Differential regulation of circulating and renal ACE2 and ACE in hypertensive mRen2.Lewis rats with early-onset diabetes

Liliya M. Yamaleyeva,1,* Shea Gilliam-Davis,2* Igor Almeida,1 K. Bridget Brosnihan,1 Sarah H. Lindsey,1 and Mark C. Chappell1

1Hypertension and Vascular Research Center, Wake Forest School of Medicine, and 2Biomedical Research Infrastructure Center, Winston-Salem State University, Winston-Salem, North Carolina

Submitted 2 December 2011; accepted in final form 28 February 2012

Yamaleyeva LM, Gilliam-Davis S, Almeida I, Brosnihan KB, Lindsey SH, Chappell MC. Differential regulation of circulating and renal ACE2 and ACE in hypertensive mRen2.Lewis rats with early-onset diabetes. Am J Physiol Renal Physiol 302: F1374–F1384, 2012. First published February 29, 2012; doi:10.1152/ajprenal.00656.2011.—We examined the impact of early diabetes on the circulating and kidney renin-angiotensin system (RAS) in male and female mRen2.Lewis (mRen2) hypertensive rats. Diabetes (DB) was induced by streptozotocin (STZ; 65 mg/kg) at 11 wk of age for 4 wk without insulin replacement. Systolic blood pressures were not increased in DB males or females compared with controls (CON). Circulating angiotensin-converting enzyme 2 (ACE2) increased ninefold (P < 0.05) in DB females and threefold (P < 0.05) in DB males, but circulating ACE and ANG II were higher in the DB groups. Serum C-reactive protein was elevated in DB females but not DB males, and the vascular responses to acetylcholine and estradiol were attenuated in the DB females. Proteinuria, albuminuria, and angiotensinojen excretion increased to a similar extent in both DB females and males. Glomerular VEGF expression also increased to a similar extent in both DB groups. Renal inflammation (CD68+ cells) increased only in DB females although males exhibited greater inflammation that was not different with DB. Cortical ACE2 did not change in DB females but was reduced (30%) in DB males. Renal neprilysin activity (>75%, P < 0.05) was markedly reduced in the DB females to that in the DB and CON males. ACE activity was significantly lower in both female (75%, P < 0.05) and male (50%; P < 0.05) DB groups, while cortical ANG II and Ang-(1-7) levels were unchanged. In conclusion, female mRen2 rats are not protected from vascular damage, renal inflammation, and kidney injury in early STZ-induced diabetes despite a marked increase in circulating ACE2 and significantly reduced ACE within the kidney.

angiotsin; kidney; neprilysin; hypertension; proteinuria

ANGIOTENSIN-CONVERTING ENZYME 2 (ACE2), a homolog of ACE, is recognized as an important enzymatic component of the renin-angiotensin system (RAS) that may regulate functional output of this hormonal system (5, 7, 8, 14, 28, 30, 51, 50). Although ACE2 was originally characterized for its ability to hydrolyze ANG I to Ang-(1-9), subsequent studies revealed a very high catalytic activity to convert ANG II to Ang-(1-7) (41, 44, 45, 56, 58). Indeed, ACE2 inhibition or genetic knockout studies reveal a heightened sensitivity to ANG II-induced increases in blood pressure, as well as an exacerbation of end-organ damage in these experimental models (31, 32, 46, 48, 52, 60). In contrast to ACE, circulating ACE2 is low to nondetectable in rodents and humans, and tissue sources of the enzyme are more likely to influence the local RAS (12, 40). Diabetic renal injury is generally associated with a reduction in the activity and/or expression of renal tissue ACE2, which may contribute to a deleterious imbalance in the relative expression of ANG II to Ang-(1-7) (21, 26, 39, 52, 60, 63). Genetic deletion of ACE2 or chronic blockade of the enzyme exacerbates diabetic renal injury (47, 48, 52, 60, 63). Moreover, recent studies demonstrate the therapeutic value of administration of either the soluble form of ACE2 or overexpression of the enzyme to attenuate renal and pulmonary injury, as well as ANG II-dependent hypertension (33, 46, 65, 66).

As premenopausal women are apparently protected from cardiovascular disease and renal injury, studies in our laboratory over the last several years have focused on the underlying mechanisms for sex differences on the extent of hypertension and end-organ damage utilizing the mRen2.Lewis congenic rat strain. Male congenic rats exhibit higher systolic blood pressure (>50 mmHg), greater levels of oxidative stress and pronounced renal injury (proteinuria), as well as higher levels of both circulating and renal ANG II compared with females (9, 10, 16, 34). Estrogen depletion (ovariectomy) markedly exacerbates hypertension in the mRen2.Lewis females (9, 10, 11, 16). This increase in blood pressure is reversed by either exogenous estrogen, the GPR30 agonist G-1, or the AT1 receptor antagonist olmesartan, suggesting that activation of the ACE-ANG II-AT1 receptor axis is negatively influenced by the GPR30 estrogen receptor in this model (9, 24). Clinical and experimental studies suggest that females are not afforded protection from diabetic pathologies including renal injury compared with males (4, 6, 19, 29, 50). The exact mechanism for the loss of protection is not known particularly in the setting of hypertension. Therefore, the current study hypothesized that early-onset diabetes would attenuate the protective arm of the RAS [ACE2-Ang-(1-7)] while activating the ACE-ANG II axis in the female mRen2.Lewis rat. The imbalance in these two arms of the RAS may exacerbate hypertension and renal injury in diabetic mRen2.Lewis females, but not in the male congenic littermates that exhibit higher pressure and more pronounced renal injury in the absence of diabetes.

