High glucose concentration stimulates intracellular renin activity and angiotensin II generation in rat mesangial cells

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Submitted 22 October 2003; accepted in final form 5 January 2004

Vidotti, D. B., D. E. Casarini, P. C. Cristovam, C. A. Leite, N. Schor, and M. A. Boim. High glucose concentration stimulates intracellular renin activity and angiotensin II generation in rat mesangial cells. Am J Physiol Renal Physiol 286: F1039–F1045, 2004. First published January 13, 2004; 10.1152/ajprenal.00371.2003.—Increased intrarenal renin-angiotensin system activity contributes to diabetic nephropathy. ANG II generation in mesangial cells (MC) is increased by high-glucose (HG) exposure. This study assessed the mechanisms involved in the glucose-induced ANG II generation in rat MC. Under basal conditions, MC mainly secreted prorenin. HG decreased prorenin secretion and induced a striking 30-fold increase in intracellular renin activity. After 72 h of HG exposure, only the mRNA levels for angiotensinogen and angiotensin-converting enzyme (ACE) were significantly elevated. However, after shorter periods of 24 h of HG stimulation the mRNA levels of the enzymes prorenin and cathepsin B, besides that for ACE, were significantly increased. The results suggest that the HG-induced increase in ANG II generation in MC results from an increase in intracellular renin activity mediated by at least three factors: a time-dependent stimulation of (pro)renin gene transcription, a reduction in prorenin enzyme secretion, and an increased rate of conversion of prorenin to active renin, probably mediated by cathepsin B. The increase in angiotensinogen mRNA in parallel to increased renin activity indicates that HG also increased the availability of the renin substrate. The consistent upregulation of ACE mRNA suggests that, besides renin, ACE is directly involved in the increased mesangial ANG II generation induced by HG.

hyperglycemia; diabetic nephropathy; glomerular sclerosis; renin-angiotensin system

DIABETIC NEPHROPATHY IS CHARACTERIZED by excessive production of mesangial matrix that contributes to glomerular sclerosis, leading to proteinuria and renal failure. Among the potential mediators of mesangial matrix expansion in diabetic nephropathy, the peptide ANG II is particularly outstanding, and many lines of evidence suggest activation of the intrarenal renin-angiotensin system (RAS) in diabetes (21). The RAS has been extensively studied in diabetes, and strong evidence indicates that the inhibition of angiotensin-converting enzyme (ACE) and/or ANG II receptors delays the progression of diabetic nephropathy (3, 37). Despite these benefits, the renoprotective effects of RAS inhibition cannot be fully explained only by the hemodynamic effects of the system causing a reduction in blood pressure. Moreover, many patients with diabetic nephropathy paradoxically present low plasma renin activity (12, 32). Thus renin suppression in this condition supports the hypothesis that, rather than systemic synthesis, increased local synthesis of ANG II in the kidney may contribute to the pathogenesis of diabetic nephropathy, although the specific site of intrarenal ANG II in this pathological condition is not completely known.

Renal tubular epithelial cells constitute a potential site of intrarenal ANG II synthesis (25, 35). In addition, it has been demonstrated that mesangial cells express mRNA for renin, angiotensinogen, and ACE (4, 5). Renin is a rate-limiting enzyme in the synthesis of ANG II (34), and we have recently demonstrated that mesangial cells in culture are able to synthesize, store, and secrete both forms of the enzyme, i.e., active renin and inactive prorenin (4).

Hyperglycemia has been recognized to be the pathogenic factor of the long-term complications of diabetes mellitus. Thus in the present study we examined whether a high glucose concentration in the culture medium is able to modify renin synthesis, the secretion of the renin/prorenin ratio, and the intracellular renin activity in mesangial cells in culture. Also, we examined whether this “hyperglycemic” condition alters the levels of mRNA expression of RAS components, including angiotensinogen, prorenin, ACE, types I and II (AT1 and AT2) angiotensin receptors, and cathepsin B, a potential enzyme involved in renin activation. Finally, we also determined whether glucose-inducing modifications in renin activity and in the expression of RAS components result in an increase in local ANG II generation.

