The Journal of Applied Pharmacology, 13, 190-197(2005)

Role of Advanced Glycation End Products in TGF-β1 and Fibronectin Expression in Mesangial Cells Cultured under High Glucose

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(Received August 8, 2005; Accepted September 20, 2005)

Abstracts – Advanced glycation end products (AGE) have been implicated in the pathogenesis of diabetic complications including nephropathy. However, the role of AGE in the activation of mesangial cells cultured under high glucose has not been elucidated. The effects of aminoguanidine, which prevents formation of AGE and protein cross-linking, on the synthesis of TGF-β1 and fibronectin by rat mesangial cells cultured under high glucose for 2 weeks were examined and compared with the effects of N^G-nitro-*L*-arginine methyl ester (NAME), a selective nitric oxide synthase inhibitor, because aminoguanidine also inhibits the inducible nitric oxide synthase. Culture of mesangial cells in 30 mM (high) glucose for 2 weeks induced 1.5-fold (ELISA) and 1.9-fold (Western blot analysis) increase in AGE in the culture media compared to 5.6 mM (control) glucose. Northern blot analysis revealed 1.5-fold increase in TGF-β1 and 1.7-fold increase in fibronectin mRNA expression in cells cultured under high glucose compared to control glucose. Increases in mRNA expression were followed by increased protein synthesis. Mink lung epithelial cell growth inhibition assay revealed 1.4-fold increase in TGF-β1 protein in high glucose media compared to control. Fibronectin protein also increased 2.1-fold that of control glucose by Western blot analysis. Administration of aminoguanidine suppressed AGE formation in a dose dependent manner and at the same time suppressed TGF-β1 and fibronectin synthesis by mesangial cells cultured in both control and high glucose. In contrast, NAME did not affect high glucose-induced changes. These findings support a role for AGE in high glucose-induced upregulation of TGF-β1 and fibronectin synthesis by mesangial cells.

Keywords □ advanced glycation end products, aminoguanidine, transforming growth factor-β1, fibronectin, mesangial cells

INTRODUCTION

High glucose-induced nonenzymatic glycosylation of proteins involves a complex cascade of condensation, rearrangements, fragmentations, and oxidative modifications that lead to sugar-amino acid adducts collectively termed advanced glycation end products (AGE). AGE accumulate in tissues as a function of time and sugar concentration and have been implicated in the pathogenesis of diabetic complications and aging (Brownlee et al., 1988; Brownlee, 1995).

A causal relationship between AGE and diabetic nephropathy has been established based on the observations that AGE accumulate in diabetic kidneys of both animals (Tomino *et al.*, 1991; Mitsuhashi *et al.*, 1993; Yang *et al.*, 1995; Lee *et al.*, 1997; Soulis *et al.*, 1997) and humans (Miyata and Monnier, 1992; Makino *et al.*, 1995), that administration of AGE prepared in vitro induces genes implicated in diabetic glomerular injury in normal rats (Yang *et al.*, 1994; Yang *et al.*, 1995) as well as cultured mesangial cells (Skolnik *et al.*, 1991; Doi *et al.*, 1992; Ha H *et al.*, 1997; Pugliese *et al.*, 1997) and reproduces glomerulosclerosis and albumiuria in normal animals (Vlassare *et al.*, 1994), and that aminoguanidine ameliorates all the manifestations of diabetic nephropathy (Lee *et al.*, 1997; Soulis *et al.*, 1997; Yang *et al.*, 1994).

Long-term exposure of mesangial cells to high glucose significantly increases transforming growth factor (TGF)-β1 and extracellular matrix (ECM) production (Pricci *et al.*, 1996; Pugliese *et al.*, 1996; Craven *et al.*, 1997) which are two prominent features of diabetic nephropathy (Steffes *et al.*, 1989; Ziyadeh and Han, 1997). Unlike initial proposal that AGE are formed on long-lived ECM macromolecules, high glucose rapidly increases intracellular AGE in cultured endothelial cells

