A novel mechanism for angiotensin II formation in streptozotocin-diabetic rat glomeruli

Rekha Singh, Ashok K. Singh, and David J. Leehey. A novel mechanism for angiotensin II formation in streptozotocin-diabetic rat glomeruli. Am J Physiol Renal Physiol 288: F1183–F1190, 2005. First published February 8, 2005; doi:10.1152/ajprenal.00159.2003.—Recent evidence suggests that the intrarenal renin-angiotensin system (RAS) may play an important role in the development of glomerular changes associated with diabetic nephropathy. In this study, the glomerular RAS was examined in male Sprague-Dawley rats made diabetic with streptozotocin (STZ), and the findings compared with those obtained in control nondiabetic rats. In diabetic rat glomerular extracts, angiotensinogen and angiotensin II (ANG II) levels were increased significantly by 2.2- and 1.9-fold, respectively, compared with nondiabetic controls. No significant differences in ANG I and angiotensin-converting enzyme (ACE) levels were observed between these groups. The HPLC analysis of the glomerular extracts demonstrated that exogenous ANG I was converted into various ANG peptides including ANG II, ANG(1–9), and ANG(1–7). A significant increase in formation of ANG II from exogenous ANG I was observed in STZ rats compared with control rats. Preincubation of glomerular extracts with captopril resulted in a 20–30% decrease in ANG II conversion from exogenous ANG I in diabetic and control rats. The possible role of ANG(1–9) in formation of ANG II was examined by HPLC. Exogenous ANG(1–9) in glomerular extracts was converted into ANG II, this conversion being significantly higher in STZ rats than in control rats. These findings provide new information that ANG II receptors were downregulated (3, 12, 24), suggesting an increase in glomerular ANG II levels. Previous studies with glomerular mesangial cells from our laboratory showed that addition of high glucose to the culture media resulted in increased ANG II production in these cells (19). In addition, we recently demonstrated that high glucose stimulates mesangial ANG II production by increasing its substrates angiotensinogen and ANG I (20). In the same study, it was also noted that other ANG peptides such as ANG(1–9) could be converted into ANG II and that this conversion was also stimulated under high-glucose conditions in mesangial cells (20). Because these findings indicated the existence of additional non-ACE mechanisms for ANG II production in mesangial cells, we tested the in vivo relevance of these mechanisms in diabetic rats. Thus the present study was designed to determine the mechanisms involved in diabetes-induced ANG II production in the rat glomeruli.

MATERIALS AND METHODS

Diabetic Rat Model

Male Sprague-Dawley rats (200–300 g) were made diabetic by a single injection of STZ (65 mg/kg body wt; Sigma, St. Louis, MO) administered into the tail vein. Control animals received an equal volume of vehicle. The diabetic state of the animal was confirmed by the demonstration of a fasting blood glucose level >250 mg/dl 24 h after STZ injection. Blood glucose measurements were carried out twice a week throughout the 4-wk study period. Food and water intake were ad libitum throughout the study. No insulin was administered to STZ-diabetic rats.

Preparation of Glomerular Extracts

Four weeks after STZ (or vehicle) injection, rats were anesthetized with pentobarbital sodium (60 mg/kg ip). Kidneys were rapidly removed, weighed, and minced on ice. Because the state of kidney tissue (fresh vs. frozen) and the extraction time can significantly affect endogenous levels of ANG I and ANG II (9), fresh kidneys were used for the preparation of glomerular extracts with rapid removal and processing of the kidney tissue. Glomeruli were isolated by sequential sieving in PBS at 4°C. Glomerular suspensions were composited of >95% glomeruli as determined by light microscopy. The suspensions were then centrifuged, and pellets were resuspended in PBS and sonicated (Microscan ultrasonic cell disruptor, Heat Systems, Farmingdale, NY) to rupture cell membranes, followed by centrifugation at 13,000 g for 20 min at 4°C. The supernatant was collected and used for various measurements.

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Because glomeruli contain many peptidase enzymes that can metabolize ANG peptides during the extraction procedure, glomerular extracts from normal and STZ-diabetic rats were also prepared in PBS containing peptidase enzyme inhibitors cocktail (Sigma). The supernatants were used for measurements of endogenous levels of ANG I and ANG II.

