA, AGE, AND CINNAMALDEHYDE (CIN) ON NO PRODUCTION IN HUMAN RENAL TUBULAR CELLS

To investigate whether AGE could affect NO production in human renal tubular (HK-2) cells, we treated these cells with BSA, AGE, or cinnamaldehyde and analyzed NO production by the Griess method. We found that raising the ambient AGE concentration causes a dose-dependent decrease in nitrite production compared with control (5% FBS) when the incubation period was for 48 h (Fig. 1A). However, the addition of non-glycated BSA did not significantly decrease nitrite production. On the other hand, raising the ambient cinnamaldehyde concentration (0.1, 1, 10, or 100 μM) causes a dose-dependent increase in AGE (500 μg/ml)-inhibited NO production when compared with AGE alone (Fig. 1B). As shown in Supplementary Fig. S1, we also observed that treatments of AGE and cinnamaldehyde for short period of time (2 h) revealed the same effects on NO production in these cells.

EFFECTS OF BSA, AGE, AND CINNAMALDEHYDE ON CGMP SYNTHESIS AND PKG ACTIVATION

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. We then examined whether cinnamaldehyde increased cGMP synthesis. First, AGE (500 μg/ml) significantly decreased cGMP synthesis at 0.5, 12, 24, 48, and 72 h when compared with non-glycated BSA (500 μg/ml) or control (Fig. 2A). After exposure of cultured cells to cinnamaldehyde (10 μM), AGE-inhibited cGMP synthesis was markedly suppressed. Next, we found that cinnamaldehyde (10 μM) markedly suppressed AGE (500 μg/ml)-inhibited PKG protein synthesis (Fig. 2B) and PKG activation (Fig. 2C) at 48 h. Cinnamaldehyde (10 μM) slightly increased cGMP synthesis and PKG synthesis/activation when cells were incubated in medium containing 5% FBS. To be noticed, we also found that treatments of AGE and cinnamaldehyde for short period of time (2 h) revealed the same effects on PKG activity (Supplementary Fig. S2). These observations indicated that the major action of AGE on the NO/cGMP/PKG pathway is reversed by cinnamaldehyde.

EFFECTS OF NO DONOR, JAK2 INHIBITOR, AND CINNAMALDEHYDE ON AGE–INHIBITED NOS PROTEIN LEVELS AND NO PRODUCTION

To investigate if exogenously added NO and the JAK2-STATs inactivation could affect AGE-inhibited NOS-NO pathway in human renal tubular cells, we treated these cells with the NO donor SNAP or the JAK2 inhibitor AG490 and examined iNOS and eNOS protein levels and nitrite production in the presence of AGE (500 μg/ml). Figure 3A illustrates that AGE (500 μg/ml) decreased iNOS and eNOS protein levels at 48 h when compared with non-glycated BSA (500 μg/ml) or control. Interestingly, cinnamaldehyde dose-dependently reduced the inhibitory effect of AGE on iNOS and eNOS protein levels (Fig. 3B). The NO donor SNAP (10 μM) and the JAK2 inhibitor AG490 (5 μM) markedly suppressed AGE-inhibited iNOS and eNOS protein levels (Fig. 3A) and nitrite production (Fig. 3C). Furthermore, iNOS and eNOS protein levels and NO production were abolished by L-NAME (10 μM), a
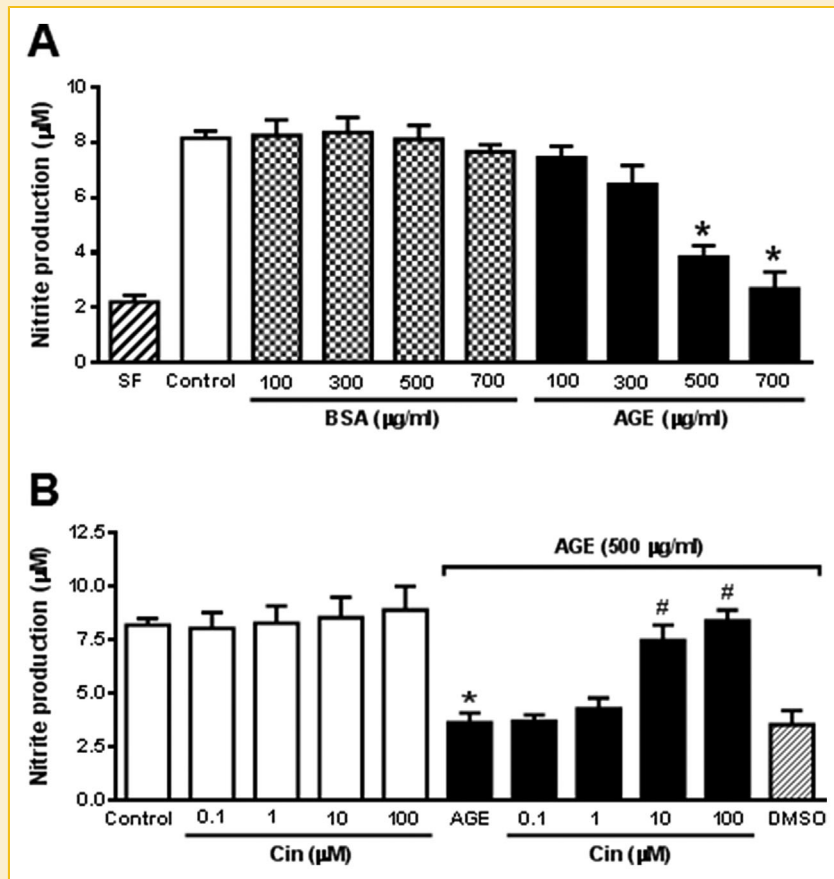
Fig. 1. Effects of BSA, AGE, and cinnamaldehyde (Cin) on NO production in human renal tubular cells. (A) Cells were treated with serum–free (SF) medium, BSA (100, 300, 500, and 700 μg/ml) or AGE (100, 300, 500, and 700 μg/ml) in the presence of 5% FBS (control) for 48 h. (B) Cells were treated with 5% FBS (control), AGE (500 μg/ml), and Cin (0.1, 1, 10, and 100 μM) in the presence of 5% FBS or AGE (500 μg/ml) for 48 h. DMSO was added as solvent control. NO production was determined by monitoring nitrite production by Griess assay as described under "Materials and Methods". Results were expressed as the mean ± SEM (n = 6). These are representative experiments, each performed at least four times. *$P < 0.05$ versus control; #$P < 0.05$ versus AGE (500 μg/ml).