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(Giardino *et al.*, 1994; Stitt *et al.*, 1995; Giardino *et al.*, 1996; Shinohara *et al.*, 1998). These studies suggest possible involvement of AGE in TGF-β1 and ECM mRNA and protein expression in mesangial cell cultured under high glucose for a long period (Pricci *et al.*, 1996; Pugliese *et al.*, 1996; Craven *et al.*, 1997). However, the role of AGE in high glucose-induced mesangial cell activation has not been elucidated. The present study assessed the role of AGE in TGF-β1 and fibronectin synthesis by rat mesangial cells. AGE were measured in mesangial cell conditioned media by ELISA. Aminoguanidine was employed as a pharmacological maneuver to inhibit AGE formation. Since aminoguanidine is also a highly potent, relatively selective inhibitor of inducible nitric oxide synthase (Corbett and McDaniel, 1996), the effect of aminoguanidine was compared with that of N^G-nitro-*L*-arginine (NAME), a selective inhibitor of nitric oxide synthase in order to determine the contribution of nitric oxide inhibition in the effect of aminoguanidine on high glucose-induced TGF-β1 and fibronectin synthesis by rat mesangial cells.

METHODS

All chemicals and tissue culture plates, unless otherwise stated, were obtained from Sigma Chemical Company (St. Louis, MO, USA) and Becton Dickinson Labware (Lincoln Park, NJ, USA).

Mesangial cell culture

Mesangial cells were obtained by culturing glomeruli isolated from kidneys of male Sprague-Dawley rats by conventional sieving methods as previously described (Harper *et al.*, 1984; Oh *et al.*, 1998). Cells between passages 6 and 10 were used for experiment. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 20% fetal bovine serum (FBS; GIBCO BRL, Gaitherburg, MD, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 μg/ml fungizone, 44 mM $NaHCO_3$, and 14 mM N-2-hydroxy-ethylpiperazine-N'-2-ethane sulfonic acid (HEPES). $2x10^5$ cells were plated in 100mm culture dish and grown in media containing 5.6 (control) or 30 mM (high) glucose in the presence or absence of aminoguanidine or NAME for 2 weeks. Media were changed every 24 h to maintain control or high glucose concentration. When cells were confluent, media were changed to serum-free DMEM for 48 h to detect fibronectin produced in the absence of preformed fibronectin in the serum. Cell viability was not affected by our experimental conditions as examined by lactate dehydrogenase release. The media were then collected, centrifuged to remove cell debris, and used for bioassay of TGF-β1 and immunoblot analysis of fibronectin and AGEs after measuring the concentration of protein by the Bradford method (Bradford 1976) using the Bio-Rad assay (Richmond, CA, USA). After removing the media, cells were washed with phosphate buffered saline (PBS), then lysed using 1 ml of a mixture consisting of 4 M guanidium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol for RNA isolation.

Northern blot analysis

Standard Northern blot was performed as previously described (Oh *et al.*, 1998) after isolation of total RNA using the method of Chomczynski and Sacchi (1989). Twenty ìg of total RNA was electrophoresed through a 1.2% agarose gel with 2.2 M formaldehyde. The RNA from the gel was transferred onto nylon membranes using a capillary transfer and covalently cross-linked to the membrane with UV light using a gene-linker (Bio-Rad, Richmond, CA, USA).

Hybridization was conducted for 20 h at 42°C using excised cDNA inserts as probes after [^{32}P]dCTP-labeling by a random primer extension method (Pharmacia Biotech, Uppsala, Sweden). The membranes were washed three times for 30 min: first in 2x sodium chloride sodium citrate (SSC; 1x SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) with 0.1% sodium dodecyl sulfate (SDS) at room temperature; second in 0.2x SSC with 0.1% SDS at room temperature; and third in 0.2x SSC with 0.1% SDS at 55°C. Autoradiography was performed by exposing the blots on Kodak X-Omat K XK-1 X-ray film with intensifying screens at -70°C for one to five days. Quantitation of mRNA signals was performed by densitometry using MCID (Imaging Research.,St. Catharines, Ontario, Canada) and normalized with GAPDH mRNA signals.