Measurement of Angiotensinogen

Antibodies to angiotensinogen were prepared using renin substrate tetradecapeptide [angiotensinogen(1–14)] (Sigma) as the immunizing antigen and the specificity of the antibody to rat kidney angiotensinogen was confirmed by Western blot analysis as described previously (20). Angiotensinogen levels in glomerular extracts were measured by ELISA (20). In brief, a 96-well plate was coated overnight at 4°C with 4 μg/ml of angiotensinogen(1–14), the immunizing antigen. Incubation was carried out with standards or samples mixed with anti-angiotensinogen antibody (1:10,000) for 2 h at room temperature followed by incubation with a secondary antibody (anti-rabbit IgG-peroxidase, 1:1,000) for 1 h. The reaction was developed using TMB substrate and after 20 min terminated with 2 N HCl. The absorbance was read at 450 nm using ELISA reader (Molecular Devices). The color intensity that develops depends on the quantity of biotinylated peptide bound to the immobilized antibody so a higher amount of peptide present in the sample will allow less binding of biotinylated peptide with the limited amount of antibody resulting in production of less color by the substrate. ANG I or ANG II standard curves were run with each assay and levels of these peptides in the samples were calculated.

Measurement of ACE Levels in Glomerular Extracts

ACE protein levels in glomerular extracts from STZ and control rats were measured by ELISA using commercially available ELISA kits (Chemicon International, Temecula, CA). In addition, ACE protein levels were also determined by Western blot analysis. Glomerular extracts were separated by 8% SDS-PAGE using a Bio-Rad Mini-

HPLC. First, standard ANG peptides (Bachem Biosciences, King of Prussia, PA) were injected into the HPLC column and analyzed on a C18 μBond reverse-phase column using an ultraviolet detector set at 214 nm (Isco, Lincoln, NE). The flow rate was maintained at 1 ml/min. Mobile phase A consisted of water with 0.00005% trifluoroacetic acid and mobile phase B consisted of 100% acetonitrile containing 0.00005% trifluoroacetic acid. The gradient program was set as 0–5 min 90% A:10% B, 5–32 min 60% A: 40% B, 32–40 min 90% A: 10% B to elute the products. After each sample analysis, a wash cycle was run to prevent contamination from any residual peptide left from the previous run. A sample chromatogram showing different ANG peptide peaks and their elution times is shown in Fig. 1.

Separation of endogenous ANG I and ANG II in glomerular extracts was carried out using HPLC followed by ELISA. First, a glomerular extract sample from a control rat was injected into the HPLC column and fractions (1–30) were collected at 1-min intervals. Fractions were air dried, reconstituted in assay buffer (supplied with ANG peptide ELISA kit), and assayed for ANG II (fractions 1–23) and ANG I (fractions 19–30) immunoactivity by ELISA (Peninsula Laboratories, Belmont, CA). As shown in Fig. 2, the peak immunoreactivity to ANG II was detected in the 20-min fraction that coincided with the elution time of standard ANG II, and the peak immunoreactivity to ANG I was detected in the 22-min fraction that coincided with the elution time of standard ANG I. In subsequent experiments, glomerular extracts of normal and STZ-diabetic rats were subjected to HPLC separation and only 20- and 22-min fractions were used for ANG II and ANG I measurements by ELISA, respectively.

ANG I and ANG II ELISA. ANG I and ANG II levels were measured by a competitive ELISA using ELISA kits purchased from Peninsula Laboratories. Briefly, ELISA plates precoated with goat anti-rabbit IgG were used for the assays. Samples were mixed with rabbit antibodies to ANG I or ANG II and biotinylated ANG I or ANG II peptides, respectively, and incubated in the wells for 2 h at room temperature. After incubation and washings, streptavidin-conjugated horseradish peroxidase (SA-HRP) was added and allowed to bind to the immobilized primary antibody/biotinylated peptide complex in the wells. The final reaction was developed with TMB and H2O2 substrate and after 20 min terminated with 2 N HCl. The absorbance was read at 450 nm using ELISA reader (Molecular Devices). The standard curve was run with each assay and levels of these peptides in the samples were calculated.