MATERIALS AND METHODS

Primary culture of rat mesangial cells. Mesangial cells were cultured by standard techniques using collagenase isolation by differential sieving (17). Briefly, glomerular mesangial cells were cultured from kidneys freshly removed from normal adult male Wistar rats. The kidney cortex was macroprepared, fragmented, forced through a graded series of stainless steel sieves (60, 100, and 200 mesh), and rinsed with RPMI 1640 culture medium. The glomeruli were then collected from the surface of the third sieve and forced through a 25 × 7-gauge needle for full decapsulation. Mesangial cells were obtained from collagenase-treated, isolated glomeruli to remove the epithelial cell component. Glomeruli were plated at a density of ~300 glomeruli/cm onto RPMI 1640 containing 10 mM d-glucose and supplemented with 20% fetal calf serum, 50 U/ml penicillin, 2.6 g acid HEPES, and 2 mM glutamine. Culture flasks were kept in a 95% air-5% CO2 humidified environment at 37°C. The culture medium was replaced every 36 h. After 3 wk, cells were harvested by trypsin and the subcultures were grown in the same culture medium. Cells were used between the third and fifth subculture and characterized by classic methods using the following criteria: morphological appearance of stellate cells, immunofluorescence staining of the extracellular matrix for type IV collagen and fibronectin, negative immunofluorescence staining for human factor VIII antigens (glomerular endothelial...
cells) and cytoketerin (parietal epithelial cells), and positive immuno-fluorescence staining for actin and myosin. In particular, the presence of contaminating juxtaglomerular cells was excluded on the basis of their characteristic phenotype including their spherical shape and the presence of a high density of granules.

**Experimental protocol.** At subconfluence, mesangial cells were divided into three groups as follows: control, where cells were kept in RPMI 1640 medium supplemented with FBS, containing normal glucose concentration of 10 mM d-glucose (NG group); high glucose, where cells were cultured in RPMI 1640 supplemented with 30 mM d-glucose; and mannitol, where cells were submitted to the same procedure as used for the cell culture in the presence of glucose, but 30 mM mannitol was added instead of glucose and served as osmolarity control (M group). Cells were exposed to 30 mM d-glucose or mannitol for a total of 72 h (16). After 48 h, cells were rinsed twice with PBS and the culture medium was replaced with a medium containing the respective normal- or high-glucose and mannitol concentrations, but no FBS, to keep the cells in the G0 phase of the cell cycle and without any interference from serum proteins. The culture medium was then collected over the last 24 h and stored at −70°C until use. Cells were rinsed with PBS, lysed with 1 mM Tris/HCl buffer, pH 7.5, and stored at −70°C until use. An additional group of cells were exposed to high glucose concentration for another 24 h.