ELISA

AGE in the mesangial cell conditioned media were measured by a competitive ELISA as described by Makita et al (1992). In brief, 96-well microtiter plates (Nunc Immunoplate, GIBCO BRL) were coated with AGE-BSA (10 μg/ml, dissolved in PBS) and incubated overnight at 4°C. Wells were washed three times with 0.15 ml of a solution containing PBS, 0.05% Tween 20, and 1 mM NaN_3 (PBS-Tween). Wells were then blocked with Super Block (Pierce, Rockford, IL, USA), a blocking buffer in PBS, for 1 h at room temperature. After washing with PBS-Tween, 50 μl of competing antigen was

added, followed by 50 μl of polyclonal anti-AGE antibody (gift from Drs. Helen Vlassara and Richard Bucala, Picower Institute for Medical Research, Manhasset, NY, USA; final dilution 1:1,000). This polyclonal antiserum to an AGE epitope(s) which forms in vitro after incubation of glucose with ribonuclease has been shown not to recognize known AGE structures such as 4-furnyl-2-furoyl-1H-imidazole, 1-alkyl-2-formyl-3,4-di- glycosylpyrrol, pyrraline, carboxymethyllysine, or pentosidine (Makita etla l., 1992). Plates were incubated for 2 h at room temperature. Wells were then washed with PBS-Tween and reacted with an alkaline phosphatase-linked anti-rabbit IgG (Organon Tenika Corperation, Durham, NC, USA) for 1 h at 37°C followed by development utilizing 3,3',5,5'-tetramethylbenzidine (Kirkegaard & Perry Laboratory Inc, Gaithersburg, MD, USA) as the colorimetric substrate. AGE- BSA standard 25 μg/ml inhibited antiserum binding by 50% under our experimental condition. Obtained values of AGE were normalized by equal concentration of protein in the media.

Bioassay for TGF-β activity

Preliminary experiments using Western blot analysis revealed increased TGF-β1 protein in the supernatant of rat mesangial cells cultured under high glucose. To determine the biological activity of TGF-β1 present in the conditioned media, the mink lung epithelial cell (MLEC) growth inhibition assay was performed as described previously (Oh *et al.*, 1998). Human recombinant TGF-β1 (Genzyme; 0.64-10,000 pg/ml) was used to generate a standard curve. Preliminary study revealed detectable TGF-β activity in the conditioned media without activation. Thus, the activation of latent TGF-β was not performed. In brief, MLEC CCL64 cells American Type Culture Collection (ATCC; Rockville, MD, USA) were grown in 96-well culture plates with RPMI containing 5% FBS and conditioned media diluted to contain concentrations of TGF-β1 within the detection range (20 to 2000 pg/ml) of the assay for 96 h. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) was then added and cells were incubated for another 8 h. Elution buffer was added and cells were incubated for additional 48 h and optical density was measured at 526 nm. Unconditioned media containing 30 mM glucose, 5 mM aminoguanidine, or 5 mM NAME did not exhibit discernible effect on MLEC growth.

Western blot analysis

Immunoblot analysis was performed to determine the synthesis and secretion of fibronectin and AGE into rat mesangial cell conditioned media (Oh *et al.*, 1998). Aliquots of conditioned media having equal amount of protein were mixed with sample buffer containing SDS and β-mercaptoethanol and heated at 95°C for 15 min. Samples were applied to 5% polyacrylamide gel and electrophoresed. A prestained SDS-PAGE standard (broad range, Bio-Rad) was also electrophoresed as a molecular weight marker. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane using a transblot chamber with Tris buffer. Western blots were incubated with appropriate antibodies (rabbit anti-mouse fibronectin, rabbit anti-AGE) for 2 h at room termperature. The membranes were washed with PBS-Tween 20 for 1 h and incubated with peroxidase-conjugated secondary antibody (rabbit anti-IgG) for 2 h at room temperature. After washing, the membranes were incubated with a color reagent (50 mg 3,3'-diaminobenzidine, 0.1 ml H_2O_2 in 100 ml PBS) for 15 min for AGE. Fibronectin immune complexes were visualized using an ECL kit (Amersham International, Amersham, UK). To quantify the difference in fibronectin and AGE amount, semiquantitative Western analysis was performed by comparing the control sample with experimental groups.