NOS inhibitor. Consistently, we also observed that treatments of AGE and cinnamaldehyde for 2 h showed the same effects in human renal tubular cells (Supplementary Fig. S3). Thus, these results suggest that sustained NO generation was regulated by persistent activation of NOS and cinnamaldehyde inhibits AGE-induced effects maybe involve the NOS/NO-dependent mechanism.

EFFECTS OF BSA, AGE, AND CINNAMALDEHYDE ON CELL GROWTH IN HUMAN RENAL TUBULAR CELLS

To explore whether BSA, AGE, or cinnamaldehyde could affect cell growth in human renal tubular cells, we treated these cells with BSA, AGE, or cinnamaldehyde and analyzed cell viability and cell number. The MTT assay (Fig. 4A) and cell number analysis (Fig. 4B) showed that AGE (500 μg/ml) or non-glycated BSA (500 μg/ml) had no significant effect on cell growth compared with control when the incubation period was for 48 h. Additionally, the JAK2 inhibitor AG490 (5 μM), cinnamaldehyde (10 μM), or the NO donor SNAP (10 μM) did not significantly affect AGE-mediated cell growth when compared with AGE alone.

EFFECTS OF BSA, AGE, AND CINNAMALDEHYDE ON CYTOTOXICITY IN HUMAN RENAL TUBULAR CELLS

To determine the effects of BSA, AGE, or cinnamaldehyde on cytotoxicity in human renal tubular cells, we treated these cells with BSA, AGE, or cinnamaldehyde and analyzed cytotoxicity by the LDH assay. Figure 5 showed that AGE (500 μg/ml), non-glycated BSA (500 μg/ml), or AG490 (5 μM), cinnamaldehyde (10 μM), and SNAP (10 μM) in the presence of AGE had no significant effect on cytotoxicity compared with control or AGE when the incubation period was for 48 h.

EFFECTS OF CINNAMALDEHYDE ON AGE–MEDIATED THE JAK2-STATS ACTIVATION

Moreover, we examined whether cinnamaldehyde and the JAK2-STATs pathway played roles in AGE-mediated signaling cascades. We found that AGE (500 μg/ml) clearly induced tyrosine phosphorylation of JAK2, STAT1, and STAT3 (but not STAT5) at 2 h when compared with control (Fig. 6). The specific JAK2 inhibitor AG490 (5 μM) and cinnamaldehyde (10 μM) significantly reduced phospho-JAK2, phospho-STAT1, and phospho-STAT3
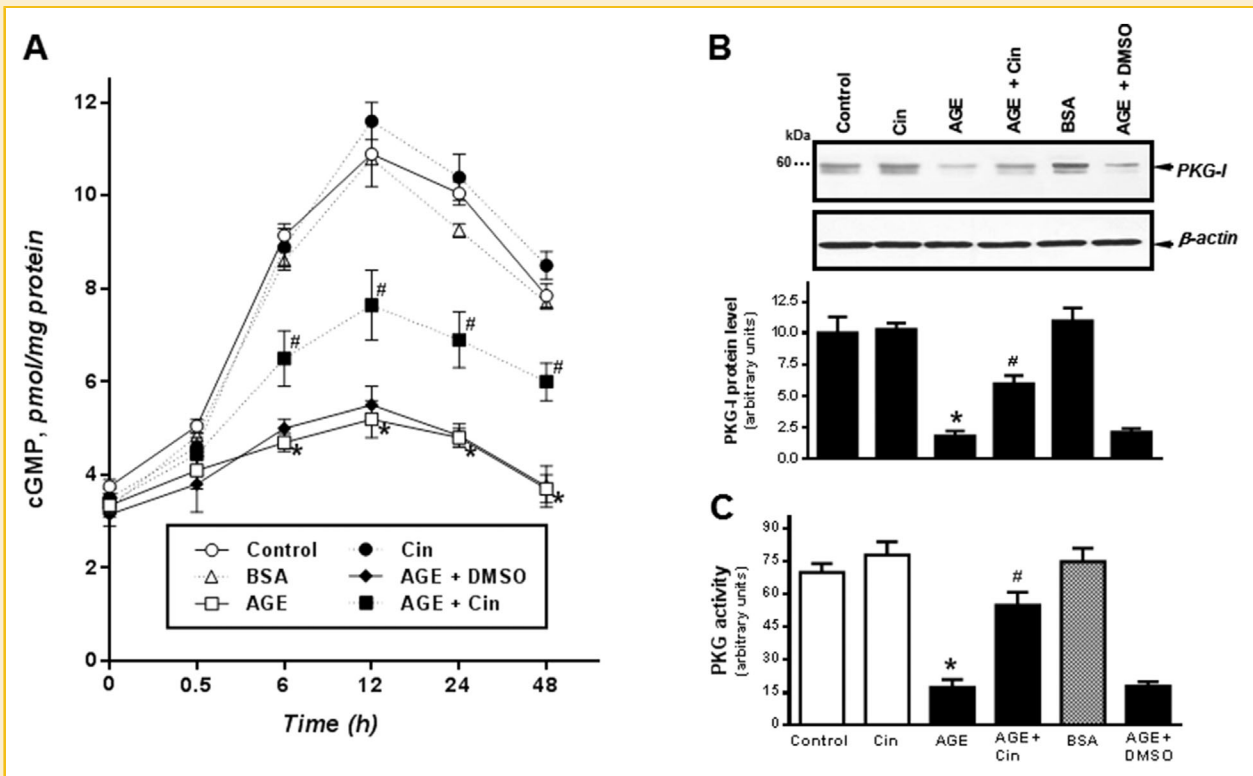
Fig. 2. Effects of BSA, AGE, and cinnamaldehyde on cGMP synthesis, PKG-I protein synthesis, and PKG activity. Cells were treated with 5% FBS (control), BSA (500 μg/ml), AGE (500 μg/ml), and Cin (10 μM) in the presence of 5% FBS or AGE for 0, 0.5, 6, 12, 24, and 48 h, and then assayed for cGMP synthesis (A). Total cell lysates from cells treated with AGE, BSA, or Cin in the presence of 5% FBS or AGE for 48 h were subjected to analysis for PKG-I protein synthesis (B) PKG activity (C). Results were expressed as the mean ± SEM (n = 4). $^*P < 0.05$ versus control; $^\#P < 0.05$ versus AGE.
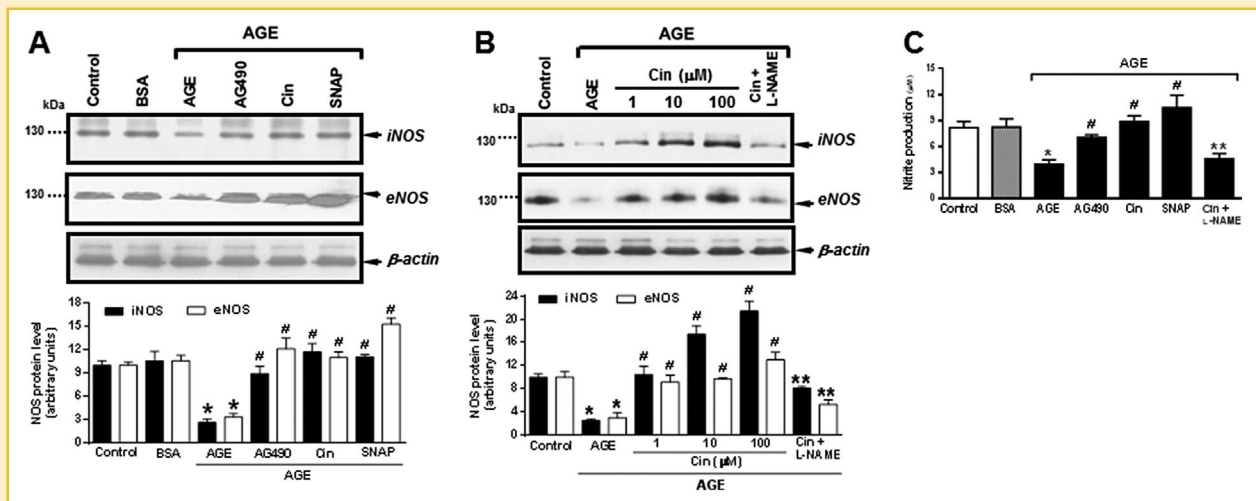

