Role of Angiotensin II in Glucose-Induced Inhibition of Mesangial Matrix Degradation

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Accumulation of mesangial matrix in diabetic nephropathy is caused by increased synthesis and decreased degradation. We have previously demonstrated that incubation in high-glucose medium decreases mesangial cell collagenase activity (*Diabetes* 44:929–935, 1995). Because angiotensin II (AII) is involved in the pathogenesis of diabetic nephropathy, the present studies were performed to determine if AII mediates glucose-induced *1*) inhibition of mesangial collagenase activity, *2*) mesangial matrix accumulation, and *3*) in-crease in transforming growth factor (TGF)-β1 secretion in mesangial cells. The direct effect of high glucose on AII generation in mesangial cells was also determined. Primary mesangial cells from normal Sprague-Dawley rats were used in all studies. Collagenase activity in cell medium was determined using three methods: *1*) zymography; *2*) quantitative assay using fluoresceinated gelatin as substrate; and *3*) a new enzyme-linked immunosorbent assay (ELISA) that specifically measures 72-kDa collagenase (MMP-2), the principal collagenase synthesized by mesangial cells. Matrix accumulation was estimated by immunoperoxidase assay on cell layers using anti–glomerular basement membrane (GBM) antibodies. TGF-β1 and AII levels were determined by ELISA. Exposure of mesangial cells to 30 mmol/l glucose (high glucose) vs. 5 mmol/l glucose (normal glucose) for 5 days resulted in a significant decrease in collagenase activity (25%) that was normalized by 10^{-4} mol/l losartan, a type 1 angiotensin II (AT_1) receptor antagonist. High glucose increased anti-GBM binding compared with normal glucose; this effect of glucose was reversed by losartan. Incubation of cells with 30 mmol/l glucose increased total TGF-β1 secretion, which was also normalized by losartan. Addition of AII (10^{-6} mol/l) for 24 h to the culture medium inhibited collagenase activity by 33%; losartan (10^{-4} mol/l) blocked this inhibition of enzyme activity. Also, AII decreased collagenase (MMP-2) levels but stimulated TGF-β1 secretion in mesangial cells. Finally, glucose increased mesangial AII generation in a concentration-dependent manner, with incubation in 30 mmol/l glucose increasing AII by 25% compared with 5 mmol/l glucose. We conclude that glucose increases AII production by mesangial cells, which results in stimulation of TGF-β1 secretion, decreased matrix degradation, and increased matrix accumulation. These effects of AII are mediated by the AT_1 receptor. *Diabetes* 48:2066–2073, 1999

Diabetic nephropathy is characterized by accumulation of extracellular matrix in the glomeruli, which eventually leads to proteinuria and renal failure. Increased synthesis and accumulation of extracellular matrix components such as collagen have been demonstrated in diabetic glomeruli (1) and mesangial cells incubated in high-glucose medium (2). Recent data from our laboratory and those of other investigators indicate that matrix degradation and degradative enzyme (e.g., collagenase) activity are also impaired by elevated glucose, suggesting that degradative mechanisms are inadequate to prevent accumulation of matrix material (3,4). Moreover, the changes in matrix degradation may be more marked than the increase in matrix protein synthesis, suggesting that degradative mechanisms could be as important in regulation of matrix accumulation (5).

The mediators of mesangial expansion in diabetic nephropathy have not been fully identified. A prominent role for the peptide angiotensin II (AII) has been suggested by the beneficial effects of ACE inhibitors in clinical diabetic renal disease (6,7) and AII receptor antagonists in experimental diabetes (8,9). The ACE inhibitor enalapril inhibits gene expression of extracellular matrix proteins in diabetic rats (10), suggesting an important role for AII in the development of glomerular sclerosis. In mesangial cell culture, several studies have reported that AII increases synthesis of matrix proteins such as collagen type I and fibronectin (11–13); these effects of AII are mediated via the type 1 angiotensin II (AT_1) receptor (13). The cytokines AII and transforming growth factor (TGF)-β1 have both been implicated in the pathogenesis of increased matrix protein synthesis under high-glucose conditions (14,15). However, the role of AII in impaired mesangial matrix degradation in mesangial cell culture has not previously been investigated.

The aim of these studies was to determine if AII has modulatory effects on matrix degradation under high-glucose conditions, because both synthesis and degradation are key factors in net accumulation of mesangial matrix in diabetic nephropathy. Studies were undertaken to examine if AII

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AII, angiotensin II; ANOVA, analysis of variance; AT_1, type 1 angiotensin II; B-AII, biotinylated angiotensin II; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; GBM, glomerular basement membrane; HG, high glucose (30 mmol/l); HM, 5 mmol/l glucose + 25 mmol/l mannitol; HRP, horseradish peroxidase; MMP, matrix metalloproteinase; NG, normal glucose (5 mmol/l); PBS, phosphate-buffered saline; TGF, transforming growth factor; TMB, 3,3′,5,5′-tetramethyl benzidine dihydrochloride.

mediates high glucose–induced inhibition of collagenase (degradative) enzyme activity and increased collagen accumulation in mesangial cells.

Furthermore, because glucose is known to stimulate TGF-β1 secretion in mesangial cells (16), we examined if this effect of high glucose is mediated by AII. Finally, because a direct effect of glucose on AII generation in renal cells has not previously been reported, we determined the effect of high glucose on AII generation in mesangial cells.

RESEARCH DESIGN AND METHODS

Culture of mesangial cells. All experiments were done using primary mesangial cells obtained from normal rat glomeruli as described below. Glomeruli were isolated from kidneys of male Sprague-Dawley rats (100–150 g) (Harlan, Indianapolis, IN) using a graded-sieving method using stainless steel and nylon meshes under sterile conditions, described previously (4). Primary culture of mesangial cells was established by plating the washed glomerular cores in RPMI 1640 tissue culture medium containing 12% fetal calf serum, 0.67 U/ml insulin, penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were grown at 37°C in 5% CO_2, 95% air in 24-well cell culture plates. The identity of mesangial cells was confirmed by phase contrast microscopy according to the morphologic criteria (stellate-shaped cell bodies with irregular cytoplasmic projections). All experiments were performed after a period of 4–5 weeks when the cells were confluent.