Analysis of data

All results are expressed as means±SE. Analysis of variance was used to assess the differences between multiple groups. If the F statistic was significant, the mean values obtained from each group were then compared by Fisher's least significant difference method. $P<0.05$ was used as the criterion for a statistically significant difference.

RESULTS

High glucose-induced AGE formation by rat mesangial cells and effect of aminoguanidine

Western blot analysis of the conditioned media after cell culture for 2 weeks demonstrated several protein bands between 48 and 118 kDa that were immunoreactive with anti-AGE antibody (figure 1). The amount of AGE-modified proteins did not increase in the mesangial cell conditioned media during the first 1 week of exposure to high glucose. However, after culture for 2 weeks 1.9-fold increase in total AGEs and 2.6-fold increase in 60 kDa AGEs were observed in high glucose media as compared to control glucose media. Aminoguanidine inhibited high glucose-induced AGE formation in a dose-dependent manner (figure 1a). Levels of 60 kDa AGEs in the media from high glucose in the presence of aminoguanidine were 2.6-fold

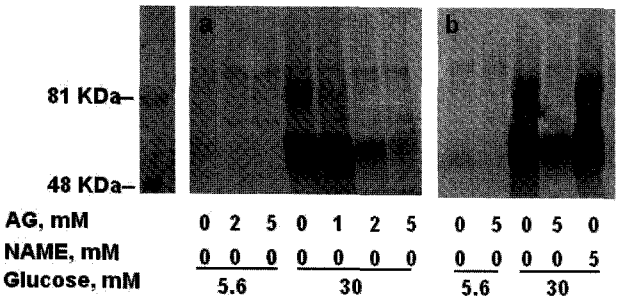

Fig. 1. Immunoblot of AGEs from rat mesangial cell conditioned media. All lanes were loaded with 2 μg of total proteins. The immune complexes were visualized by using horseradish peroxidase-linked anti-rabbit IgG. Mesangial cells cultured under 30 mM glucose for 2 weeks produced several proteins immunoreactive with anti-AGE antibody, all of which were inhibited by aminoguanidine (AG) in a dose-dependent manner (a). NAME did not inhibit high glucose-induced AGE formation by rat mesangial cells (b).

that of control glucose at 1 mM, 1.5-fold at 2 mM, and 1.1-fold at 5 mM. Five mM aminoguanidine which nearly completely blocked AGE formation under high glucose was used for subsequent studies. On the other hand, 5 mM NAME did not inhibit high glucose-induced AGE formation (figure 1b).

High glucose-induced AGE formation and inhibition by aminoguanidine were also quantitated by ELISA as summarized in figure 2. The levels of AGE expressed as % of total AGE formed in control glucose (100%) alone were 59±16, 147±16, and 71±5% for control glucose in the presence of 5 mM aminoguanidine, high glucose alone, and high glucose in the presence of 5 mM aminoguanidine, respectively. Protein

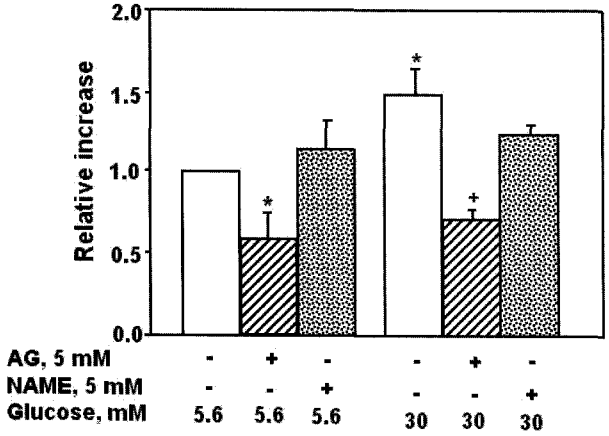

Fig. 2. Inhibitory effect of aminoguanidine (AG) on AGE formation by rat mesangial cells as examined by ELISA. Values are expressed as means±S.E. from three to five experiments. *P< 0.05 vs. 5.6 mM glucose; +P<0.05 vs. 30 mM glucose.

