Mechanism of Increased Angiotensin II Levels in Glomerular Mesangial Cells Cultured in High Glucose

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Abstract. Previous studies have shown that glucose increases angiotensin II (AngII) levels in rat glomerular mesangial cells and that AngII mediates the inhibitory effects of high glucose on matrix degradation in these cells. The present study addresses the following questions: (1) What are the mechanisms for the generation of AngII in mesangial cells? (2) What are the effects of glucose on AngII generation by these mechanisms? Experiments employed primary mesangial cells from normal Sprague-Dawley rats. The levels of immunoreactive angiotensinogen (AGT), angiotensin I (AngI), and angiotensin II (AngII) were measured by ELISA. AGT mRNA expression was determined by Northern blot analysis. Incubation of cells for 24 h in high glucose (30 mM) increased AngI levels by 1.5-fold and increased AGT mRNA expression; this was accompanied by a 1.5-fold increment in AngI and 1.7-fold increment in AngII levels.

Recent studies have provided considerable evidence for the presence of an independent renin-angiotensin system (RAS) in kidney and its involvement in the pathogenesis of diabetic nephropathy. It is now well established that angiotensin II (AngII) is locally produced in the kidney and that it plays an important role in the development of diabetic glomerulosclerosis. Angiotensin-converting enzyme (ACE) inhibitors and AngII receptor blockers (ARBs) have produced beneficial effects in attenuating the progression of glomerulosclerosis in animals (1,2) and in humans (3). In cultured glomerular mesangial cells, several studies have reported that AngII increases synthesis of matrix proteins such as collagen type I and fibronectin (4,5) mediated by the type I angiotensin II (AT₁) receptor (6). In addition, AngII has been shown to decrease matrix degradation by inhibiting the activity of matrix metalloproteinases-2 (MMP-2) in rat mesangial cells; this effect is mediated via the AT₁ receptor because it can be blocked by losartan (7).

Regulation of the intrarenal RAS appears to be independent of the plasma RAS. Renal vasodilation in response to ARBs is enhanced in diabetic patients despite low plasma renin activity, suggesting that the renal vascular RAS is activated despite suppression of the circulating RAS (8). AngII levels in several intrarenal compartments, including glomeruli, have been found to be several-fold higher than those found systemically (9). In diabetic rats, plasma AngII levels were found to be decreased, yet AngII receptors in glomeruli were downregulated, implying an increase in glomerular AngII levels (10). Evidence suggests that the intrarenal RAS within glomeruli and proximal tubules may be activated by hyperglycemia, leading to stimulation of local AngII production (7,11). Indeed, high glucose has been shown to stimulate angiotensinogen gene expression in rat proximal tubules via activation of the p38 mitogen-activated protein kinase pathway (12). The existence of a separate RAS within the mesangial cells is supported by recent reports showing that mesangial cells themselves contain angiotensinogen, renin, AT₁ receptors, and ACE (13,14). In cultured mesangial cells, stretch/relaxation increases angiotensinogen gene expression and levels with a concurrent increase in AT₁ receptor expression (13). We have previously demonstrated that exposure to high glucose resulted in a significant increase in immunoreactive AngII levels in mesangial cells (7). However, the mechanisms by which high glucose increases mesangial AngII generation are not yet clear. Therefore, the present study was designed to determine the mechanisms involved in increasing AngII generation under high-glucose conditions.
Materials and Methods

Culture of Mesangial Cells

All experiments were conducted on primary mesangial cells obtained from 100 to 150-g male Sprague-Dawley rats (Harlan, Indianapolis, IN) as described previously (7). In brief, glomeruli were isolated from kidney using a graded-sieving technique with stainless steel and nylon meshes under sterile conditions. Primary culture of mesangial cells was established by plating isolated glomeruli in RPMI 1640 tissue culture medium containing 12% fetal calf serum (FCS), 0.67 U/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were allowed to grow at 37°C in 5% CO2 and 95% air in 24-well cell culture plates or cell culture flasks as required. The identity of mesangial cells was confirmed by differential immunofluorescence staining using antibodies against cytokeratin (negative), actin (positive), and Factor VIII (negative). Cells were used between passage 1 to 3.