Fig. 1. Representative chromatogram showing elution times of various standard ANG peptides that were analyzed by HPLC on a C18 μBond reverse-phase column using an ultraviolet detector set at 214 nm. The flow rate was maintained at 1 ml/min and the gradient was set as 0–5 min 90% A:10% B, 5–32 min 60% A: 40% B, 32–40 min 90% A: 10% B to elute the products (solvent A = water + 0.00005% trifluoroacetic acid and solvent B = 100% acetonitrile + 0.00005% trifluoroacetic acid).

Fig. 2. Immunoreactive angiotensin in HPLC fractions eluted from a control rat glomerular extract. A sample of glomerular extract was analyzed using HPLC as described in Fig. 1 and fractions (1 ml) were collected at 1-min intervals for 30 min. Samples were air dried, reconstituted in assay buffer, and assayed for ANG II (fractions 1–23) and ANG I (fractions 19–30) immunoactivity by ELISA. The peak immunoreactivity to ANG II was detected in the 20-min fraction that coincided with the elution time of standard ANG II, and the peak immunoreactivity to ANG I was detected in the 22-min fraction that coincided with the elution time of standard ANG I.
Table 1. Elution time of various angiotensin peptides

<table>
<thead>
<tr>
<th>Breakdown Products of ANG(1–10) and ANG(1–8)</th>
<th>ANG Peptides Identified in Glomerular Extracts Incubated with ANG(1–10)</th>
<th>Breakdown Products of ANG(1–9)</th>
<th>ANG Peptides Identified in Glomerular Extracts Incubated with ANG(1–9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANG(1–10)</td>
<td>22.6 ± 0.16</td>
<td>ANG (1–7)</td>
<td>11.1 ± 0.08</td>
</tr>
<tr>
<td>ANG(2–10)</td>
<td>21.3 ± 0.16</td>
<td>ANG (1–9)</td>
<td>17.2 ± 0.14</td>
</tr>
<tr>
<td>ANG(3–10)</td>
<td>22.9 ± 0.03</td>
<td>ANG (1–8)</td>
<td>20.0 ± 0.04</td>
</tr>
<tr>
<td>ANG(4–10)</td>
<td>21.2 ± 0.14</td>
<td>ANG(2–9)</td>
<td>15.4 ± 0.29</td>
</tr>
<tr>
<td>ANG(5–10)</td>
<td>18.9 ± 0.06</td>
<td>ANG(3–9)</td>
<td>16.8 ± 0.17</td>
</tr>
<tr>
<td>ANG(2–8)</td>
<td>18.7 ± 0.12</td>
<td>ANG(4–9)</td>
<td>14.3 ± 0.14</td>
</tr>
<tr>
<td>ANG(3–8)</td>
<td>21.0 ± 0.05</td>
<td>ANG(4–8)</td>
<td>19.9 ± 0.02</td>
</tr>
<tr>
<td>ANG(1–7)</td>
<td>11.6 ± 0.11</td>
<td>ANG(1–7)</td>
<td>11.3 ± 0.15</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 5 experiments for each peptide). Angiotensin peptides identified in glomerular extracts incubated with ANG(1–10) or ANG(1–9) were separable by >1.0 min. ANG(1–10), ANG I; ANG(1–8), ANG II.

Protein Cell (Bio-Rad Laboratories, Hercules, CA) and transferred to a polyvinylidene difluoride membrane (Bio-Rad) overnight at 4°C. Membranes were incubated in blocking buffer (PBS containing 0.1% Tween 20 and 5% nonfat milk protein) for 2 h, washed, and then incubated with 1:100 of primary antibody to ACE (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature. Further incubation was carried out with a HRP-conjugated secondary antibody (1:000) for 1 h. The specific proteins on the membranes were detected by chemiluminescence using the ECL detection system (Amersham Biosciences, Piscataway, NJ).