concentration in the conditioned media varied in rat mesangial cells that were exposed to control glucose (range 0.07 to 0.13 mg/ml of media) or high glucose (range 0.09 to 0.13 mg/ml of media) because the cells used were in different passage and/or from different origin of cell line. When the levels of protein in the media were expressed as % of the paired sample maintained in control glucose alone (100%), they were 92% in control glucose in the presence of 5 mM aminoguanidine, 86% in control glucose in the presence of 5 mM NAME, 106% in high glucose alone, 120% in high glucose in the presence of 5 mM aminoguanidine, and 89% in high glucose in the presence of 5 mM NAME. This finding suggests that total protein produced by mesangial cells were not altered by different experimental condition.

High glucose-induced TGF-β1 mRNA expression and protein synthesis by rat mesangial cells and effect of aminoguanidine

As shown in figure 3, TGF-β1 mRNA levels of rat mesangial cells increased by 1.5-fold that of control glucose after 2-week exposure to high glucose. Five mM aminoguanidine, but not NAME, effectively inhibited high glucose-induced TGF-β1 mRNA expression. On MLEC growth inhibition assay high glucose induced 1.4-fold increase in TGF-β protein synthesis and secretion into the media (figure 4). High glucose-induced TGF-β production was effectively inhibited by treatment with 5 mM aminoguanidine but not by NAME. Aminoguanidine at 5 mM also blocked TGF-β production by rat mesangial cells

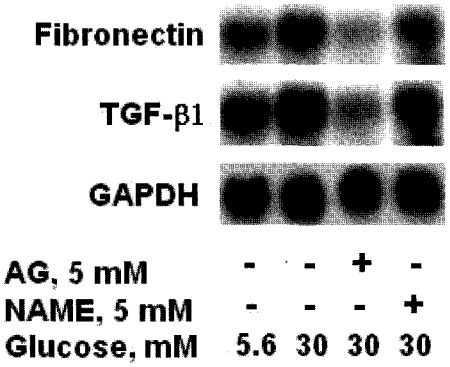

Fig. 3. Effects of aminoguanidine (AG) on high glucose-induced mesangial cell TGF-β1 and fibronectin mRNA expression. The upper panel shows a representative Northern blot. Values are means±S.E. from three experiments. 1, 5.6 mM (control) glucose; 2, 30 mM (high) glucose; 3, high glucose in the presence of 5 mM aminoguanidine; 4, high glucose in the presence of 5 mM NAME. *P<0.05 vs. 5.6 mM glucose; +P< 0.05 vs. 30 mM glucose.

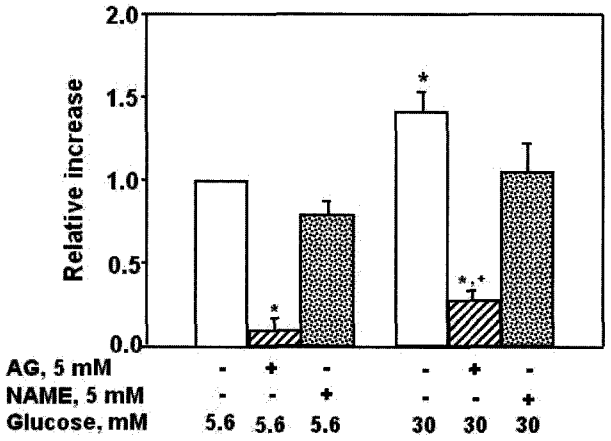

Fig. 4. Measurement of TGF-β like activities in the mesangial cell conditioned media using CCL64 cell growth inhibition assay. Values are means±S.E. from three to five experiments. *P <0.05 vs. 5.6 mM glucose; +P<0.05 vs. 30 mM glucose. Control values are 53.6±21.4 pg/mg of protein.

under control glucose.