Experimental Conditions

To glucose-free RPMI 1640 media (Life Technologies BRL, Life Technologies, Grand Island, NY), either 5 mM glucose (NG) or 30 mM glucose (HG) was added. Mesangial cells were incubated in this media (without serum and insulin) for a period of 1 to 5 d. Either 5 mM glucose + 25 mM 2-deoxy-D-glucose (2-DG) or 5 mM glucose + 25 mM mannitol (HM) were used as osmotic controls. The cells remained healthy after 5 d of culture in the absence of serum under these experimental conditions. At the end of each experiment, cells were washed, scraped in ice-cold phosphate-buffered saline (PBS), and sonicated for 2 min on ice. This was followed by centrifugation at 13,000 × g for 20 min. The supernatant was collected and stored at −70°C until used for various determinations.

Measurement of AGT Levels in Mesangial Cells

Antibody to AGT was prepared as follows. Rabbits were immunized with 500 μg of antigen (porcine AGT; Sigma Chemical Co., St. Louis, MO) emulsified in complete Freund adjuvant in several intracutaneous sites on the back. After 4 wk, a booster injection of 250 μg of antigen in incomplete Freund adjuvant was administered intramuscularly in the flanks. The animals were bled after 2 wk, and the serum was tested for antibodies. Antibody reactions toward rat endogenous AGT as determined by Western blot analysis and shown in Figure 1. No cross-reaction of anti-AGT antibodies toward AngI and AGH was observed when tested by ELISA (data not shown). The anti-AGT antibody was used to measure AGT levels in mesangial cells by a competitive ELISA as follows. A 96-well plate was sensitized overnight with AGT (10 μg/ml) in 50 mM borate buffer (pH 10.5). The first incubation was carried out with standards (porcine AGT) or samples and anti-AGT antibody (1:10,000) for 2 h at room temperature followed by washing and then incubation with a secondary antibody (anti-rabbit IgG-peroxidase, 1:1000) for 1 h. The final reaction was developed using TMB and H2O2 as substrate and measured by reading absorbance at 450 nm after termination with 2N HCl. The amount of immunoreactive AGT in samples was calculated from a standard curve.

Figure 1. A Western blot analysis showing the reactivity of anti-angiotensinogen (AGT) antibody against rat endogenous AGT. Samples of rat plasma and extracts of rat liver and kidney were electrophoresed on 8% SDS gel and transferred electrophoretically to nitrocellulose membrane. Membranes were incubated in blocking buffer, washed, and then incubated with anti-AGT antibody (1:100) for 2 h at room temperature. This was followed by incubation with a peroxidase conjugated anti-rabbit antibody (1:200) for 1 h. Reaction was developed using DAB and H2O2 as a substrate. Membranes were washed and stored in H2O. Reaction bands were observed near 50 kD for all samples, which coincides with the reported molecular weight for rat AGT (lane 1, molecular weight marker; lane 2, rat kidney; lane 3, rat liver; lane 4, rat plasma).

Northern Blot Analysis of AGT mRNA Expression

Total RNA was isolated from mesangial cells using a commercial RNA isolation kit (Promega, Madison, WI). A sample of total RNA was separated on a 1.2% agarose gel and transferred onto a Hybond XL nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was sequentially probed with 32P-labeled cDNA of AGT and β-actin. The membrane was subsequently washed and exposed to autoradiography. The band densities for AGT and β-actin were determined using image analysis.