Treatment with AII receptor antagonist, AII, or both. To study the role of AII in mediating the effects of high glucose on mesangial matrix degradation, cells were exposed to 30 mmol/l glucose (high glucose) with or without 10^{-4} mol/l losartan for 5 days. Mesangial cells incubated in 5 mmol/l glucose (normal glucose) under similar conditions served as controls. At the end of the treatment period, cell media were collected for determination of collagenase activity; cells were used for estimation of collagen accumulation. All experiments were performed under serum-free conditions and in the absence of insulin.

The effect of exogenous AII on mesangial collagenase activity was studied by incubating the cells in normal glucose medium containing 10^{-6} mol/l AII in the presence or absence of the AII receptor antagonist, losartan (10^{-4} mol/l). Incubations were terminated after 24 h, after which cell media were collected and collagenase activity was determined. In separate experiments, effects of different AII concentrations (10^{-8} to 10^{-6} mol/l) on collagenase (matrix metalloproteinase-2 [MMP-2]) and TGF-β1 levels in mesangial cell medium were also examined.

Zymography. The presence of collagenase IV (gelatinase) activity in mesangial cell culture medium was determined by SDS substrate gel electrophoresis (4). In brief, samples of cell media were dialyzed, lyophilized, and reconstituted at a known protein concentration. Gelatin (1 mg/ml) as substrate was incorporated into a 8% polyacrylamide gel. Sample protein (25 µg) was mixed with sample buffer (lacking β-mercaptoethanol) and electrophoresed under nonreducing conditions. After removal of SDS by washing in 2.5% Triton X-100, gels were incubated at 37°C overnight in 50 mmol/l Tris-HCl (pH 8.0) containing 5 mmol/l $CaCl_2$ and 1 µmol/l $ZnCl_2$. Gels were stained with Coomassie blue, and gelatin-degrading enzymes were identified by their ability to degrade gelatin at a given molecular weight.

Determination of free collagenase activity. Collagenase activity was determined by a fluorometric assay previously described (4). Briefly, media collected from cultured cells were dialyzed, lyophilized, and reconstituted at a known protein concentration. Samples (25 µl) were preincubated with 4.2 mU/ml of purified plasmin (Sigma, St. Louis, MO) at 37°C for 40 min to activate latent collagenase. A gelatin-fluorescein conjugate (1:2 molar ratio) was diluted 1:100 in 100 mmol/l Tris-HCl (pH 7.4) containing 5 mmol/l $CaCl_2$ and added to each sample at a concentration of 10 µg gelatin/sample. Samples were incubated at 37°C for 1 h, after which 4 ml distilled water was added to each sample. The fluorescein in each sample tube was measured using excitation and emission wavelengths of 480 and 525 nm, respectively. Collagenase activity was calculated as level of gelatin degraded compared with maximal degradation by clostridial collagenase, included as standard control in the assay.

Measurement of collagenase (MMP-2). Concentration of collagenase (MMP-2) in mesangial cell medium was measured by a new enzyme-linked immunosorbent assay (ELISA) (Amersham Pharmacia Biotech, Piscataway, NJ). This assay specifically measures the amount of free MMP-2 present in the samples (the older ELISA detects MMP-2 that is free and bound to tissue inhibitors of metalloproteinases). Measurement of active MMP-2 is made possible by its reaction with a detection enzyme that, in the presence of a substrate (supplied in the kit), generates a product with an absorbance of 405 nm detectable by spectrophotometry. In brief, samples and standards were incubated in microtiter wells precoated with anti–MMP-2 antibody at 4°C for 24 h. To measure the total (active plus latent) MMP-2 content, latent MMP-2 was activated by the addition of p-aminophenylmercuric acetate (APMA) to each sample and the standard. After repeated washing, detection enzyme and the substrate were added to each well and plates were incubated at 37°C for 4 h. Absorbances were read at 405 nm using an ELISA microtiter plate reader, and the levels of MMP-2 in each sample were determined from the standard curve of human pro–MMP-2, included in the assay.

Estimation of collagen accumulation by immunoperoxidase assay. The amount of collagen that accumulated in the mesangial cell layer was measured by a quantitative immunoperoxidase assay using antibody to glomerular basement membrane (GBM). Although anti-GBM can react to other GBM proteins, it is predominantly directed toward collagen IV (17,18). Antibodies were obtained from rabbit serum immunized with purified rat GBM. Cell layers were washed with phosphate-buffered saline (PBS) and incubated with a 1:200 dilution of anti-GBM antibody in PBS containing 1% bovine serum albumin (BSA) for 1 h at room temperature. An identical dilution of normal rabbit serum was used as a control. After three washes with PBS, cells were incubated with a 1:2,000 dilution of peroxidase-linked goat anti-rabbit IgG (Pierce, Rockford, IL) in PBS containing 1% BSA for 1 h at room temperature. Finally, cells were washed with PBS and incubated with 0.02% o-phenylenediamine dihydrochloride (OPD) (Sigma) and 0.024% hydrogen peroxide for 20 min at room temperature. The reaction was terminated by the addition of 5N sulfuric acid, and color intensity was measured at 495 nm using a spectrophotometer (Beckman, Allendale, NJ). Bound peroxidase conjugate was estimated from a standard curve of peroxidase conjugate versus enzyme activity. The molar equivalent of antibody was derived assuming that peroxidase and conjugate are present in a 1:1 molar ratio and that the second antibody reacted with the first antibody in a 1:1 molar ratio. Bound antibody was calculated and expressed as micrograms per milligram cell protein.