Fig. 3. Effects of cinnamaldehyde, the NO donor, the JAK2 inhibitor, and the iNOS inhibitor on AGE-mediated NOS expression and NO production. (A) Total cell lysates from cells treated with 5% FBS (control), BSA (500 μg/ml), AGE (500 μg/ml), and Cin (10 μM), SNAP (10 μM), or AG490 (5 μM) in the presence of AGE for 48 h were subjected to analysis for iNOS and eNOS protein levels as described under "Materials and Methods". (B) Total cell lysates from cells treated with 5% FBS (control), AGE (500 μg/ml), or Cin (1, 10, 100 μM) and Cin (10 μM) + L-NAME (10 μM) in the presence of AGE for 48 h were subjected to analysis for NOS protein levels. (C) Cells were treated with 5% FBS (control), BSA (500 μg/ml), AGE (500 μg/ml), or Cin (10 μM), SNAP (10 μM), AG490 (5 μM), and Cin (10 μM) + L-NAME (10 μM) in the presence of AGE for 48 h. NO production was determined by monitoring nitrite production by Griess assay. Results were expressed as the mean ± SEM (n = 6). These are representative experiments, each performed at least three times. $^*P < 0.05$ versus control; $^\#P < 0.05$ versus AGE.
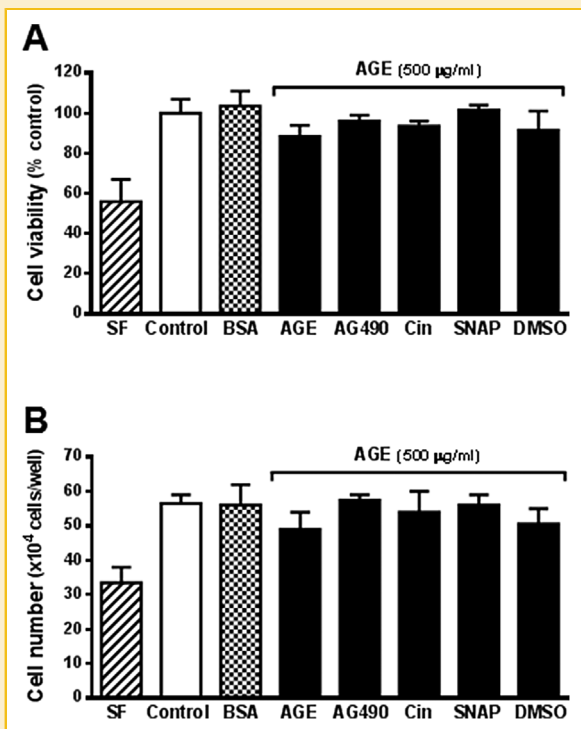
Fig. 4. Effects of BSA, AGE, and cinnamaldehyde on cell growth in human renal tubular cells. Cells were treated with serum–free (SF) medium, 5% FBS (control), BSA (500 μg/ml), AGE (500 μg/ml), or AG490 (5 μM), Cin (10 μM), and SNAP (10 μM) in the presence of AGE for 48 h, and then assayed for cell viability (A) and cell numbers (B) as described under "Materials and Methods". Results were expressed as the mean ± SEM (n = 6).

without affecting JAK2, STAT1, and STAT3 protein levels in AGE-treated cells. Surprisingly, AGE and cinnamaldehyde did not significantly affect tyrosine phosphorylation of STAT5. Taken together, these observations demonstrate that the JAK2-STAT1/STAT3 pathway may be important signal mediators in the
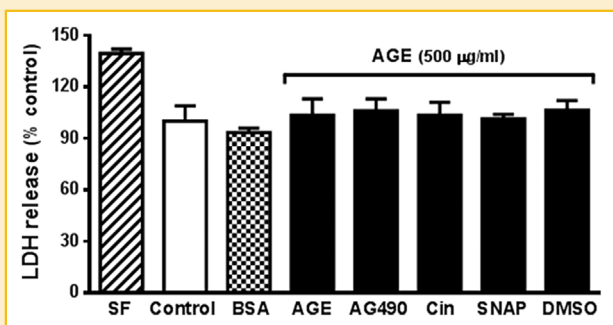

Fig. 5. Effects of BSA, AGE, and cinnamaldehyde on cytotoxicity in human renal tubular cells. Cells were treated with serum–free (SF) medium, 5% FBS (control), BSA (500 μg/ml), AGE (500 μg/ml), or AG490 (5 μM), Cin (10 μM), and SNAP (10 μM) in the presence of AGE for 48 h, and then assayed for cytotoxicity as described under "Materials and Methods". Results were expressed as the mean ± SEM (n = 6).