The cDNA probes for AGT and β-actin were synthesized by RT-PCR using total RNA from mesangial cells and the following primers: AGT, 5’ CTGACCCAGTTCTTGTCGCC-3’ (forward), 5’ TGGGGTTATACCTGCGCC-3’ (reverse) (13); β-actin, 5’ TCATGAAGTGAGCAGTTGACATCCGT-3’ (forward), 5’ CCTAGAAGCATTGGCCGTGACGATG-3’ (reverse) (15). The RT-PCR products were 720 bp for AGT and 285 bp for β-actin as expected from primer positions. The bands were excised from the gel and eluted by centrifugation. The probes were labeled by the random primer method (Stratagene, Cedar Creek, TX) using alpha 32P-dCTP as the label and purified by ethanol precipitation.

Measurement of Renin Activity

Renin activity was measured as AngI formed in the presence of excess renin substrate. For these experiments, we utilized commercially available porcine AGT (Sigma Chemical Co., St. Louis, MO), because it is known that rat renin can cleave porcine AGT. Samples were incubated with or without excess of AGT (1 μM) at 37°C for 1 h followed by measurement of AngI levels using a commercial ELISA kit (Peninsula Laboratories, Belmont, CA).

Western Blot Analysis for ACE

Sample proteins from mesangial cell extracts were separated on 8% SDS-PAGE under reducing conditions and transferred to PVDF membrane (Bio-Rad Lab., Hercules, CA) overnight at 4°C using transfer buffer containing 25 mM Tris-HCl, 192 mM glycine, 20% wt/vol methanol. After incubation in blocking buffer and washings, membranes were incubated with anti-ACE antibody (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA) followed by incubation with a HRP-conjugated secondary antibody (1:1000; Santa Cruz Biotechnology Inc.). Specific proteins on the membranes were detected by chemiluminescence using the ECL detection system (Amersham Biosciences, Piscataway, NJ).

Measurement of ACE Levels and Activity

Mesangial cells were incubated in NG, HG, or 2-DG for a period of 1 to 5 d. Cell extracts were prepared and analyzed for ACE levels...
Conditions were compared using the experiments where cells were subjected to two or more than two test of experiments for a given test condition. Results obtained from P respectively. A AngI and AngII, respectively. The amount of AngI or AngII was calculated from a standard curve for as shown in Figure 3A. The increase in AGT levels was observed within 24 h of incubation in HG and was maintained after 2 and 5 d of incubation (Figure 3B). The effect of HG on AGT mRNA expression is shown in Figure 4. In the presence of HG for a 24-h period, there was an increase in AGT mRNA expression in mesangial cells compared with that in the presence of 5 mM glucose (Figure 4A). Densitometric analysis of the bands showed a significant increase in the ratio of AGT mRNA/β-actin mRNA in HG compared with NG (Figure 4B).

Statistical Analyses
Experiments were conducted using primary mesangial cells derived from pooled glomeruli obtained from a group of three to four rats. Values are expressed as mean ± SEM, and n denotes the total number of experiments for a given test condition. Results obtained from experiments where cells were subjected to two or more than two test conditions were compared using the t test or one-way ANOVA, respectively. A P-value of <0.05 was considered to be significant.

Results
High Glucose Increases AGT in Mesangial Cells
Incubation of mesangial cells in media containing 30 mM glucose (HG) produced a significant 1.5-fold increase in AGT levels compared with 5 mM glucose (NG) (Figure 2). In contrast, incubation in 30 mM 2-deoxy-D-glucose (2-DG) did not increase AGT levels (Figure 2). Concentrations of glucose ≥ 15 mM produced similar effects on AGT in mesangial cells as shown in Figure 3A. The increase in AGT levels was observed within 24 h of incubation in HG and was maintained after 2 and 5 d of incubation (Figure 3B). The effect of HG on AGT mRNA expression is shown in Figure 4. In the presence of HG for a 24-h period, there was an increase in AGT mRNA expression in mesangial cells compared with that in the presence of 5 mM glucose (Figure 4A). Densitometric analysis of the bands showed a significant increase in the ratio of AGT mRNA/β-actin mRNA in HG compared with NG (Figure 4B).