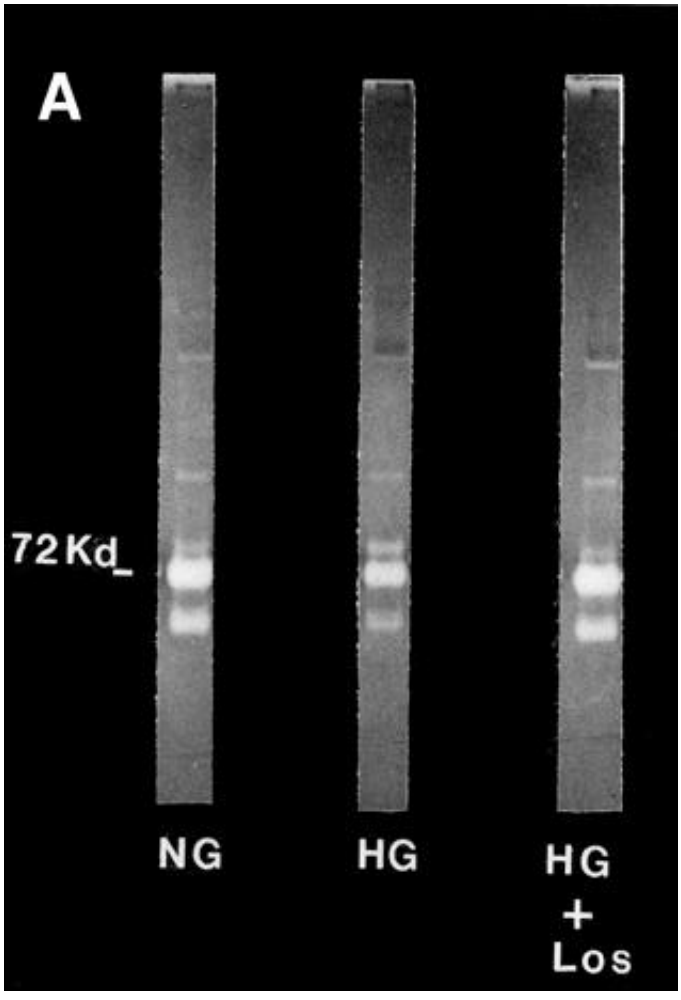
Measurement of TGF-β1. TGF-β1 levels were measured using a sandwich ELISA that employs a primary anti-human TGF-β1 antibody as a capture antibody and the avidin-biotin peroxidase detection system (R&D Systems, Minneapolis, MN). In brief, cell supernatants were dialyzed, lyophilized, and reconstituted at a known protein concentration. The 96-well plates (Costar, Cambridge, MA) were coated with 100 µl monoclonal anti-human TGF-β1 antibody (4 µg/ml) in PBS, pH 7.4, for 24 h at 4°C. Latent TGF-β1 was activated by treating the samples with 1.0N HCl for 15 min at room temperature and then neutralizing with 1.2N NaOH, 0.5 mol/l HEPES. Plates were incubated with 100 µl sample or standard for 90 min at 37°C followed by incubation with 100 µl/well of biotinylated anti-human TGF-β1 antibody (100 ng/ml) for 90 min at 37°C. After three washes, 100 µl streptavidin-horseradish peroxidase (HRP) (Zymed, South San Francisco, CA) was added to each well and incubated for 20 min at room temperature. The reaction was carried out by using a mixture of 1:1 3,3′,5,5′-tetramethyl benzidine dihydrochloride (TMB) and hydrogen peroxide as substrate for 30 min; the reaction was stopped by the addition of 0.5N H_2SO_4. Absorbances at 450 nm were read using an ELISA microtiter plate reader, and total TGF-β1 concentrations in each sample were determined from the standard curve of recombinant human TGF-β1, included in the assay.

Measurement of AII. Angiotensin II levels were determined in cell extracts prepared from cultured mesangial cells. To harvest cells, plates were washed twice with cold PBS followed by the addition to each well of 0.4 ml of the following extraction buffer: 20 mmol/l [Tris(hydroxymethyl)aminomethane] hydrochloride, pH 7.4; 10 mmol/l EDTA; 5 mmol/l ethylene glycol-bis-*N*,*N*,*N*′,*N*′-tetraacetic acid; 5 mmol/l β-mercaptoethanol; 50 µg/ml phenylmethyl sulfonyl fluoride; and 0.1 µg/ml aprotinin (Sigma). Cells were scraped in the extraction buffer and dounce homogenized. Samples were centrifuged to pellet insoluble fraction for 3–5 min in a microfuge. Supernatants were collected and passed through a Centricon-10 filter with a cutoff of >10,000 Da (Amicon, Beverly, MA). The filtrate was used for AII measurements and also for protein content. AII in the samples was determined by ELISA using the avidin-streptavidin method of Pennisula Laboratories (Belmont, CA), available as a commercial kit. Briefly, samples or standards were incubated with anti-AII antibody and biotinylated angiotensin II (B-AII) in 96-well plates coated with *S. aureus* Protein A. After incubation, the unbound B-AII was removed by washing, and the bound B-AII was determined by reaction of streptavidin-HRP in the wells using TMB and H_2O_2 as substrate. Reaction was terminated with 2N HCl, and the color intensity in each well was read at 450 nm using an ELISA microtiter plate reader. The AII amount in each well was calculated from the standard curve. This method is sensitive in measuring 10^{-9} to 10^{-11} mol/l concentrations of AII.

Statistical analysis. Data obtained from cells subjected to more than two test conditions were compared using analysis of variance (ANOVA) followed by posttest comparisons between groups (Student-Newman-Keuls). A P value <0.05 was considered to be significant. Results are presented as means ± SE.