AGE-mediated biological responses in human renal tubular cells. Treatment with cinnamaldehyde might be an effective strategy for reducing AGE-enhanced activation of the JAK2-STAT1/STAT3 signaling cascade.

EFFECTS OF BSA, AGE, AND CINNAMALDEHYDE ON PROTEIN EXPRESSION OF SOCS AND RAGE

SOCS proteins have been identified as crucial negative regulators of various cytokines employing JAK-STAT signaling [Linossi et al., 2013]. To annex a better understanding of mechanisms involved in AGE/RAGE-induced JAK2-STAT1/STAT3 activation in human renal tubular cells, we further examined the effects of cinnamaldehyde on AGE-mediated SOCS-1/SOCS-3 and RAGE protein expression. As depicted in Figure 7, BSA (500 μg/ml), AGE (500 μg/ml), and cinnamaldehyde (10 μM) did not significantly affect SOCS-1 protein levels for 48 h. However, cinnamaldehyde significantly reversed AGE (500 μg/ml)-blocked SOCS-3 protein expression. Next, AGE-induced RAGE protein expression was suppressed by cinnamaldehyde. The results indicated that inhibition of SOCS-3 protein expression is the underlying mechanism by AGE to promote activation of the JAK2-STAT1/STAT3 pathway. Cinnamaldehyde negatively regulated AGE/RAGE-induced these effects.

EFFECTS OF CINNAMALDEHYDE, THE NO DONOR, AND THE JAK2 INHIBITOR ON AGE–MEDIATED PROTEIN EXPRESSION OF P27^{KIP1} AND COLLAGEN IV AND CELLULAR HYPERTROPHY

Previous studies have indicated that cell cycle regulatory molecules and ECM proteins are required for the development of growth factors/cytokines-mediated cellular growth and hypertrophy [Heineke and Molkentin, 2006], but interference with the expression of one of these proteins may attenuate hypertrophy. To annex a better understanding of mechanisms involved in AGE-induced renal hypertrophy and fibrosis, we further examined the effects of cinnamaldehyde and the NO donor on AGE-mediated protein synthesis of p27^{Kip1} and collagen IV. As depicted in Figure 8A, cinnamaldehyde (10 μM) significantly reversed AGE (500 μg/ml)-enhanced protein expression of p27^{Kip1} and collagen IV for 5 days. Hypertrophy is the result of a greater increase in cell protein than DNA and represents a phenomenon of increasing cell size [Heineke and Molkentin, 2006]. At a cellular level, we measured quantitatively by assessing the ratio of cell protein to cell number (Fig. 8B), as hypertrophy is characterized by larger cells with higher protein content than nonhypertrophic cells. The results indicated that AGE markedly enhanced hypertrophy index in human renal tubular cells after 5 days of incubation when compared with control cells. Moreover, we found that cinnamaldehyde, SNAP, and AG490 markedly reversed AGE-induced cellular hypertrophy. BSA and DMSO did not significantly affect the hypertrophic growth in these cells.

DISCUSSION

Several studies have shown that cinnamaldehyde possesses a wide variety of bioactive properties [Chao et al., 2005, 2008; Cheng et al., 2006; Tung et al., 2008; Zhang et al., 2008]. Many reports concerning
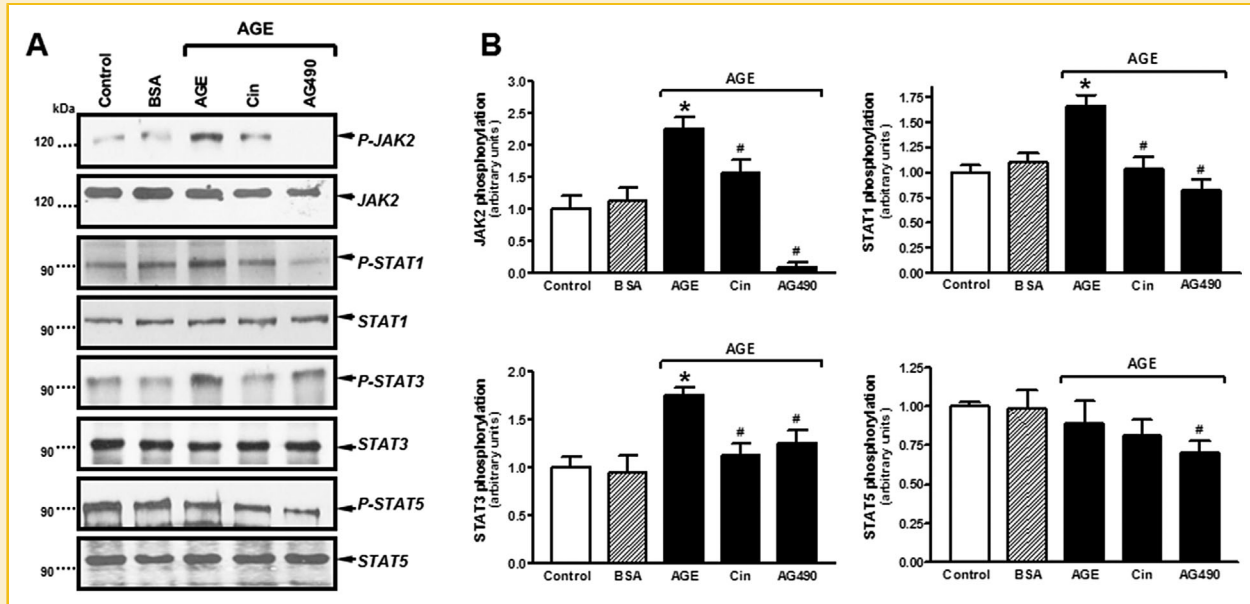
Fig. 6. Effects of cinnamaldehyde, the NO donor, and the JAK2 inhibitor on AGE–mediated JAK2–STATs phosphorylation. Total cell lysates from cells treated with Cin (10 μM) or AG490 (5 μM) in the presence of AGE (500 μg/ml) for 2 h. (A) Proteins were separated by polyacrylamide gels and immunoblotted with antiphospho–JAK2 (P–JAK2), antiphospho–STAT1 (P–STAT1), antiphospho–STAT3 (P–STAT3), and antiphospho–STAT5 (P–STAT5) antibodies (upper panels) or antibodies corresponding to the above antibodies (lower panels). The concentration of BSA was 500 μg/ml. This is a representative experiment independently performed three times. (B) Laser densitometry of the gels showed in (A) and two additional phosphorylation experiments. $^*P < 0.05$ versus control; $^\#P < 0.05$ versus AGE.

the effects of cinnamaldehyde on immunomodulation and anti-cancer activity, but there is no report about the effect of cinnamaldehyde on NOS/NO signaling. On the basis of the current results, we found that the JAK2-STAT1/STAT3 cascade is an important AGE/RAGE-induced signaling pathway contributing to overproduction of p27^{Kip1} and collagen IV, ECM accumulation, and cellular hypertrophy in renal tubular cells.