High Glucose Does Not Increase Renin Activity
Renin activity in mesangial cells was measured as AngI formation in the presence of excess exogenous AGT (1 µM). AngI formation was increased by more than fivefold in mesangial cells incubated in media containing AGT compared with cells incubated in media without AGT. However, the increase in AngI levels in the presence of AGT was similar between NG and HG. In mesangial cells incubated in HG, AngI formation from exogenous AGT was 2.5 ± 0.3 ng/mg protein per h compared with 2.8 ± 0.4 ng/mg protein per h in cells incubated in NG (P = NS).

High Glucose Increases AngI and AngII Levels in Mesangial Cells
In the presence of HG for a 24-h incubation period, AngI levels in mesangial cells were increased significantly compared with NG or 2-DG (Figure 5A). The increase in mesangial AngI...
A significant increase in AngII level was also observed in mesangial cells incubated in HG for 24 h in contrast to cells incubated in NG or 2-DG (Figure 5B). After 5 d of incubation, AngII levels were 167 ± 11% in mesangial cells incubated in HG compared with 100% NG control ($P < 0.05$, $n = 3$). These results demonstrate that AngI and AngII formation is increased in mesangial cells in the presence of high levels of glucose.

**High Glucose Does Not Affect ACE Levels or Activity**

ACE in mesangial cells is present as a membrane-bound ectoenzyme and also in a secreted form; therefore, cell extracts and conditioned media were analyzed for ACE activity and levels. After a 24-h incubation period, ACE activity in media from cells incubated in HG was $22 ± 0.6$ pmol/mg protein per min in media from cells incubated in NG. Similarly, ACE levels as determined by ELISA were similar in media from NG-treated and HG-treated cells (NG: 8.5 ng/mg protein versus 8.4 ng/mg protein in HG). Western blot analysis of the conditioned media from cells incubated in the presence of high levels of glucose.
of NG, HG, and 2-DG confirmed that ACE is secreted into the culture medium by mesangial cells. As shown in Figure 6A, a distinct band at 145 kD corresponding to the ACE protein was identified by Western analysis. However, no significant differences in the band density were observed among NG-, HG-, and 2-DG-treated cells (Figure 6A). ACE levels in mesangial cell extracts remained unchanged after 24 h or 5 d of incubation in HG (Figure 6B).

**Figure 5.** Effect of high glucose on IR-AngI (A) and IR-AngII (B) in mesangial cells incubated in 5 mM glucose (NG), 30 mM glucose (HG), or 5 mM glucose + 25 mM 2-deoxy-D-glucose (2-DG) for 24 h. Immunoreactive AngI and AngII levels were measured by ELISA. Values are mean ± SEM of four experiments. *P < 0.05 compared with NG and 2-DG.

**Figure 6.** (A) Western blot analysis showing angiotensin-converting enzyme (ACE) protein in media from mesangial cells incubated in 5 mM glucose (NG), 30 mM glucose (HG), or 5 mM glucose + 25 mM 2-deoxy-D-glucose (2-DG) for 24 h. A distinct band at 145 kD molecular weight was identified as that of ACE protein. (B) Effect of high glucose on ACE levels in mesangial cells incubated in NG, HG, or 2-DG for 1 to 5 d. Values are mean ± SEM of five experiments.

**Formation of AngII from Exogenous AngI in Mesangial Cell Extracts**

Formation of AngII and other Ang peptides from AngI was examined by HPLC. First, stability of exogenous AngI was tested in incubation buffer (PBS) at 37°C. AngI (10^{-4} M) was incubated in PBS buffer at 37°C for a period of 1 to 4 h. At the end of each hour, a 25-μl sample was injected into the column, and peak absorbance at 214 nm was calculated from the AngI peak height. AngI peak absorbances were 1.6 units at 1, 2, 3, and 4 h. No other Ang peptide peaks were detected at any time point. These observations indicated that incubation of AngI with PBS buffer resulted in stability of the AngI with no appearance of metabolites.