RESULTS

Losartan reverses high glucose inhibition of collagenase activity. To determine if AII has a role in mediating the inhibitory effects of high glucose on collagenase activity, mesangial cells were coincubated with normal glucose (NG)



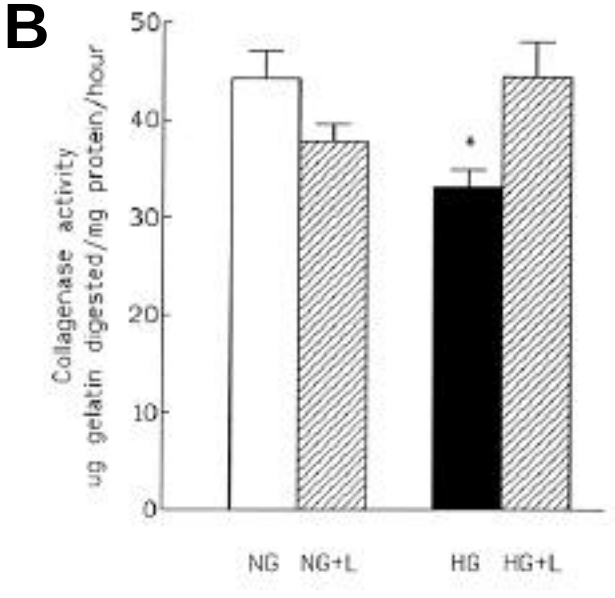


FIG. 1. ***A*****: Effect of losartan on 72-kDa collagenase activity in glucose-loaded cells. A representative zymogram shows the effect of 5 days of incubation with 5 mmol/l glucose (NG), 30 mmol/l glucose (HG), or 30 mmol/l glucose + 10^{-4} mol/l losartan (HG + Los) on collagenase activity. The decrease in collagenase activity observed with HG was blocked by losartan.** ***B*****: Quantitation of free collagenase activity by fluorometric assay from mesangial cells incubated under the experimental conditions in** ***A*****. Data shown are the means ± SE of nine experiments. *$P < 0.05$ compared with NG and HG + Los.**

or high glucose (HG) and losartan, an AII (AT_1) receptor antagonist. As shown in Fig. 1*A*, incubation of mesangial cells in HG resulted in a decrease in collagenase activity that was reversed by the addition of 10^{-4} mol/l losartan. Measurement of collagenase activity using fluorometric assay revealed similar results. There was a significant reduction in enzyme activity under high glucose compared with normal glucose (HG, 33 ± 1.8, vs. NG, 44 ± 2.8 µg gelatin digested · h^{-1} · mg $protein^{-1}$; $P < 0.05$) (Fig. 1*B*). Addition of 10^{-4} mol/l losartan to cell medium resulted in a complete restoration of collagenase activity under high glucose conditions and no significant change in enzyme activity under normal glucose conditions (Fig. 1*B*). In parallel experiments, matrix accumulation was assessed by anti-GBM antibody binding to the cell layers. Incubation of mesangial cells in high versus normal glucose resulted in an increase in anti-GBM binding that was reversed by losartan (NG, 2.5 ± 0.05; HG, 3.0 ± 0.11; HG + losartan, 2.7 ± 0.05 µg bound/mg protein; $P < 0.05$ by ANOVA) (Fig. 2).

Losartan blocks high glucose stimulation of TGF-β1. Incubation of mesangial cells in HG versus NG significantly increased total TGF-β1 secretion, which was blocked by 10^{-4} mol/l losartan as shown in Fig. 3 (NG, 1.1 ± 0.2; HG, 2.2 ± 0.3; HG + losartan, 1.4 ± 0.2 ng/ml; $P < 0.05$ by ANOVA). Additional experiments were carried out to examine the effects of 10^{-4} mol/l losartan on TGF-β1 secretion under 5 mmol/l (normal) glucose. Although losartan treatment resulted in a slight increase in total TGF-β1 secretion under normal glucose conditions, it was not significant compared with control (NG + losartan, 1.5 ± 0.8, vs. NG, 1.2 ± 0.4 ng/ml). In subsequent experiments, mesangial cells were exposed to 10^{-6} mol/l AII for 24 h under high-glucose conditions, and total TGF-β1 levels were measured. Interestingly, addition of AII to high-glucose medium did not produce any further increase in total

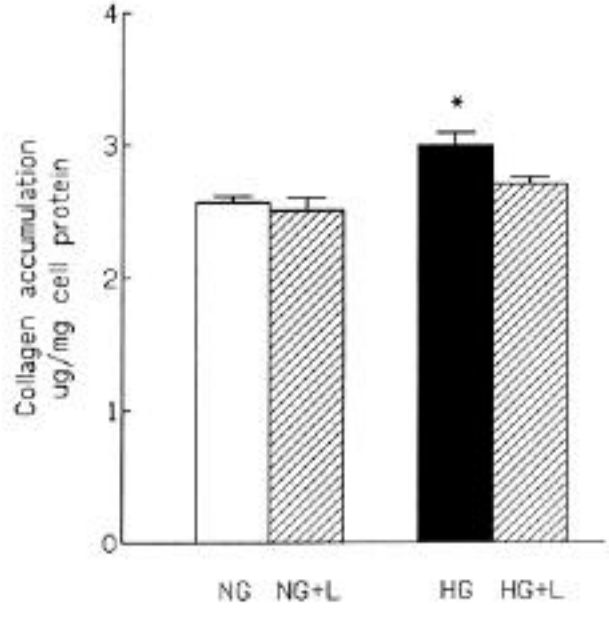


FIG. 2. Quantitation of collagen accumulation in mesangial cells. Incubation in 30 mmol/l glucose (HG) compared with 5 mmol/l glucose (NG) resulted in an increase in collagen content of the cells. This effect of HG was reversed by 10^{-4} mol/l losartan (HG + Los). Values shown are means ± SE of 10 experiments. *$P < 0.05$ compared with NG and HG + Los.