High glucose-induced fibronectin mRNA expression and protein production by rat mesangial cells and effect of aminoguanidine

Changes of fibronectin mRNA expression by rat mesangial cells in response to high glucose, aminoguanidine, and NAME were qualitatively similar to the changes observed in TGF-β synthesis. Exposure of mesangial cells to high glucose for 2 weeks induced 1.7-fold increase in the level of fibronectin mRNA. Five mM aminoguanidine, but not NAME, effectively inhibited these changes (figure 3).

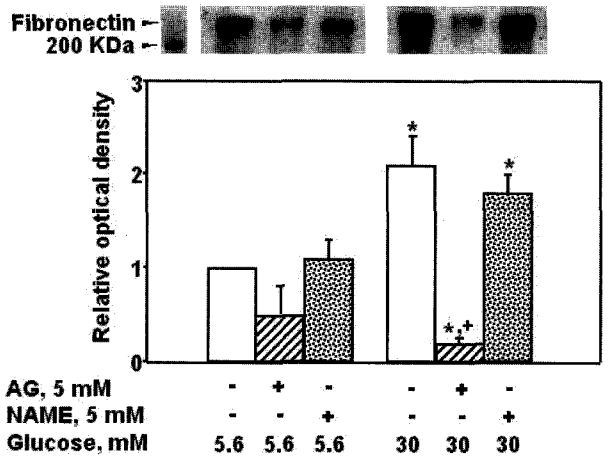

Fig. 5. Effect of aminoguanidine (AG) on high glucose-induced fibronectin protein production by rat mesangial cells. All lanes were loaded with 200 ng of total proteins. The immune complexes were visualized by ECL. The upper panel shows a representative Western blot. Values are means±S.E. from three to five experiments. *P<0.05 vs. 5.6 mM glucose; +P<0.05 vs. 30 mM glucose.

Western blot analysis of mesangial cell conditioned media showed that incubation of cells with high glucose increased the intensity of immunoreactive bands, an index of enhanced fibronectin protein synthesis and secretion into media (figure 5). Quantitative scanning revealed 2.1-fold increase under high glucose and this was effectively inhibited by addition of 5 mM aminoguanidine but not by NAME. Aminoguanidine at 5 mM also reduced fibronectin production by rat mesangial cells under control glucose.

DISCUSSION

Our present study demonstrated that AGE are formed and secreted during 2-week culture of rat mesangial cells under 30 mM glucose and that AGE play an important, if not exclusive, role in the high glucose-induced upregulation of TGF-β1 and fibronectin mRNA and protein expression in mesangial cells cultured under high glucose.

Rat mesangial cells cultured in high glucose for 2 weeks under our experimental condition produced significantly more immunoreactive AGE than cells cultured in control glucose. Compared to accumulation of cytosolic AGE in endothelial cells cultured under high glucose (Giardino *et al.*, 1994; Giardino *et al.*, 1996; Shinohara *et al.*, 1998), the increment of AGE in the present study was much less (less than 3-fold vs. 14-fold that of control glucose) and slower (2 weeks vs. 1 week). These differences may be due to the fact that we measured AGE which were formed within mesangial cells and secreted into media or which may have been formed within the media but not cytosolic AGE. Although intracellular AGE formed in rat mesangial cells cultured under high glucose has not been directly demonstrated, time-related cytosolic AGE formation was postulated to activate mesangial cell leading to ECM accumulation (Pricci *et al.*, 1996). Since aminogunidine also inhibited AGE in the media of rat mesangial cells cultured under control glucose, cells continuously exposed to 5.6 mM glucose for 2 weeks without subculturing appear to produce appreciable amount of AGE.