**Determination of active and inactive renin (prorenin) content and renin activity.** The renin content of the active and inactive forms of renin was estimated by measuring the amount of ANG I present in the cell lysate and culture medium by HPLC, as described below. Total renin content included active renin and the inactive prorenin forms. Prorenin was activated by adding 10 μl of trypsin (50 μg/ml) for 16–18 h at 37°C to both the cell lysate and culture medium. Active renin was evaluated in the absence of trypsin. To prevent further cleavage of prorenin, angiotensinogen, and ANG I, a pool of enzymatic inhibitors was added to the cell homogenate and culture medium: 50 mM EDTA, 1 mM OPhε, 32 mM PMFS, and 200 mM DTT. These inhibitors act efficiently on a broad range of metallo-, serine, and cysteine proteases, respectively. Renin activity was estimated by ANG I generation when the cell lysate and culture medium (in the presence and absence of trypsin) were incubated with 10 μl of 1 mg/ml synthetic tetradecapeptide substrate (Sigma, St. Louis, MO) for 4 h at 37°C, as previously described (24). The reaction was stopped by adding 10 μl of 50% H3PO4. One hundred microliters of each sample were filtered and injected into the HPLC system. The released ANG I peptide was quantified by reverse-phase HPLC using an aquaporine ODS 300 column equilibrated with 0.1% phosphoric acid containing 5% acetonitrile (vol/vol). ANG I was separated by isocratic elution for 5 min, followed by a 20-min linear gradient of 5–35% acetonitrile in 0.1% phosphoric acid (vol/vol) at 1.5 ml/min. The chromatographic profile of each sample was compared with that obtained for standard samples containing angiotensinogen and ANG I at an absorbance of 214 nm. Peptide fragments were identified by elution position and peak height (Rf). The results were corrected by the peptide solution to correct for small differences in retention time for ANG I peptide was quantified by ANG I peptide was quantified by ELISA (model PTC-100, MJ Research, Watertown, MA) using 2 μl of reverse-transcribed cDNA in a total volume of 20 μl containing 1.0–2.5 mM MgCl2 (optimized for each primer pair), 0.5 mM of each primer, 0.5 mM dNTP mix, and 0.5 U Taq DNA polymerase (Pharmacia Biotech). Primer sequences for amplification of angiotensinogen, prorenin, and ACE, and AT1 and AT2 receptors were designed using appropriate software (PCR-Designer, Research Genetics) based on each cDNA sequence obtained from GenBank. Primer sequences and the amplification conditions for each primer are shown in Table 1. Negative controls were included in each PCR set where cDNA was replaced with water as a control for contamination from exogenous sources. In addition, the RT enzyme was omitted in some samples as a negative control for amplification of genomic DNA. Positive controls included kidney cortex cDNA and the efficiency of the RT reaction was monitored by the amplification of the constitutively expressed gene for β-actin. PCR products were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining under UV light.

**Expression levels of RAS components by quantitative real-time PCR analysis.** Because the presence of RAS components was confirmed by RT-PCR, the expression levels of each mRNA were estimated by real-time RT-PCR using the ABI GeneAmp 5700 Sequence Detection System SDS (ABI Prism 7700, Applied Biosystems), which was developed to determine gene expression with high sensitivity and specificity. The cDNA was synthesized from 1 μg of total RNA extracted from each group of cells as described above. Real-time PCR product accumulation was monitored using the intercalating dye SYBR Green I (Molecular Probes), which exhibits a higher fluorescence on the binding of double-strand DNA. Relative gene expression was calculated using conditions at the end of PCR, when amplification was logarithmic and thus could be correlated to the initial copy number of gene transcripts. The reactions were cycled 40 times under the conditions previously determined by conventional PCR. Fluorescence for each cycle was quantitatively analyzed by ABI Prism 7700 SDS (Applied Biosystems). At the end of the PCR, the temperature was increased from 60 to 95°C at a rate of 2°C/min, and fluorescence was measured every 15 s to construct the melting curve. A nontemplate control was run with each assay. The relative amount of each experiment per group was reported as relative expression normalized with the GAPDH housekeeping gene, used as an endogenous control, and expressed in arbitrary units.

**ANG II measurement by ELISA.** ANG II levels in the cell lysate were determined by ELISA using the avidin-streptavidin method as previously described (38). Five micrograms of protein samples were

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Primer Sequence</th>
<th>Product Size, bp</th>
</tr>
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<tbody>
<tr>
<td>Angiotensinogen</td>
<td>5′-CGGCTGTTGCTGGTTAGCTCT-3′</td>
<td>398</td>
</tr>
<tr>
<td>Prorenin</td>
<td>5′-GGGCGTGCAGCTGTGG-3′</td>
<td>295</td>
</tr>
<tr>
<td>ACE</td>
<td>5′-GTGGCCACCTGCTGACCAAAGTT-3′</td>
<td>228</td>
</tr>
<tr>
<td>AT1B receptor</td>
<td>5′-ATGGCGAGTGGTGGTGGGC-3′</td>
<td>244</td>
</tr>
<tr>
<td>AT2 receptor</td>
<td>5′-GGGCGTGCAGCTGTGG-3′</td>
<td>275</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>5′-GGGGGAAAACTCTCAAAGAATT-3′</td>
<td>388</td>
</tr>
</tbody>
</table>