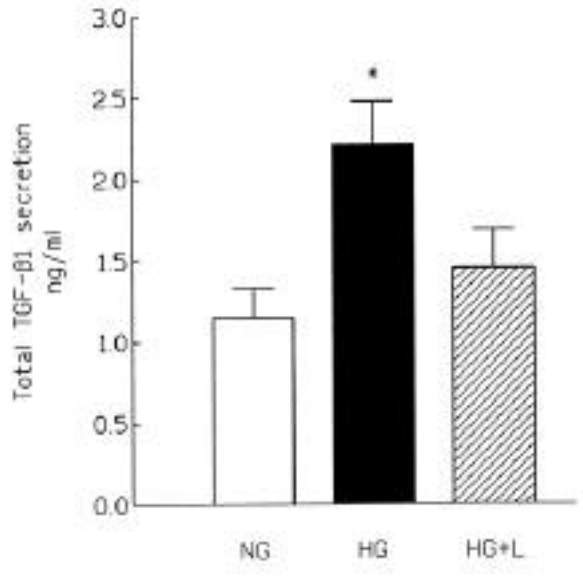


FIG. 3. Total TGF-β1 secretion in medium from mesangial cells incubated in 5 mmol/l glucose (NG), 30 mmol/l glucose (HG), and HG + 10^{-4} mol/l losartan (HG+L) for 5 days. Incubation in high glucose produced a significant increase in total TGF-β1 that was blocked by losartan treatment. Values shown are the means ± SE of six experiments. *P < 0.05 vs. NG and HG+L.

TGF-β1 secretion (HG, 2.3 ± 0.6, vs. HG + AII, 2.2 ± 0.6 ng/ml) (Fig. 4), although it did stimulate TGF-β1 significantly under normal glucose (NG + AII, 1.6 ± 0.2, vs. NG, 0.84 ± 0.08 ng/ml; P < 0.05) (Fig. 4).

Direct effect of AII on collagenase activity. To further investigate if AII is the mediator of glucose-induced inhibition of collagenase, we examined the effects of exogenous AII on collagenase activity. Addition of AII (10^{-6} mol/l) to the culture medium containing normal glucose for 24 h under serum- and insulin-free conditions resulted in a decrease in collagenase activity. Figure 5*A* shows a typical zymogram depicting a decrease in 72-kDa collagenase in the presence of AII, which was restored to normal when the cells were coincubated with 10^{-6} mol/l AII and 10^{-4} mol/l losartan. Results from the fluorometric assay for collagenase activity also demonstrated a significant decrease in enzyme activity in the pres-

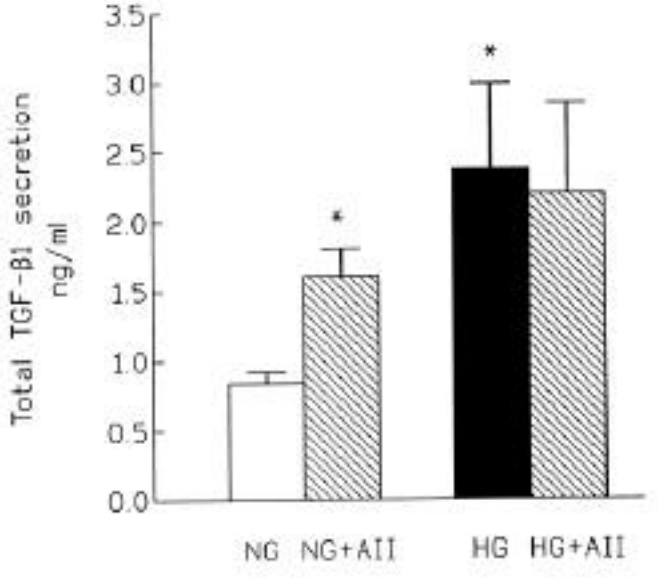
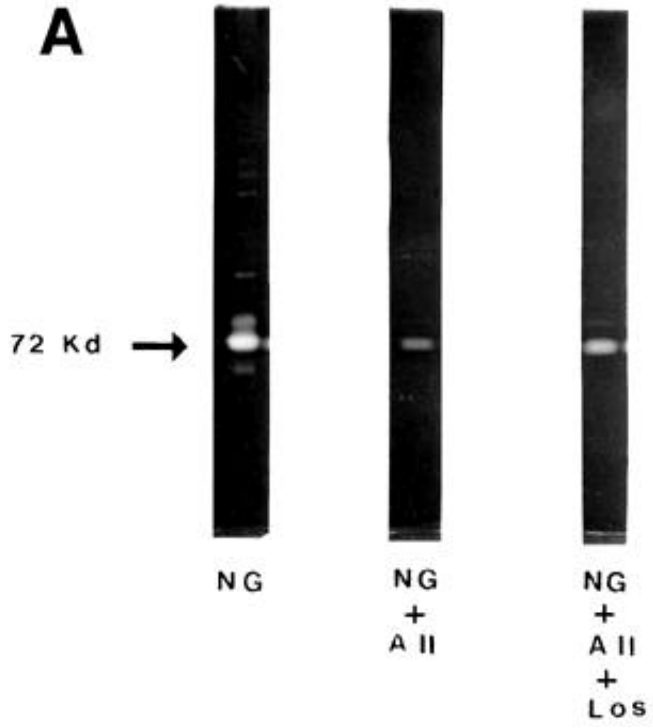


FIG. 4. Total TGF-β1 secretion in medium from mesangial cells incubated with 5 mmol/l glucose (NG) or 30 mmol/l glucose (HG) and in the presence of 10^{-6} mol/l AII for 24 h. Both AII and HG increased TGF-β1 compared with NG (*P < 0.05), but addition of AII to high-glucose medium did not further enhance TGF-β1 secretion. Values expressed are the means ± SE of seven experiments.