Analysis of ANG II Formation from Exogenous ANG I or ANG(1–9) Using HPLC

Formation of ANG II from exogenous ANG I or ANG(1–9) was studied using HPLC as described previously (20). First, to identify ANG peptide peaks generated by glomerular extracts, the elution times for various ANG peptides and their breakdown products were established by running their standards. ANG I, ANG II, ANG(2–8) [ANG III], ANG(3–8) [ANG IV], ANG(1–9), and ANG(1–7) were purchased from Bachem Biosciences; breakdown products of ANG I [ANG(2–10), ANG(3–10), ANG(4–10), ANG(5–10)] and ANG(1–9) [ANG(2–9), ANG(3–9), and ANG(4–9)] were custom synthesized (Dr. B Wakim, Medical College of Wisconsin, Milwaukee, WI). These standard peptides were run individually and their elution times were recorded and presented in Table 1. After each sample analysis, a wash cycle was run to prevent contamination from any residual peptide left from the previous run. Most of the ANG peptides were separable by >1.0 min though elution times of ANG(2–8) and ANG(3–8) were found to be overlapping with elution times of ANG(5–10) and ANG(4–10), respectively (Table 1). Glomerular extracts were incubated with 10–4 M of exogenous ANG I or ANG(1–9) for 1 h at 37°C. A 12-μg sample of glomerular extract from a STZ or control rat was injected into the HPLC column and analyzed using the same conditions described above. Absorbance at 214 nm for each peptide was calculated from the peptide peak height and used to determine the representative chromatograms shown in RESULTS. Quantification of peptide peaks was carried out as described above, and results were presented as the ratio of ANG peptides to ANG II.

Statistics

Values are expressed as means ± SE, and n denotes the number of animals for each experiment. Results were compared using Student’s t-test. In experiments where more than one determinant was present, one-way ANOVA was applied. A P value of <0.05 was considered to be significant.

RESULTS

Effect of Diabetes on Blood Glucose, Body Weight, and Kidney Weight

A significant increase in blood glucose levels of diabetic rats was observed as early as 24 h after STZ injection that was maintained throughout the 4-wk study period. At the time of euthanasia, blood glucose levels were significantly higher in STZ-diabetic rats compared with control animals (STZ: 480 ± 27 vs. 114 ± 6 mg/dl in control rats; P < 0.0001, n = 7). Body weights of diabetic rats were significantly lower compared with control rats (STZ: 243 ± 18 g vs. control: 330 ± 6 g; P < 0.001, n = 7). In STZ-diabetic rats, kidney weights increased significantly compared with controls. The kidney weight to body weight ratio was 11 ± 0.7 g/kg in STZ rats compared with 6.6 ± 0.1 g/kg in control rats (P < 0.001, n = 7).

Effect of Diabetes on Glomerular Angiotensinogen, ANG I, and ANG II

In STZ rats, immunoreactive total angiotensinogen levels in glomerular extracts were increased significantly by 2.2-fold compared with control rats (Table 2). Also, glomerular ANG II levels in STZ rats were significantly increased by 1.9-fold compared with control rats (Table 2). In contrast, no significant

Table 2. Effect of diabetes on angiotensinogen and ANG peptides in glomerular extracts

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>STZ-diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensinogen, pmol/mg protein (n = 8)</td>
<td>208 ± 48</td>
<td>460 ± 37*</td>
</tr>
<tr>
<td>ANG I, fmol/mg protein (n = 4)</td>
<td>33 ± 7</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>ANG II, fmol/mg protein (n = 4)</td>
<td>31 ± 8</td>
<td>59 ± 4*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Numbers in parentheses indicate number of rats in each group. *P < 0.05 compared with control group.
differences were observed in ANG I levels between STZ and control rats (Table 2).

Similar results were obtained when glomerular extracts were prepared in PBS containing protease enzyme inhibitors. ANG II levels in STZ rats were 63 ± 8 vs. 39 ± 2 fmol/mg protein in control rats (n = 4; P < 0.05), thus showing an increase by 1.6-fold, whereas no significant differences in ANG I levels were observed between the two groups (STZ: 29 ± 2 vs. control: 28 ± 1 fmol/mg protein; n = 4; P = NS). Interestingly, the presence of inhibitors in the extraction buffer did not result in any significant increases in measured ANG I or ANG II levels in either STZ or control glomerular extracts. Thus it appears that preparation of glomerular extracts promptly from fresh kidney tissues at 4°C (as carried out in experiments without inhibitors) can be an effective method to prevent excessive loss of ANG peptides due to degradation.