ACE, angiotensin-converting enzyme.
added to the wells of a microtiter plate in duplicate. Protein was allowed to adsorb for 1 h at 37°C and then blocked with 200 μl of PBS-Tween 20 containing 0.5% casein for 2 h at 37°C, and the anti-ANG II antibody (1:100 dilution) was added to the respective well and incubated for 1.5 h at 37°C. Plates were washed three times with PBS-Tween 20, and 150 μl of biotinylated anti-mouse IgG (1:500 dilution, Amersham Life Science) were added to each well and incubated for 1 h at 37°C. The final wash was followed by development using o-phenylenediamine dihydrochloride (Abbott Laboratories) and hydrogen peroxidase reagents for 20 min at room temperature. Absorbance was recorded at 495 nm, and ANG II concentrations were calculated from the standard curve.

Statistical analysis. Results are expressed as means ± SE. Data were analyzed by one-way ANOVA followed by the Tukey or Newman-Keuls test. Nonparametric data were analyzed by the Dunn or Kruskal-Wallis test. P < 0.05 was considered significant.

RESULTS

Total renin content, including active renin and prorenin, was measured in the cell lysate and culture medium. Renin content was estimated in the presence of an excess of substrate. In the cell lysate it corresponds to the amount accumulated over 72 h, and the content in the culture medium corresponds to the amount accumulated over the last 24 h of the experimental protocol. The amount of prorenin was calculated from the difference between the content of total and active renin. The content of renin was estimated from renin activity, obtained with an excess of substrate. Figure 1 represents the content of active and inactive renin in both cell lysate and culture medium. High glucose concentration induced an impressive elevation in the intracellular content of active renin as well as in the prorenin content compared with the control groups (NG and M). In contrast, in the culture medium the content of active renin was similar for the three groups, but the amount of prorenin was reduced by HG compared with control cells.

Figure 2 shows renin activity expressed as nanograms of ANG I per milligram protein per hour in the cell lysate and culture medium. Besides increasing renin content, a high glucose concentration also induced a significant 30-fold increase in the renin activity measured in the cell lysate compared with the control and M groups but did not change renin activity in the culture medium.

The expression of mRNAs for angiotensinogen, cathepsin B, prorenin, ACE, and AT1 and AT2 receptors was detected in mesangial cells by RT-PCR (data not shown). After optimization of the PCR conditions for each primer pair, specific for each component, cDNA samples were used to quantify the mRNAs by real-time PCR. Figure 3 shows a representative standard curve and amplification curves for ACE in the three groups. A threshold was determined for each PCR run to match the logarithmic phase of the curve, and the amount of each cDNA was estimated from the standard curve. As can be observed in this representative curve for ACE, the control samples (NG and M) were closer to the most diluted points (1:100 and 1:10), whereas the HG samples coincided with the undiluted point.

Figure 4 shows the relative quantification of mRNAs for all RAS components. Data are expressed in arbitrary units considering GAPDH as the internal control. The mRNA expression levels detected after 72 h of high-glucose exposure were unchanged for all components except angiotensinogen, 1.8-fold higher than in the NG group, and mainly ACE mRNA, which presented a 15-fold increase compared with the NG group. In addition, the mRNA expression levels of the RAS enzymes including prorenin, cathepsin B, and ACE were further estimated after 24 h of high-glucose exposure. Figure 5 shows that after this shorter period the mRNA expression...
levels for the three enzymes were significantly elevated in the HG group compared with the NG group.

ANG II concentration in mesangial cells was determined by ELISA. As shown in Fig. 6, glucose-stimulated cells presented a significant increase in ANG II generation compared with control cells. Mannitol-exposed cells also presented an increase in ANG II, but the mean value obtained did not differ from control cells.