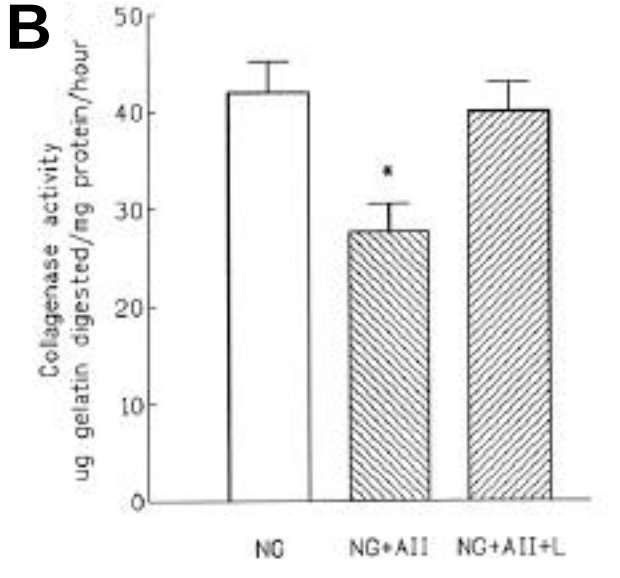


FIG. 5. *A*: Effect of 10^{-6} mol/l AII on mesangial 72-kDa collagenase activity: representative zymogram of media collected from mesangial cells incubated for 24 h in 5 mmol/l glucose (NG) alone or in the presence of 10^{-6} mol/l AII (NG + AII) or 10^{-6} mol/l AII plus 10^{-4} mol/l losartan (NG + AII + Los). The decrease in collagenase activity observed in the presence of AII was reversed by coincubation with losartan. *B*: Quantitation of free collagenase activity by fluorometric assay from mesangial cells incubated under the experimental conditions in *A*. Data shown are the means ± SE of eight experiments. *P < 0.05 compared with NG and NG + AII + Los.

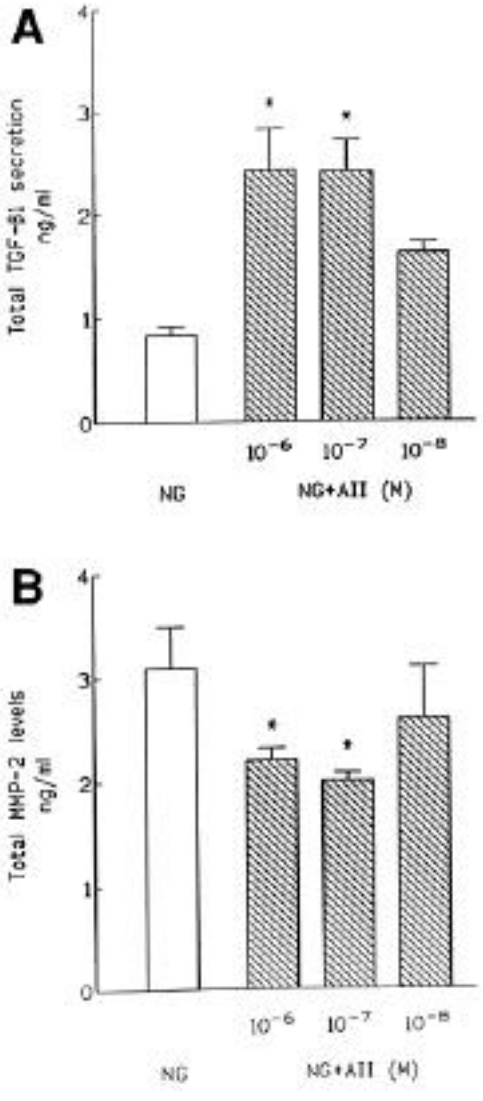


FIG. 6. Effect of AII on TGF-β1 and collagenase (MMP-2) levels in mesangial cells incubated with 5 mmol/l glucose (NG) or 5 mmol/l glucose + 10^{-8} to 10^{-6} mol/l AII for 24 h. Cell media were analyzed for total TGF-β1 and MMP-2 levels by ELISA. AII significantly increased TGF-β1 secretion with concurrent decrease in MMP-2 levels at 10^{-6} and 10^{-7} mol/l compared with NG ($P < 0.05$). However, 10^{-8} mol/l AII did not produce any significant changes in either TGF-β1 secretion or MMP-2 levels in mesangial cells. Graphs are the means ± SE of three experiments.

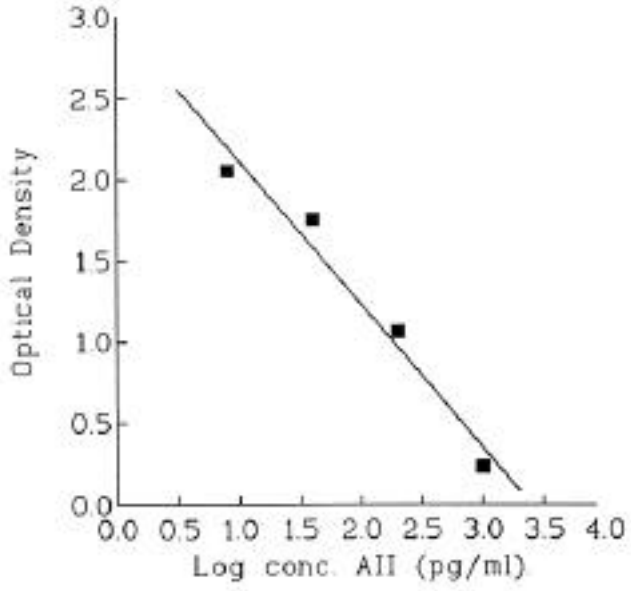


FIG. 7. A typical standard curve for AII measurement by ELISA. Anti-human AII antibody was used as a capture antibody.

ence of 10^{-6} mol/l AII, which was restored by treatment with 10^{-4} mol/l losartan (NG, 42 ± 3.2; NG + AII, 28 ± 3.0; NG + AII + losartan, 40 ± 3.1 µg gelatin digested · h^{-1} · mg $protein^{-1}$; $P < 0.05$) (Fig. 5*B*).