Effect of Diabetes on Glomerular ACE

Western blot analysis of glomerular extracts showed a distinct band at 75 kDa corresponding to the molecular mass for cellular ACE in both STZ and control rats. However, no significant differences in ACE protein were observed between the two groups (data not shown). Similarly, immunoreactive ACE levels in glomerular extracts did not differ between STZ and control rats (STZ: 28.6 ± 2 vs. control: 26 ± 4 ng/mg protein).

Formation of ANG II from Exogenous ANG I in Glomerular Extracts

First, to test whether exogenous ANG I was stable in incubation buffer (PBS), ANG I (10⁻⁴ M) was incubated in PBS buffer at 37°C for a period of 1–4 h. At the end of each hour, a 25-μL sample was injected into a HPLC column and analyzed. The ANG I peak was identified and peak absorbance at 214 nm was calculated from the ANG I peak height. ANG I 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 ANG I was stable in PBS buffer with no appearances of metabolites for up to 4 h of incubation.

Next, a 12-μg protein sample of glomerular extract from a STZ-diabetic or control rat was incubated in the presence of exogenous ANG I (10⁻⁴ M) for 1 h at 37°C and then injected into the HPLC column. As shown in Fig. 3, a representative chromatogram from a control and STZ rat, exogenous ANG I was converted to ANG II and other ANG peptides such as ANG(1–9) and ANG(1–7). The identity of another peak recorded at 19 min could not be established with certainty because both ANG(2–8) and ANG(5–10) peptides eluted at this time. This peak is shown as an unidentified peak in Fig. 3. The ANG II/ANG I ratio increased significantly in diabetic glomerular extracts indicating increased formation of ANG II from exogenous ANG I in diabetic rats compared with controls (Fig. 4A). There were trends toward an increase in the ANG(1–9)/ANG I ratio in diabetic rats suggesting increased formation of ANG(1–9) from exogenous ANG I and a decrease in ANG(1–7)/ANG I ratio, suggesting a decrease in formation of ANG(1–7) from exogenous ANG I in diabetic rats (Fig. 4A).

Effect of ACE Inhibition on Conversion of Exogenous ANG I to ANG Peptides in Glomerular Extracts

Preincubation of glomerular extracts with captopril (10⁻⁴ M) for 30 min reduced ANG II generation from exogenous ANG I in both STZ and control rats (data not shown).

Role of ANG(1–9) in ANG II Formation in Glomerular Extracts

The possibility of ANG II formation from the intermediary angiotensin peptide ANG(1–9) was examined by HPLC. Glomerular extract samples from control and STZ-diabetic rats were incubated with exogenous ANG(1–9) (10⁻⁴ M) for 1 h at 37°C and analyzed by HPLC. Figure 5, a representative chromatogram from a control and STZ rat, shows conversion of exogenous ANG(1–9) to ANG(1–7) and ANG II. In STZ glomerular extracts, the ANG(1–7)/ANG I or ANG(1–9)/ANG I ratios was observed in either control or STZ diabetic glomerular extracts (data not shown).
Increased accumulation of ANG II in the presence of exogenous ANG(1–9) in STZ rat glomerular extracts could be influenced by decreased breakdown of ANG II. Therefore, STZ and control glomerular extracts were prepared in PBS containing EDTA (to inhibit ANG II breakdown) and STZ-diabetic (filled bars) rat glomerular extracts. The ratio of ANG II/ANG I was reduced by captopril (hatched bars) in both STZ-diabetic and control glomerular extracts but it remained higher in STZ glomerular extracts compared with control extracts (P = NS; n = 5). Values are means ± SE.