The significance of high glucose-induced AGE formation by rat mesangial cells was evaluated in terms of TGF-β1 and fibronectin mRNA and protein expression. In agreement with previous studies where mesangial cells were exposed to high glucose for up to 4 weeks (Pricci *et al.*, 1996; Pugliese *et al.*, 1996; Craven *et al.*, 1997), high glucose increased TGF-β1 and

fibronectin mRNA and protein expression. Aminoguanidine inhibited high glucose-induced changes at the concentration that nearly completely blocked AGE formation, strongly suggesting that AGE play an important role in high glucose-induced ECM synthesis by mesangial cells. These findings support the results of previous in vivo studies (Lee *et al.*, 1997; Soulis *et al.*, 1997; Yang *et al.*, 1994) which suggested an important pathogenic role for AGE in diabetic nephropathy. Since exogenously administered AGE have been shown to upregulate expression of mRNAs and proteins of growth factors and ECM by mesangial cells by binding with AGE-specific receptors (Skolinik *et al.*, 1991; Doi *et al.*, 1992; Ha *et al.*, 1997; Pugliese *et al.*, 1997; Thornally, 1998), it is not surprising to find that AGE in the media activate rat mesangial cells.

One may argue that the effect of aminoguanidine demonstrated in the present study may be attributable to inhibition of other effect of glucose than formation of AGE, since there was not enough time for accumulation of AGE in the media in the present study and aminoguanidine also inhibited AGE formation in cells cultured under control glucose. High glucose-induced activation of enzymatic glucose metabolism (Kolm-Litty *et al.*, 1998; Bleyer *et al.*, 1994), protein kinase C (King *et al.*, 1997), oxidative stress (Ha *et al.*, 1997), and transcription (Hoffmann *et al.*, 1998) are known to increase TGF-β1 and ECM synthesis by mesangial cells. Although the effects of aminoguanidine on these direct effects of high glucose are not known, aminoguanidine is a nucelophilic hydrazine derivative, has a variety of pharmacological effects due to its reducing properties, and condenses readily with aldehydes, ketones, and sugars. Inhibition of both diamine oxidase (Yu and Zuo, 1997) and the inducible nitric oxide synthase (Corbett and McDaniel, 1992) are known pharmacological effects of aminoguanidine in addition to inhibition of AGE formation. Since polyamines are essential for cell growth, proliferation, and differentiation (Heby and Persson, 1990), excessive polyamine accumulation by aminoguanidine could influence ECM accumulation.

The contribution of nitric oxide synthase inhibition to the observed effect of aminoguanidine was evaluated by examining the effect of NAME, a selective nitric oxide synthase inhibitor. The concentration of aminoguanidine used in the present study (5 mM) is far above reported IC50 values for aminoguanidine on inhibition of nitric oxide synthesis (Corvett and McDaniel, 1996). NAME had no effect on high glucose-induced AGE formation or TGF-β1 and fibronectin mRNA and protein expression by mesangial cells, suggesting that the inhibitory effect of aminoguanidine on high glucose-induced mesangial cell activation is mainly due to inhibition of AGE formation. Although nitric oxide concentration was not measured under our present experimental condition, concentration of NAME used in the present study (5 mM) is considered to be sufficient to inhibit nitric oxide production. NAME 1 mM effectively inhibited nitric oxide production in mesangial cells (Trachtman *et al.*, 1995). Lack of NAME's effect on high glucose-induced TGF-β1 and fibronectin synthesis by rat mesangial cell is in agreement with current understanding of the role of nitric oxide on diabetic nephropathy. While initial increase of nitric oxide synthesis in diabetic kidney may result in renal hyperperfusion (Tilton *et al.*, 1993), decreased nitric oxide has been suggested to have a role in mesangial expansion in later stage of diabetic nephropathy (Craven *et al.*, 1997). In fact, nitric oxide production was reduced in mesangial cells cultured under high glucose (Trachtman *et al.*, 1997) and nitric oxide reduced production of collagen and fibronectin by mesangial cells (Craven *et al.*, 1997; Trachtman *et al.*, 1995).

In conclusion, our present study demonstrated for the first time that AGE are produced during long-term culture of rat mesangial cells under high glucose and supports a role for AGE in high glucose-induced upregulation of TGF-β1 and fibronectin synthesis by rat mesangial cells.

ACKNOWLEDGMENTS

This work was supported by a grant from Korea Research Foundation (KRF-2001-041-F00109). The authors are grateful to Drs. Helen Vlassara and Richard Bucala of the Picower Institute for Medical Research, Manhasset, NY for the generous gift of anti-AGE antibody. Technical assistance from Ms. Mi-Ra Yu is acknowledged.

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