So far, there appears to be no receptor binding to and mediating effects of cinnamaldehyde. However, several studies have shown that some receptor protein levels and activities change during cinnamaldehyde exposure [Koh et al., 1998; Chao et al., 2008; Jianming et al., 2010; Takasao et al., 2012]. Treatment with cinnamaldehyde significantly increased the phosphorylation levels of the insulin-like growth factor-I receptor (IGF-IR) and its downstream signaling molecules such as insulin receptor substrate-1 and ERK [Takasao et al., 2012]. Additionally, the induction of interleukin 2 receptor α (IL-2Rα) by Con A was blocked by cinnamaldehyde [Koh et al., 1998]. This inhibition of IL-2Rα expression by cinnamaldehyde was consistent with the attenuation of lymphoproliferation and cell cycle progression. It is known that the JAK/STAT pathway is rapidly activated upon IL-2R and IGF-IR ligation [Terrell et al., 2006]. In this study, we found that cinnamaldehyde significantly reverses AGE-inhibited NO generation and induces high levels of cGMP synthesis and PKG activation in human renal proximal tubular cells. Treatments with cinnamaldehyde and the NO donor markedly reversed AGE-induced JAK2-STAT1/STAT3 activation, p27^{Kip1} and collagen IV protein levels, and cellular hypertrophy. Moreover, the ability of cinnamaldehyde to suppress AGE-induced

STAT activation was also verified by the observation that it reversed the decrease of SCOS-3 protein levels induced by AGE. Taken together, our data revealed a novel biological function of cinnamaldehyde and suggested a possible molecular mechanism for its action.

AGE was formed via a nonenzymatic reaction between reducing sugars and the free amino acid groups on proteins, lipids and nucleic acids, and accumulated in the glomeruli and tubulointerstitium, in particular in diabetes [Bohlender et al., 2005; Sato et al., 2006; Coughlan et al., 2008]. Not only hyperglycemia but also oxidative stress and inflammation contribute to the formation of AGE. On the other hand, AGE was involved in the generation of oxygen free radicals causing oxidative damage particularly to kidney [Lee et al., 2003; Niedowicz and Daleke, 2005; Coughlan et al., 2008]. AGE cause abnormally high levels of ROS by contributory autoxidation of glucose and protein glycation, and oxidative stress have been reported to be a causal factor of tubulointerstitial fibrosis in DN [Lee et al., 2003; Bohlender et al., 2005; Huang et al., 2009]. Elevated AGE and ROS, in turn, target a variety of intracellular signaling molecules that are involved in the progression of hypertrophy and apoptosis in the glomeruli and tubulointerstitium [Da Ros et al., 2004; Coughlan et al., 2008; Giacco and Brownlee, 2010]. The mechanism of the ROS pathway in conjunction with the NO signaling that promote and/or blunt the progression of DN have been the focus of many laboratories' efforts to reveal new therapeutic targets [Tan et al., 2007; Turgut and Bolton, 2010]. NOS activity is well known to be involved in the regulation of renal function [Huisman et al., 2002; Evans and Fitzgerald, 2005]. Moreover, reduced NO bioavailability is associated with increased oxidative stress and dysfunction in the
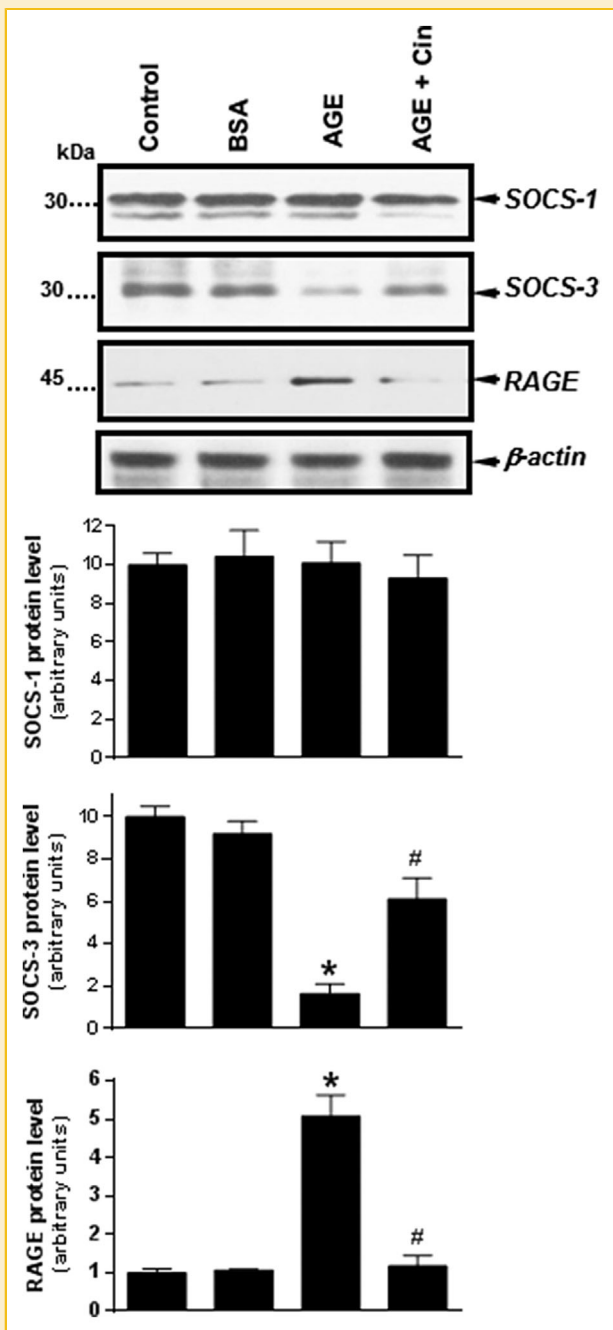
Fig. 7. Effects of BSA, AGE, and cinnamaldehyde on SOCS and RAGE protein levels. Total cell lysates from cells treated with 5% FBS (control), BSA (500 μg/ml), AGE (500 μg/ml), and Cin (10 μM) in the presence of AGE for 48 h were subjected to Western blot analysis for SOCS-1, SOCS-3, and RAGE protein levels. This is a representative experiment independently performed four times. $^*P < 0.05$ versus control; $^\#P < 0.05$ versus AGE.

failing glomeruli and tubulointerstitium [Okumura et al., 2006; Forbes et al., 2007]. Therefore, up-regulation of renal NOS and an increase in NO bioavailability is an important compensatory mechanism to prevent or delay the progression of DN induced by ROS and AGE.