Conversion of Exogenous ANG II in Glomerular Extracts

In separate experiments, when STZ and control glomerular extracts were incubated with exogenous ANG II, generation of ANG(1–6) (identified based on its elution time) and two other unidentified peaks were observed (Fig. 6). No significant differences were observed in the ANG(1–6)/ANG II ratio between the two groups (STZ: 0.078 ± 0.01 vs. control: 0.065 ± 0.01; P = NS, n = 3).

DISCUSSION

This study demonstrated that endogenous ANG II levels in STZ-diabetic rat glomeruli were significantly increased compared with control extracts (Table 3). However, the ANG(1–7)/ANG(1–9) ratio remained lower and the ratio of ANG II/ANG(1–9) remained higher in STZ glomerular extracts in the presence of captopril (Table 3). In contrast, preincubation of glomerular extracts with chymostatin (chymase inhibitor) up to 60 min before incubation with exogenous ANG(1–9) had no effect on the conversion of ANG(1–9) to ANG II and ANG(1–7) in either STZ or control rats (data not shown).

Effect of Enzyme Inhibitors on Conversion of ANG(1–9) to ANG II in Glomerular Extracts

Table 3. Effect of captopril on the conversion of exogenous ANG(1–9) to ANG(1–7) and ANG II in glomerular extracts

<table>
<thead>
<tr>
<th>Group</th>
<th>Conversion Ratio ANG(1–7)/ANG(1–9)</th>
<th>Conversion Ratio ANG II/ANG(1–9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.37±0.09</td>
<td>0.14±0.03</td>
</tr>
<tr>
<td>STZ-diabetic</td>
<td>0.18±0.02*</td>
<td>0.08±0.01†</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 5 rats in each group). *P < 0.05 vs. control −captopril. †P = 0.06 vs. control +captopril.
pared with control nondiabetic rats. The increase in glomerular ANG II was accompanied by a significant increase in total angiotensinogen levels in diabetic rat glomeruli compared with controls, although ANG I and ACE levels did not differ between the diabetic and control rats. These findings led us to examine ANG II formation from exogenous ANG I in glomerular extracts using HPLC assays. In both STZ and control glomerular extracts, exogenous ANG I was converted into various ANG peptides such as ANG(1–9), ANG(1–7), and ANG II, and the conversion of ANG I to ANG II was significantly higher in STZ rats vs. controls. Because glomerular ACE levels and protein did not differ between the diabetic and control rats, increased ANG II conversion in diabetic rats did not appear to be due to stimulation of ACE. Furthermore, captopril treatment resulted in only 20–30% inhibition of exogenous ANG I conversion to ANG II in STZ and control glomerular extracts, suggesting that there are additional mechanisms or pathways for the conversion of ANG I to ANG II that employ enzymes other than ACE. Because in the present study a large amount of ANG(1–9) was generated from ANG I, its possible role in ANG II formation was explored using HPLC. Exogenous ANG(1–9) incubated with glomerular extracts was converted to ANG II in both STZ-diabetic and control rats, and this conversion was significantly higher in STZ glomerular extracts compared with control extracts. Increased accumulation of ANG II in the presence of exogenous ANG I or ANG (1–9) in STZ glomerular extracts was unlikely to be due to increased stability of ANG II in STZ glomerular extracts because breakdown of exogenous ANG II to its metabolic products was similar in STZ and control glomerular extracts.