AII stimulates TGF-β1 but inhibits total collagenase in mesangial cells. The effects of AII on TGF-β1 and collagenase (MMP-2) levels were determined simultaneously in mesangial cells incubated with different concentrations of AII (10^{-8} to 10^{-6} mol/l) under normal glucose. After 24 h of AII exposure, cell media were collected and analyzed for TGF-β1 levels. In the same samples, the concentration of collagenase (MMP-2) was determined by a new ELISA (see METHODS). As shown in Fig. 6*A*, AII produced a significant increase in total TGF-β1 secretion at concentrations of 10^{-6} and 10^{-7} mol/l (NG, 0.83 ± 0.08; NG + 10^{-6} mol/l AII, 2.42 ± 0.4; NG + 10^{-7} mol/l AII, 2.40 ± 0.3 ng/ml; $P < 0.05$ by ANOVA). In conjunction, a significant decrease in total MMP-2 levels in cell medium was observed at the same AII concentrations (NG, 3.2 ± 0.4; NG + 10^{-6} mol/l AII, 2.2 ± 0.1; NG + 10^{-7} mol/l AII, 2.0 ± 0.08 ng/ml; $P < 0.05$) (Fig. 6*B*). However, no significant effects on total TGF-β1 secretion or total collagenase (MMP-2) levels were observed at lower AII concentrations (10^{-8} mol/l) (Fig. 6*A* and *B*).

Glucose increases AII generation in mesangial cells. In these experiments, the direct effect of high glucose on AII generation in mesangial cells was examined. AII was measured by ELISA using anti-human angiotensin II antibody. A typical standard curve obtained by this method is shown in Fig. 7. Mesangial cells were exposed to medium containing 5 mmol/l glucose (NG), 30 mmol/l glucose (HG), and 5 mmol/l glucose + 25 mmol/l mannitol (HM) (osmotic control) without serum or insulin for 24 h, after which cells were analyzed for AII by ELISA. As shown in Fig. 8, exposure to high glucose resulted in a significant increase in AII compared with normal glucose or mannitol control (HG, 197 ± 16; NG, 159 ± 4; HM, 141 ± 11 pg/mg cell protein). In separate experiments, mesangial cells were incubated for 24 h with various concentrations of glucose (5–60 mmol/l). Figure 9 shows a concentration-dependent effect of glucose on AII secretion in the cells, with a ~20% increase in cells treated with 15 mmol/l glucose and a 45% increase in cells treated with 60 mmol/l glucose compared with the 5 mmol/l glucose control.

DISCUSSION

Our results indicate that glucose-induced inhibition of collagenase activity and increase in collagen accumulation is mediated by AII via the AT_1 receptor, since these effects could be reversed by the specific AT_1 receptor antagonist losartan. The stimulatory effect of glucose on TGF-β1 secretion in mesangial cells is also blocked by losartan, suggesting that this effect of glucose is also mediated by AII. Exogenous AII inhibited collagenase activity, decreased levels of the major collagenase produced by mesangial cells (MMP-2),

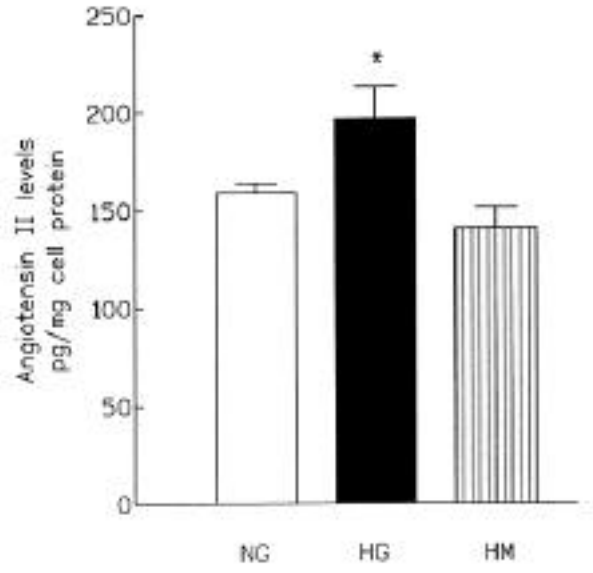


FIG. 8. Effect of high glucose on AII in rat mesangial cells incubated with 5 mmol/l glucose (NG), 30 mmol/l glucose (HG), and 5 mmol/l glucose + 25 mmol/l mannitol (HM) for 24 h. There was a significant increase in AII secretion in HG-loaded cells compared with either NG or HM (*$P < 0.05$). Values shown are the means ± SE of five experiments.

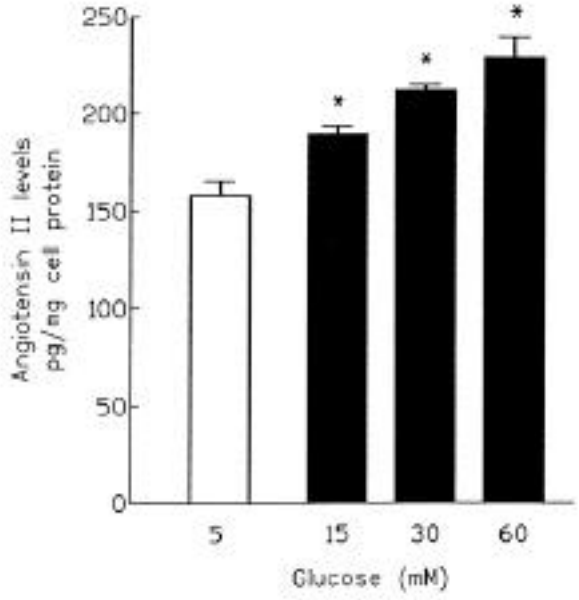


FIG. 9. AII in mesangial cells incubated with varying concentrations of glucose (5–60 mmol/l) for 24 h. Glucose loading increased AII production in a concentration-dependent manner (*$P < 0.05$ vs. 5 mmol/l glucose). Data shown are the means ± SE of four experiments.

and increased TGF-β1 secretion in mesangial cells. Taken together, these data suggest that glucose stimulates AII, which in turn increases TGF-β1 secretion, leading to inhibition of collagenase activity. This supposition is supported by our finding that high glucose increased AII production in mesangial cells. Thus, AII appears to be the proximate mediator of the inhibitory effects of glucose on matrix degradation.