Recent studies reported that cinnamaldehyde displays an exciting anti-diabetic efficacy and its mechanism involve the retinol binding protein 4 (RBP4)-glucose transporter 4 (GLUT4) system, during which the serum RBP4 levels are lowered and the expression of tissue GLUT4 protein is up-regulated [Zhang et al., 2008]. It enhances the antioxidant defense against ROS produced under hyperglycemic conditions and thus protects pancreatic β-cells against their loss [Subash et al., 2014]. In addition, cinnamaldehyde significantly decrease glycosylated hemoglobin level and at the same time markedly increase plasma insulin levels in STZ-induced diabetic rats [Subash et al., 2007]. On the other hand, cinnamaldehyde prevents development of hypertension in insulin deficiency and insulin resistance through normalization of NO generation and vascular contractility [El-Bassossy et al., 2011]. Interestingly, the present study demonstrates that cinnamaldehyde significantly reversed AGE-inhibited NO generation and induces high levels of cGMP synthesis and PKG activation. Treatments with cinnamaldehyde for longer (48 h) and shorter (2 h) time periods markedly attenuated AGE-inhibited NOS protein synthesis and NO generation (Fig. 3 and Supplementary Fig. S3). Moreover, AGE-induced the JAK2-STAT1/STAT3 activation, RAGE/p27^{Kip1}/collagen IV protein synthesis, and cellular hypertrophy were reversed by cinnamaldehyde. These results suggested a possibility that cinnamaldehyde may interfere AGE-inhibited NO signaling and improve the aggravation of DN.

Reduced bioavailability of endogenous NO is a central pathophysiological event in renal and cardiovascular diseases [Heineke and Molkentin, 2006; Cerra and Pellegrino, 2007; Prabhakar et al., 2007]. Impaired NO release in chronic renal failure has been implicated in the pathogenesis of hypertension and the progression of renal insufficiency. Recently, it was demonstrated that nitrate treatment almost completely prevented proteinuria and histological signs of renal injury, and the cardiac hypertrophy and fibrosis were attenuated [Carlström et al., 2011]. NOS gene delivery in rats with renal failure improves NO release, which likely prevents the aggravation of renal failure and injury [Savard et al., 2012].

In normal renal physiology, the renal tubulointerstitium-derived NO is one of the most potent endogenous immunomodulators and, by its anti-inflammatory and anti-hypertrophic properties, it is widely recognized as an endogenous renoprotective agent in diabetes [Okumura et al., 2006; Tan et al., 2007; Sucher et al., 2010; Arellano-Mendoza et al., 2011]. In the present study, we suggest that one of the mechanisms of AGE/RAGE-induced cellular hypertrophy in renal tubular cells is by blocking the NO signaling and enhancing the JAK2-STAT1/STAT3 activation. The ability of cinnamaldehyde to attenuate cellular hypertrophy was verified by the observation that it reversed AGE/RAGE-mediated these effects. Indeed, renal tubular dysfunction with reduced NO production and/or bioavailability is a common feature in patients with apparent coronary atherosclerosis with diabetes and may contribute to the development and progression of DN [Sucher et al., 2010; Crabtree and Channon, 2011]. Thus, preservation of NO level and activity is an ideal therapeutic target for DN. However, excess local production

Fig. 8. Effects of cinnamaldehyde, the NO donor, and the JAK2 inhibitor on AGE-mediated p27^{Kip1} and collagen IV protein expression and cellular hypertrophy. Total cell lysates from cells treated with 5% FBS (control), BSA (500 μg/ml), AGE (500 μg/ml), or Cin (10 μM), SNAP (10 μM), and AG490 (5 μM) in the presence of AGE (500 μg/ml) for 5 days were subjected to Western blot analysis for p27^{Kip1} and collagen IV protein levels (A) and assay for hypertrophy index (B). DMSO was added as solvent control. These are representative experiments, each performed at least three times. $^*P < 0.05$ versus control; $^#P < 0.05$ versus AGE.

of NO derived from NOS aggravates ROS production and excess administration with exogenous NO donor induces tissue damage as well [Evans and Fitzgerald, 2005; Garvin et al., 2011]. This implies that NO acts as a double-edged sword in renal complications in diabetes.

In conclusion, the present study demonstrates that cinnamaldehyde is able to stimulate NO generation, cGMP synthesis, and PKG activation in AGE-treated renal tubular cells. This novel pathway downstream of NOS/NO stimulation may be beneficial to the tubulointerstitium under hyperglycemia or AGE accumulation.

REFERENCES

Arellano-Mendoza MG, Vargas-Robles H, Del Valle-Mondragon L, Rios A, Escalante B. 2011. Prevention of renal injury and endothelial dysfunction by chronic L-arginine and antioxidant treatment. Ren Fail 33:47–53.

Bohlender JM, Franke S, Stein G, Wolf G. 2005. Advanced glycation end products and the kidney. Am J Physiol Renal Physiol 289:645–659.

Carlström M, Persson AE, Larsson E, Hezel M, Scheffer PG, Teerlink T, Weitzberg E, Lundberg JO. 2011. Dietary nitrate attenuates oxidative stress, prevents cardiac and renal injuries, and reduces blood pressure in salt-induced hypertension. Cardiovasc Res 89:574–585.

Cerra MC, Pellegrino D. 2007. Cardiovascular cGMP-generating systems in physiological and pathological conditions. Curr Med Chem 14:585–599.

Chang ST, Chen PF, Chang SC. 2001. Antibacterial activity of leaf essential oils and their constituents from Cinnamomum osmophloeum. J Ethnopharmacol 77:123–127.

Chao LK, Hua KF, Hsu HY, Cheng SS, Lin IF, Chen CJ, Chen ST, Chang ST. 2008. Cinnamaldehyde inhibits pro-inflammatory cytokines secretion from monocytes/macrophages through suppression of intracellular signaling. Food Chem Toxicol 46:220–231.