DISCUSSION

Many lines of evidence suggest a role for intrarenally formed ANG II in the pathogenesis of diabetic nephropathy (12, 21, 32). It has been shown that glucose and ANG II are able to increase the synthesis of collagen types I and IV and other matrix proteins in mesangial cells, as well as to decrease the levels of proteases involved in matrix degradation, resulting in the matrix expansion observed in diabetic nephropathy (19, 37). It has been shown that increasing glucose concentrations cause proportional increases in ANG II generation in mesangial cells (38). In the present study, we obtained further evidence that mesangial cells may contribute to the enhancement of intrarenal ANG II observed in diabetic kidneys.

We have shown that mesangial cells are able to synthesize, store, and spontaneously secrete both the active and inactive forms of renin, as previously demonstrated by us in rat and mouse mesangial cells (4) and by Chansel et al. (10) in human mesangial cells. Moreover, it was shown that the secretion rate of active and inactive renin changes in response to the external stimulus induced by high glucose concentration in the culture medium. In control and mannitol-stimulated cells, prorenin was predominant in the culture medium, indicating that under basal conditions most of the renin secreted is in the inactive
form, as also described for juxtaglomerular cells and other renin-secreting cells (9, 15, 18). The reason mesangial cells secrete large quantities of inactive renin is not known, but the presence of renin and prorenin receptors in the kidney (29) and mesangial cells (28) suggests an exciting possibility of autocrine and paracrine functions for prorenin and renin secreted by mesangial cells.

High glucose induced a reduction in renin content in the culture medium, mainly in prorenin secretion, roughly three times that observed in control cells, resulting in the accumulation of prorenin in the intracellular compartment. It was recently demonstrated that the mesangial renin receptor binds renin and prorenin, and the binding of renin to this receptor, in human mesangial cells, induced a fourfold increase in the efficiency of angiotensinogen conversion to ANG I (29) compared with renin in the soluble phase. Moreover, the renin/prorenin binding to the receptor would not be detected in the culture medium but in the cell lysate fraction. Whether the increased prorenin content in the intracellular compartment observed in the present study was a result of reduced secretion and/or a binding to the receptor deserves further investigation. In addition, intracellular prorenin accumulation was also a result of increased prorenin gene transcription, observed after a short period of high-glucose stimulation. Actually, one of the most important events observed in the present study was an impressive rise in intracellular renin content and activity induced by high-glucose exposure, suggesting that the accumulated prorenin was converted to active renin inside the cell, and thus high glucose also induced an increase in the rate of prorenin-to-renin conversion. This result is in contrast to that recently described by Singh and co-workers (38). They found that renin activity, measured as ANG I generation in the presence of an excess of angiotensinogen, was not altered by high glucose. We have no explanation for this discrepancy, but maybe some differences between the protocols used to measure renin activity would be considered. For instance, the aforementioned study does not mention whether the protease inhibitors were added to the renin assay, as in the present study, and this is particularly important because renin appears to be rapidly inactivated in vitro and probably by proteases (13).

The conversion of prorenin to active renin results from the proteolytic cleavage of 43 amino acids from the pro-segment of prorenin. Although many enzymes have been implicated in this mechanism, cathepsin B has been suggested to be the primary one involved in the activation of prorenin in vivo (27). The presence of cathepsin B-like activity was recently demonstrated in rat mesangial cells, in parallel to its ability to hydrolyze prorenin (1), making this enzyme a potential candidate involved in the conversion of prorenin to active renin in mesangial cells. We found a significant increase in cathepsin B mRNA expression levels after 24 h of high-glucose exposure, possibly indicating that the increase in the conversion of prorenin to active renin induced by high glucose was, at least initially, mediated by cathepsin B. On the other hand, cathepsin B mRNA returned to control levels after the more prolonged period of glucose stimulation. Actually, previous reports have shown that the diabetic milieu is mostly associated with a decrease in the degradative enzymes, including cathepsin B, in glomeruli and mesangial cells (22, 23, 39). Taken together, these data raise the possibility that glucose may interfere with cathepsin B expression through a time-dependent pathway, initially stimulating and then suppressing cathepsin B gene transcription.

Similar to cathepsin B mRNA expression, high glucose induced an initial increase in prorenin mRNA, detected after 24 h, with a return to control levels after 72 h of glucose exposure, suggesting that renin synthesis by mesangial cells is probably subjected to the usual negative feedback control
induced by ANG II as classically observed in juxtaglomerular cells (26).