Next, a 25-μg sample of cell extract from mesangial cells incubated in NG, HG, or HM was incubated in the presence of exogenous AngI (10^{-4} M) for 1 h at 37°C and then injected...
into a HPLC column. The peak height for each Ang peptide was measured and used for calculating the peak absorbances at 214 nm for each Ang peptide. Figure 7A shows a sample chromatograph reconstructed from the peak absorbance obtained for each Ang peptide in one such experiment using an NG-treated cell extract. As shown in this figure, AngII and other Ang peptides, including Ang(1–9), Ang(1–7), and Ang(3–8), were produced from exogenous AngI. These peptides accumulated in the reaction mixture in a time-dependent manner up to the maximum time (4 h) tested (data not shown). The ratio of Ang peptides/AngI is shown in Figure 7B. There was a significant increase in the ratio of AngII/AngI in cells incubated in HG compared with cells incubated in NG or HM (Figure 7B). These results suggest that AngII formation in the presence of exogenous AngI is stimulated by HG. Production of Ang(1–9) from exogenous AngI did not differ significantly among NG, HG, and HM (Figure 7B). Also, HG did not alter Ang(1–7) and Ang(3–8) generation from exogenous AngI (data not shown). Of note, HPLC of cell extracts without exogenous AngI did not show peaks for angiotensin metabolites.

Pre-incubation of mesangial extracts with enalaprilat (10^{-4} M) for 30 min resulted in a decrease of 30 to 40% in AngII generation from exogenous AngI in cells incubated in NG, HG, or HM. These findings indicated that conversion of AngI to AngII in mesangial cell extracts is only partly inhibited by ACE inhibitors.

Role of Ang(1-9) in AngII Formation in Mesangial Cells

The possibility of AngII formation from the intermediary Ang peptide Ang(1–9) was examined by HPLC analysis. In these experiments, mesangial cell extracts from NG-, HG-, or HM-treated cells were incubated with exogenous Ang(1–9) (10^{-4} M) for 1 h at 37°C and analyzed by HPLC. The result of one such experiment using NG cell extracts is shown in Figure 8A. The ratio of AngII/Ang(1–9) obtained in NG-, HG-, or HM-treated cells is presented in Figure 8B. There was a significant increase in AngII/Ang(1–9) ratio in cells incubated in HG, suggesting that formation of AngII from Ang(1–9) is increased under high glucose conditions. Pre-incubation with enalaprilat produced a decrease of about 30% in the formation of AngII from exogenous Ang(1–9); this effect being similar in cells incubated in NG, HG, or HM.

Discussion

The present studies demonstrate that high levels of glucose stimulate AGT production in rat glomerular mesangial cells. The level of AGT was significantly increased by 1.5-fold in mesangial cells incubated in high glucose (30 mM) media compared with cells incubated in normal glucose (5 mM) media. This glucose-induced increase in AGT was accompanied by a significant increase in AGT mRNA expression in mesangial cells. The stimulatory effect of glucose on AGT in mesangial cells indicated that glucose increases the availability of substrate for the ultimate formation of AngII in mesangial cells.

Previous studies have demonstrated in rat mesangial cells that AGT mRNA expression is increased by mechanical stretch (13). Also, in rat proximal tubular cells, high glucose was shown to increase AGT gene expression via stimulation of the p38 mitogen-activated protein kinase pathway (12). In our study, high glucose increased AGT mRNA expression as well as AGT levels in mesangial cells. However, no significant change in renin activity was observed in these cells, suggesting that the increased AngI levels observed are due to increase in AGT (renin substrate) rather than renin activity. Of note,
mechanical stretch was also found to stimulate AGT production in rat mesangial cells without any significant effect on renin activity (13). Incubation of mesangial cells in high glucose also increased AngII levels 1.7-fold, consistent with our previously reported findings (7).