Chao LK, Hua KF, Hsu HY, Cheng SS, Liu JY, Chang ST. 2005. Study on the antiinflammatory activity of essential oil from leaves of Cinnamomum osmophloeum. J Agric Food Chem 53:7274–7278.

Cheng SS, Liu JY, Hsui YR, Chang ST. 2006. Chemical polymorphism and antifungal activity of essential oils from leaves of different provenances of indigenous cinnamon (*Cinnamomum osmophloeum*). Bioresour Technol 97:306–312.

Cheng SS, Liu JY, Tsai KH, Chen WJ, Chang ST. 2004. Chemical composition and mosquito larvicidal activity of essential oils from leaves of different Cinnamomum osmophloeum provenances. J Agric Food Chem 52:4395–4400.

Chertin B, Rolle U, Farkas A, Puri P. 2002. The role of nitric oxide in reflux nephropathy. Pediatr Surg Int 18:630–634.

Coughlan MT, Mibus AL, Forbes JM. 2008. Oxidative stress and advanced glycation in diabetic nephropathy. Ann NY Acad Sci 1126:190–193.

Crabtree MJ, Channon KM. 2011. Synthesis and recycling of tetrahydrobiopterin in endothelial function and vascular disease. Nitric Oxide 25:81–88.

Da Ros R, Assaloni R, Ceriello A. 2004. Antioxidant therapy in diabetic complications: What is new. Curr Vasc Pharmacol 2:335–341.

Daroux M, Prévost G, Maillard-Lefebvre H, Gaxatte C, D'Agati VD, Schmidt AM, Boulanger E. 2010. Advanced glycation end-products: implications for diabetic and non-diabetic nephropathies. Diabetes Metab 36:1–10.

Demple B. 2002. Signal transduction by nitric oxide in cellular stress responses. Mol Cell Biochem 235:11–18.

El-Bassossy HM, Fahmy A, Badawy D. 2011. Cinnamaldehyde protects from the hypertension associated with diabetes. Food Chem Toxicol 49:3007–3012.

Evans RG, Fitzgerald SM. 2005. Nitric oxide and superoxide in the renal medulla: A delicate balancing act. Curr Opin Nephrol Hypertens 14:9–15.

Forbes JM, Fukami K, Cooper ME. 2007. Diabetic nephropathy: Where hemodynamics meets metabolism. Exp Clin Endocrinol Diabetes 115:69–84.

Friedman EA. 1999. Advanced glycation end-products in diabetic nephropathy. Nephrol Dial Transplant 14(Suppl.3):1–9.

Garvin JL, Herrera M, Ortiz PA. 2011. Regulation of renal NaCl transport by nitric oxide, endothelin, and ATP: Clinical implications. Annu Rev Physiol 73:359–376.
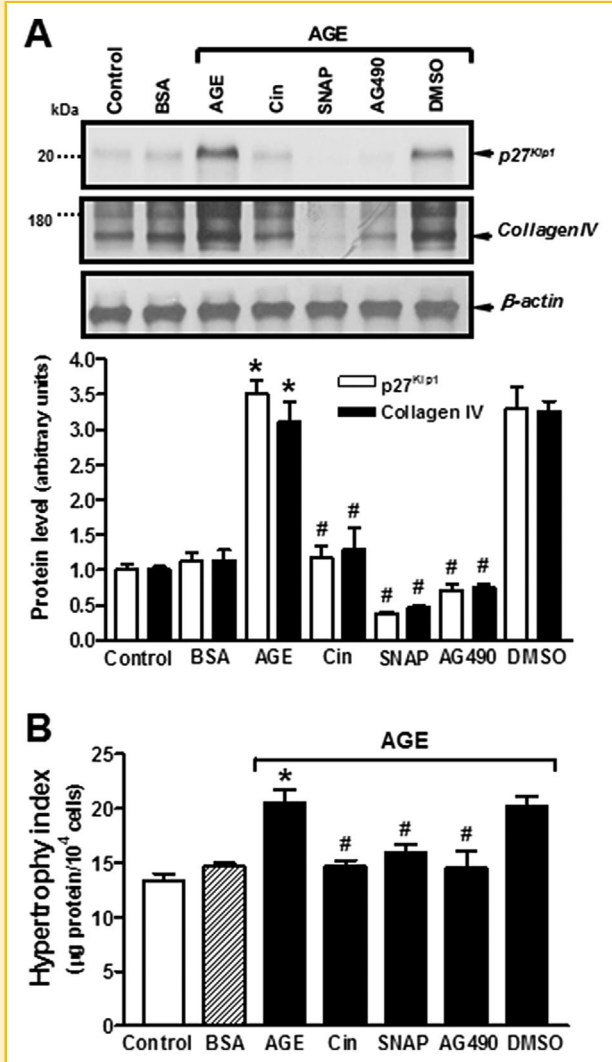
Giacco F, Brownlee M. 2010. Oxidative stress and diabetic complications. Circ Res 107:1058–1070.

Heineke J, Molkentin JD. 2006. Regulation of cardiac hypertrophy by intracellular signalling pathways. Nat Rev Mol Cell Biol 7:589–600.

Huang JS, Chuang LY, Guh JY, Huang YJ. 2009. Effects of nitric oxide and antioxidants on advanced glycation end products-induced hypertrophic growth in human renal tubular cells. Toxicol Sci 111:109–119.

Huisman A, Vos I, van Faassen EE, Joles JA, Gröne HJ, Martasek P, van Zonneveld AJ, Vanin AF, Rabelink TJ. 2002. Anti-inflammatory effects of tetrahydrobiopterin on early rejection in renal allografts: Modulation of inducible nitric oxide synthase. FASEB J 16:1135–1147.

Ito K, Chen J, El Chaar, Stern M, Seshan JM, Khodadadian SV, Richardson JJ, Hyman I, Vaughan MJ, Felsen DP. 2004. Renal damage progresses despite improvement of renal function after relief of unilateral ureteral obstruction in adult rats. Am J Physiol Renal Physiol 287:F1283–F1293.

Jianming L, Keqiang Z, Sangkil N, Richard AA, Richard J, Wei W. 2010. Novel angiogenesis inhibitory activity in cinnamon extract blocks VEGFR2 kinase and downstream signaling. Carcinogenesis 31:481–488.

Ka H, Park HJ, Jung HJ, Choi JW, Cho KS, Ha J, Lee KT. 2003. Cinnamaldehyde induces apoptosis by ROS-mediated mitochondrial permeability transition in human promyelocytic leukemia HL-60 cells. Cancer Lett 196:143–152.