The conversion of ANG I to ANG(1–9) may occur through a specific carboxypeptidase that has been recently identified from a human heart failure ventricle cDNA library (7) and from a human lymphoma cDNA library (21). This enzyme has been termed ACE2 or ACEH and although having a similar catalytic domain to ACE, it is present in only heart, kidney, and testis. Recent studies using ACE2 knockout mice demonstrated that ACE2 plays an important role in regulation of ANG II levels in kidney and heart (6). As a carboxypeptidase, ACE2 is also known to remove a COOH-terminal amino acid from various other angiotensin peptides (6, 22). Although it is not known whether this enzyme is present in the glomeruli, our recent studies (20) and the present study suggest that this enzyme is present in rat cultured mesangial cells as well as in glomeruli. Moreover, conversion of ANG(1–9) to ANG II points to the existence of yet another unidentified carboxypeptidase in the rat glomeruli that can convert ANG(1–9) to ANG II (Fig. 7). Because not much is known about the specificity of the enzyme involved in this conversion, the possible role of ACE or chymase in conversion of ANG(1–9) to ANG II was tested in the present study. Preincubation of glomerular extracts with chymostatin (chymase inhibitor) did not block formation of ANG II in the presence of exogenous ANG(1–9) in either STZ or control rats. These results indicate that chymases are probably not involved in the conversion of ANG(1–9) to ANG II, at least in the glomeruli. Also, chymostatin did not affect conversion of ANG(1–9) to ANG(1–7) in either STZ or control glomerular extracts. On the other hand, captopril (ACE inhibitor) produced a significant inhibition of ANG(1–7) generation from exogenous ANG(1–9) in both STZ and control glomerular extracts, indicating that ACE is involved in the conversion of ANG(1–9) to ANG(1–7). In addition, captopril inhibited conversion of exogenous ANG(1–9) to ANG II by >50% in both STZ and control glomerular extracts. This result was surprising, as ANG(1–9) is converted to ANG II by removal of a single amino acid possibly via carboxypeptidase activity; therefore, inhibition of this enzyme by captopril (a dipeptidase inhibitor) cannot be explained at this time. It appears that captopril may have nonspecific effects on enzymes other than ACE in rat glomeruli or that this enzyme may be another kind of ACE-related carboxypeptidase that, unlike ACE2, is susceptible to ACE inhibitors. Although captopril reduced the ANG II/ANG(1–9)
ratio in both STZ and control glomerular extracts, the ANG II/ANG(1–9) ratio remained higher in STZ glomerular extracts in the presence of captopril. These results emphasize that the conversion of exogenous ANG(1–9) to ANG II, at least in part, employs an enzyme other than ACE and its activity is stimulated in diabetic rat glomeruli.

Our findings that immunoreactive angiotensinogen and ANG II levels are increased in 4-wk STZ-diabetic glomerular extracts suggest early changes in the RAS in diabetic rat glomeruli. Previously, angiotensinogen mRNA levels were noted in one study to be increased in 8-wk STZ-diabetic kidney (1), decreased in 4-wk STZ-diabetic kidney (5); and yet another study reported no change in angiotensinogen mRNA levels in the kidney of 2-wk STZ-diabetic rats (12). More recently, Wehbi et al. (23) reported no change in angiotensinogen mRNA in glomeruli obtained from 2-wk STZ-diabetic rats. It is known that protein levels can change without changes in the mRNA levels by virtue of increased stability and/or increased translational efficiency of the mRNA. This could explain our findings of increased total angiotensinogen levels in diabetic glomeruli vs. no change in angiotensinogen mRNA as reported by Wehbi et al. Of note, in the present study, the anti-angiotensinogen antibody used for ELISA may also react with des-ANG I angiotensinogen in addition to intact angiotensinogen mRNA in glomeruli obtained from 2-wk STZ-diabetic rats. Although a recent study that it may be an endogenous antagonist of ANG II (17). In our study, the F1189

Our study of the converting activities of diabetic glomerular extracts in the presence of exogenous ANG I and ANG(1–9) have yielded interesting information on other ANG peptides whose roles are being increasingly recognized. One such finding was the decreased formation of ANG(1–7) from both exogenous ANG I and ANG(1–9). ANG(1–7) has important physiological functions that are often opposed to those of ANG II (8). ANG(1–7) has diuretic and natriuretic effects (4), inhibits oxygen consumption in the rat proximal tubule (11), and attenuates ANG II-induced vasoconstriction, suggesting that it may be an endogenous antagonist of ANG II (17). In addition, ANG(1–7) has antiproliferative effects (10) and could oppose the effects of ANG II on matrix metabolism.

In conclusion, these findings support the hypothesis that diabetes is associated with stimulation of the glomerular RAS. Glomerular ANG II levels are increased by an increase in angiotensinogen substrate and by conversion of ANG I to ANG II via non-ACE mechanisms. Future studies are needed to further characterize the non-ACE mechanisms for ANG II formation so that effective inhibition of ANG II forming activities can be achieved in diabetes.

ACKNOWLEDGMENTS


GRANTS

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