ACE is the best-known enzyme that can convert AngI into AngII by removal of the dipeptide His$^9$-Leu$^{10}$ at the carboxy-terminal. In the kidney, ACE has been localized to glomerular endothelial cells and proximal tubule brush border (17). Previous studies have reported ACE activity in glomeruli (18,19) and also in cultured mesangial cells (13). However, the ability of mesangial cells to synthesize ACE has been better established recently by studies in which this enzyme has been purified and characterized from primary rat mesangial cells (14). In the present study, Western blots analysis demonstrated the presence of ACE protein in conditioned media collected from the cells, thus confirming these earlier reports. Enalaprilat decreased formation of AngII from exogenous AngI in both normal and high glucose-treated cells, suggesting that ACE plays a role in generation of AngII from AngI in mesangial cells. However, glucose did not affect ACE levels or activity in any of our studies. Thus ACE does not appear to be involved in the upregulation of mesangial AngII generation by glucose.

In the course of our HPLC experiments, we observed that addition of exogenous AngI to cell extracts resulted in formation of many other ANG peptides in addition to AngII, including Ang(1-9), Ang(1-7), and Ang(3-8). The observation that the ACE inhibitor enalaprilat only partially decreased formation of AngII from exogenous AngI suggested that AngI to AngII conversion takes place via non-ACE as well as ACE-dependent pathways in these cells. Because of our novel finding that mesangial cells generate a large amount of Ang(1-9) from exogenous AngI, we did further experiments and found that AngII can be formed from Ang(1-9), and, in contradiction to the ACE pathway, this pathway is stimulated by high glucose.

A carboxypeptidase capable of generating AngII from AngI by sequential cleavage of the carboxy-terminal His$^9$ and Leu$^{10}$ was described in human kidney over 15 yr ago (20). However, until recently there has been little study of these enzymatic pathways. A metalloproteinase that hydrolyzes the carboxy-terminal Leu$^{10}$ from AngI to generate Ang(1-9) has been recently identified from a human heart failure ventricle cDNA library and termed ACE2 (21). Other studies have also reported a human homolog of ACE named ACEH from a human lymphoma cDNA library (22). Within kidney, ACE2 has been shown by immunohistochemical methods to be present in endothelial and epithelial cells (21). Although it is not known if ACE2 is produced by mesangial cells, our results showing conversion of AngI to Ang(1-9) provide strong evidence that this enzyme or a similar enzyme is present in these cells. Furthermore, our findings point to the existence of an as yet unidentified carboxypeptidase that can convert Ang(1-9) to AngII in mesangial cells. Our preliminary findings that this enzyme can be partially inhibited by enalaprilat were surprising. Ang(1-9) is hydrolyzed to AngII by removal of a single amino acid via an unknown carboxypeptidase; therefore, inhibition of this enzyme by enalaprilat (a dipeptidase inhibitor) cannot be explained at this time. It appears that enalaprilat has nonspecific effects on enzymes other than ACE in mesangial cells.

AngII has been implicated in glomerulosclerosis because it stimulates synthesis of matrix proteins in mesangial cells (4,5). In addition, our previous studies have shown that AngII also inhibits matrix degradation, which can contribute to matrix...

Figure 8. (A) Sample chromatograph showing formation of AngII from exogenously added Ang(1-9) (10$^{-4}$ M) to cell extracts from NG. Absorbance for each peak was calculated as explained in Figure 7A. Absorbance data obtained for Ang(1-9) and AngII was used to generate a chromatograph. (B) Effect of 30 mM glucose (HG) on formation of AngII from Ang(1-9) in mesangial cell extracts. The peak absorbance for AngII and Ang(1-9) was calculated from the respective peak height and ratio of AngII/Ang(1-9) is presented. Values are mean ± SEM of three experiments. *$P < 0.05$ compared with NG.
accumulation in mesangial cells (7). Furthermore, AngII mediates high glucose-induced inhibition of matrix degradation, because these effects are blocked by the angiotensin receptor antagonist, losartan (7). These data support the hypothesis that AngII has nonhemodynamic effects on matrix metabolism that may contribute to matrix accumulation in diabetic nephropathy (23). Stimulation of AngII production by high glucose provides AngII to its receptors in mesangial cells, and the possible role of this novel pathway in the pathogenesis of diabetic nephropathy should be explored.

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**References**