Kiu H, Nicholson SE. 2012. Biology and significance of the JAK/STAT signalling pathways. Growth Factors 30:88–106.

Koh WS, Yoon SY, Kwon BM, Jeong TC, Nam KS, Han MY. 1998. Cinnamaldehyde inhibits lymphocyte proliferation and modulates T-cell differentiation. Int J Immunopharmacol 20:643–660.

Kone BC. 2004. Nitric oxide synthesis in the kidney: Isoforms, biosynthesis, and functions in health. Semin Nephrol 24:299–315.

Lee HB, Yu MR, Yang Y, Jiang Z, Ha H. 2003. Reactive oxygen species-regulated signaling pathways in diabetic nephropathy. J Am Soc Nephrol 14:S241–S245.

Linossi EM, Babon JJ, Hilton DJ, Nicholson SE. 2013. Suppression of cytokine signaling: The SOCS perspective. Cytokine Growth Factor Rev 24:241–248.

Marrero MB, Banes-Berceli AK, Stern DM, Eaton DC. 2006. Role of the JAK/STAT signaling pathway in diabetic nephropathy. Am J Physiol Renal Physiol 290:F762–F768.

Matsui F, Meldrum KK. 2012. The role of the Janus kinase family/signal transducer and activator of transcription signaling pathway in fibrotic renal disease. J Surg Res 178:339–345.

Niedowicz DM, Daleke DL. 2005. The role of oxidative stress in diabetic complications. Cell Biochem Biophys 43:289–330.

Okumura M, Masada M, Yoshida Y, Shintaku H, Hosoi M, Okada N, Konishi Y, Morikawa T, Miura K, Imanishi M. 2006. Decrease in tetrahydrobiopterin as a possible cause of nephropathy in type II diabetic rats. Kidney Int 70:471–476.

Pilz RB, Casteel DE. 2003. Regulation of gene expression by cyclic GMP. Circ Res 93:1034–1046.

Prabhakar S, Starnes J, Shi S, Lonis B, Tran R. 2007. Diabetic nephropathy is associated with oxidative stress and decreased renal nitric oxide production. J Am Soc Nephrol 18:2945–2952.

Prabhakar SS. 2001. Tetrahydrobiopterin reverses the inhibition of nitric oxide by high glucose in cultured murine mesangial cells. Am J Physiol Renal Physiol 281:179–188.

Satoh M, Fujimoto S, Arakawa S, Yada T, Namikoshi T, Haruna Y, Horike H, Sasaki T, Kashihara N. 2008. Angiotensin II type 1 receptor blocker ameliorates uncoupled endothelial nitric oxide synthase in rats with experimental diabetic nephropathy. Nephrol Dial Transplant 23:3806–3813.

Sato T, Iwaki M, Shimogaito N, Wu X, Yamagishi S, Takeuchi M. 2006. TAGE (toxic AGE) theory in diabetic complications. Curr Mol Med 6: 351–358.

Savard S, Lavoie P, Villeneuve C, Agharazii M, Lebel M, Larivière R. 2012. ENOS gene delivery prevents hypertension and reduces renal failure and injury in rats with reduced renal mass. Nephrol Dial Transplant 27:2182–2190.

Subash BP, Alshatwi AA, Ignacimuthu S. 2014. Beneficial antioxidative and antiperoxidative effect of cinnamaldehyde protect streptozotocin-induced pancreatic β-cells damage in wistar rats. Biomol Ther 22: 47–54.

Subash BP, Prabuseenivasan S, Ignacimuthu S. 2007. Cinnamaldehyde–a potential antidiabetic agent. Phytomedicine 14:15–22.

Sucher R, Gehwolf P, Oberhuber R, Hermann M, Margreiter C, Werner ER, Obrist P, Schneeberger S, Ollinger R, Margreiter R, Brandacher G. 2010. Tetrahydrobiopterin protects the kidney from ischemia-reperfusion injury. Kidney Int 77:681–689.

Suzuki D, Toyoda M, Yamamoto N, Miyauchi M, Katoh M, Kimura M, Maruyama M, Honma M, Umezono T, Yagame M. 2006. Relationship between the expression of advanced glycation end-products (AGE) and the receptor for AGE (RAGE) mRNA in diabetic nephropathy. Intern Med 45:435–441.

Takasao N, Tsuji-Naito K, Ishikura S, Tamura A, Akagawa M. 2012. Cinnamon extract promotes type I collagen biosynthesis via activation of IGF-I signaling in human dermal fibroblasts. J Agric Food Chem 60: 1193–1200.

Tan AL, Forbes JM, Cooper ME. 2007. AGE, RAGE, and ROS in diabetic nephropathy. Semin Nephrol 27:130–143.

Tanji N, Markowitz GS, Fu C, Kislinger T, Taguchi A, Pischetsrieder M, Stern D, Schmidt AM, D'Agati VD. 2000. Expression of advanced glycation end products and their cellular receptor RAGE in diabetic nephropathy and nondiabetic renal disease. J Am Soc Nephrol 11:1656–1666.

Terrell AM, Crisostomo PR, Wairiuko GM, Wang M, Morrell ED, Meldrum DR. 2006. Jak/STAT/SOCS signaling circuits and associated cytokine-mediated inflammation and hypertrophy in the heart. Shock 26:226–234.

Trengove MC, Ward AC. 2013. SOCS proteins in development and disease. Am J Clin Exp Immunol 2:1–29.

Tung YT, Chua MT, Wang SY, Chang ST. 2008. Anti-inflammation activities of essential oil and its constituents from indigenous cinnamon (*Cinnamomum osmophloeum*) twigs. Bioresour Technol 99:3908–3913.

Turgut F, Bolton WK. 2010. Potential new therapeutic agents for diabetic kidney disease. Am J Kidney Dis 55:928–940.

Zhang M, Takimoto E, Hsu S, Lee DI, Nagayama T, Danner T, Koitabashi N, Barth AS, Bedja D, Gabrielson KL, Wang Y, Kass DA. 2010. Myocardial remodeling is controlled by myocyte-targeted gene regulation of phosphodiesterase type 5. J Am Coll Cardiol 56:2021–2030.

Zhang W, Xu YC, Guo FJ, Meng Y, Li ML. 2008. Anti-diabetic effects of cinnamaldehyde and berberine and their impacts on retinol-binding protein 4 expression in rats with type 2 diabetes mellitus. Chinese Medical J 121:2124–2128.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.