Matrix proteins such as collagens have highly specialized structures, and multiple enzymatic mechanisms are involved in their catabolism. The principal enzyme systems involved are the matrix metalloproteinases (MMPs) (19) and the plasminogen/plasmin system (20). MMPs include the interstitial collagenase, stromelysins, and type IV collagenases, and among these MMP-2 (a 72-kDa protein) has been shown to be the major collagenase enzyme secreted by mesangial cells (21,22). The effects of the diabetic state on the activity and expression of MMPs have been studied. MMP-2 activity was decreased in streptozotocin-induced diabetic rats (23–25), suggesting that impaired degradative enzyme activity contributes to matrix accumulation in nephropathy. Previous studies from our laboratory have demonstrated decreased collagenase activity and mRNA expression in mesangial cells under high-glucose conditions (4). Moreover, recent studies have reported downregulation of MMP-2 mRNA expression in glucose-loaded mesangial cells (26). The relevance of these findings for clinical diabetic nephropathy is suggested by a recent publication demonstrating downregulation of the glomerular matrix MMP-2 gene in human renal biopsy specimens (27). In view of these reports, the present study focused on MMP-2 activity, since this enzyme appears to play an important role in mesangial matrix degradation.

AII has many effects that may contribute to diabetic nephropathy, including both hemodynamic and nonhemodynamic effects. The latter include stimulatory effects on extracellular matrix protein synthesis. In renal mesangial cells, AII has been shown to cause increased synthesis of mesangial matrix proteins such as collagen I and fibronectin (11–13), effects mediated by the AT_1 receptor (13). The ACE inhibitor enalapril inhibits gene expression of extracellular matrix proteins in diabetic rats (10), suggesting an important role for AII in the development of glomerular sclerosis. Recently, rat mesangial cells in culture have shown to express renin, angiotensinogen, and AT_1 receptor gene (28), indicating that locally produced AII can act on mesangial cells to promote increased matrix synthesis in diabetes. The findings of Wang et al. (29) that high glucose stimulates expression of the angiotensinogen gene in opossum kidney cells suggest that high glucose per se may increase the substrate for angiotensin formation. Our results are in agreement with those studies, since high glucose increased AII generation in mesangial cells.

Much of the evidence suggesting a role for the intrarenal renin-angiotensin system in the pathogenesis of diabetic nephropathy is based on observations of the effects of blocking AII. ACE inhibitors attenuated progression of glomerulosclerosis in clinical diabetic renal disease (6,7). Also, AII receptor antagonists have been shown to attenuate the development of renal disease in experimental diabetes (8,9). A decrease in glomerular AII receptors in the diabetic rat has been reported by several investigators (30,31). Similar results have been obtained in proximal tubules accompanied with a decrease in the mRNA expression for the AT_{1a} receptor (32). The importance of the renin-angiotensin system in diabetes is also supported by recent findings that rats transfected with the mouse *Ren-2* gene and then made diabetic both overexpress renin and develop severe glomerulosclerosis (33).

Glucose and AII have been shown to have similar effects on renal cells. Both high glucose and AII increased synthesis of collagen types I and IV and other matrix proteins in mesangial cells (2,11,12). We have shown that both glucose and AII inhibit mesangial collagenase activity (4 and this study). In addition, AII also decreased collagenase levels in mesangial cells (this

study). Moreover, both glucose and AII have been demonstrated to increase plasminogen activator inhibitor synthesis and decrease plasminogen activator activity (34,35). These alterations in the plasminogen/plasmin system would tend to cause decreased matrix degradation and increased accumulation, in part because plasmin activates collagenase (20).

Increased glomerular synthesis and deposition of the cytokine TGF-β1 have been demonstrated in diabetes (36). In a recent study employing AT_{1a} receptor–deficient mice made diabetic with streptozotocin, an increase in renal TGF-β1 mRNA levels was seen only in mice with an intact renin-angiotensin system and not in the absence of the AT_{1a} receptor (37). TGF-β1 has been shown to mediate the stimulatory effects of both glucose and AII on matrix protein synthesis in glomerular mesangium (15,12). The present data show that inhibition of collagenase activity by high glucose and AII is also mediated by TGF-β1, since both glucose and AII stimulated TGF-β1 secretion in mesangial cells with a concurrent decrease in collagenase activity. Previously, we have demonstrated that the decrease in mesangial collagenase activity due to high glucose and AII can be reversed by antibody to TGF-β1 (38,39). Thus, it appears that both glucose and AII may use TGF-β1 as the mediator of their effects on matrix degradation. Furthermore, absence of any further enhancement in TGF-β1 production when AII was added to glucose-loaded mesangial cells indicates that glucose and AII may stimulate TGF-β1 via a common pathway.

Taken together, these findings support the hypothesis that the high-glucose milieu of diabetes increases mesangial AII generation, which in turn stimulates TGF-β1 activity, leading to increased synthesis and decreased degradation of mesangial matrix proteins, resulting in the matrix expansion observed in diabetic nephropathy.

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