In contrast to time-dependent upregulation of prorenin and cathepsin B gene transcription, the expression levels of ACE mRNA were significantly elevated after both 24 and 72 h of high-glucose stimulation, suggesting that ACE is probably involved in the increased mesangial ANG II generation by glucose. However, whether ACE gene transcription is influenced by high glucose and/or by ANG I concentration deserves additional investigation.

High glucose induced an upregulation of angiotensinogen gene expression detected after 72 h. Singh et al. (38) recently demonstrated that mesangial cells incubated in high-glucose media increased both angiotensinogen mRNA and protein levels. Taken together, these data suggest that, in parallel to increased renin activity, high glucose also increased the availability of the renin substrate.

The results showed that mesangial cells in culture are able to constitutively express mRNA for both AT1 and AT2 receptors, as also previously demonstrated (6, 11, 20, 35). In contrast to intracellular components, high glucose did not change mRNA expression levels for AT1 or AT2 receptors. Conflicting results have been reported concerning the effect of glucose or diabetes on the expression of ANG II receptors in kidney cells. Down-regulation of the AT1 receptor has been demonstrated in glomeruli and proximal tubules of diabetic rats (7, 30, 40), as well as in mesangial cells chronically incubated in culture medium containing high glucose levels (2). In contrast, Chouinard and co-workers (11) recently demonstrated that high glucose did not modify AT1 mRNA expression in mesangial cells but increased the expression of AT2 receptors. On the other hand, the absence of changes in ANG II receptor mRNA observed in the present study does not rule out the possibility of an upregulation of gene transcription during shorter periods of glucose stimulation. Finally, it is important to reflect on the possibility that ANG II may not leave the cells to induce its effects. Previous data from our laboratory (unpublished observations) showed ANG II immunostaining in the nucleus of mesangial cells, which was strongly increased after exposure to high glucose levels. ANG II accumulation inside the nucleus has been found in other tissues, including myocardium, brain, smooth muscle, and adrenal glands (8, 14, 32). The presence of ANG II inside the nucleus may represent an exciting mechanism involved in the well-known effects of ANG II-inducing gene expression, particularly those involved in cell growth, metabolism, and synthesis of extracellular matrix components, manifestations that are typical of diabetic nephropathy.

In summary, the present study showed a significant increase in ANG II generation in mesangial cells exposed to a high glucose concentration. The results suggest that the mechanisms involved in the upregulation of mesangial ANG II primarily include an increase in intracellular renin activity, which resulted from at least three mechanisms, i.e., a time-dependent stimulation of prorenin gene transcription, a reduction in prorenin secretion, and an increase in the rate of prorenin conversion to active renin, probably mediated by cathepsin B. The increased renin activity was paralleled by a rise in angiotensinogen gene expression, indicating availability of the substrate to renin. In contrast to time-dependent upregulation of prorenin and cathepsin B gene transcription, the expression levels of ACE mRNA were significantly elevated after both 24 and 72 h of high-glucose stimulation, indicating that ACE upregulation is probably necessary to increase ANG II synthesis. The mRNA levels for ANG II receptors were unchanged, at least after 72 h of exposure to glucose, suggesting that ANG II production by mesangial cells under glucose stimulation may serve as an intracrine action, thus directly mediating the proliferative and inflammatory effects of ANG II, with consequent cell proliferation, matrix expansion, and inflammation contributing to the glomerular sclerosis observed in diabetic nephropathy.

ACKNOWLEDGMENTS

Part of this study was presented in preliminary form at the 35th Annual Meeting of the American Society of Nephrology, Philadelphia, PA, November 1–4, 2002.

GRANTS

This work was supported by grants from Fundação de Amapá a Pesquisa do Estado de São Paulo, Conselho Nacional de Desenvolvimento Científico e Tecnológico, Fundação Oswaldo Ramos, and Fundo de Auxílio aos Docentes e Alunos.

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