* L. M. Yamaleyeva and S. Gilliam-Davis contributed equally to this work.

Address for reprint requests and other correspondence: M. C. Chappell, Hypertension and Vascular Research Center, Wake Forest School of Medicine, Medical Center Bld., Winston-Salem, NC 27157 (e-mail: mchappel@wakehealth.edu).
MATERIALS AND METHODS

Animals. mRen2.Lewis rats were obtained from the Wake Forest University School of Medicine Hypertension Center transgenic breeding colony. Rats were housed in a temperature-controlled room (22 ± 2°C) with a 12:12-h light-dark cycle with free access to food and water in an AALAC-approved facility. At 11 wk of age, rats received either a single intraperitoneal injection of 0.1 M citrate buffer, pH 4.5 (nondiabetic; n = 9), or 65 mg/kg of streptozotocin (STZ; Sigma, St. Louis, MO) dissolved in 0.1 M citrate buffer (diabetic; n = 9) at 11 wk of age. Diabetes was verified by blood glucose levels >300 mg/dl after 24 h following the injection of STZ, and animals with 300–400 mg/dl were used in the study. Four weeks after STZ administration, rats were housed in metabolic cages (Harvard Bioscience, South Natick, MA) for 24-h urine collection and assessment of food and water intake. The rats were euthanized by decapitation without anesthesia, and trunk blood was collected into chilled Vacutainer blood collection tubes (Becton Dickinson, Sandy, UT) containing a cocktail of peptidase inhibitors for plasma, as well as a smaller serum sample collected with no inhibitors or chelators (2). All procedures were approved by the Wake Forest School of Medicine Institutional Animal Care and Use Committee.

Vascular studies. The thoracic aorta was dissected from surrounding fat, cut into 2- to 3-mm segments, and suspended from isometric force transducers (Grass Technologies, West Warwick, RI). Organ baths were filled with Krebs solution containing (in mM): 118 NaCl, 25 NaHCO3, 4.8 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, and 11 glucose (pH 7.4) and bubbled continuously with 95% O2-5% CO2 at 37°C. An optimal passive tension of 2 g was applied (24, 25). The responses to 1 μM phenylephrine, and 1 μM acetylcholine (Sigma-Aldrich) were assessed, and then rings were washed and precontracted with 1 μM phenylephrine before completion of a dose-response curve to estradiol (E2; 10−9 to 10−4 M).

Biochemical assays. Plasma and cortical tissue levels of ANG II and Ang-(1-7) were measured by RIA following solid-phase extraction and expressed as femtomoles per milliliter (picomolar; pM) or per milligram protein (34). Serum glucose was measured using the Freestyle blood glucose monitoring system (Abbott, Alameda, CA) and serum insulin by an RIA kit (Millipore, Billerica MA). Creatinine was determined by a QuantiChrom Creatinine Assay Kit (Bioassay Systems, Hayward, CA). Plasma complement-reactive protein (CRP) was determined by an ELISA kit from Alpco Diagnostics (Salem, NH). ACE, ACE2, and neprilysin activities in serum and purified cortical membranes were performed using an HPLC-determined by an ELISA kit from Alpco Diagnostics (Salem, NH). Plasma glucose was measured using the FreeStyle blood glucose monitoring system (Abbott, Alameda, CA) and insulin tended to decline following STZ treatment (Table 1).

ACE2 AND ACE IN THE DIABETIC mRen2.LEWIS RAT

Immunohistochemistry. Kidney sections (5 μm) were stained using the Avidin Biotin Complex (ABC) method as previously described (25). The staining required that antigen retrieval treatment sections be heated in sodium citrate buffer at pH 6.0 for 50 min. Monoclonal CD68—(dilution 1:100; Chemicon, Temecula, CA), VEGF (dilution 1:100, sc-152; Santa Cruz Biotechnology, Santa Cruz, CA) and secondary biotinylated anti-mouse or anti-rabbit antibodies (dilution 1:500; Vector Laboratories, Burlingame, CA) were used for the staining. Endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in 10% methanol for 30 min. Nonspecific binding was blocked in a buffer containing 5% normal goat serum, 1% bovine serum albumin, and 0.1% Triton X-100 in PBS for 30 min. Antibody binding was detected using a Vectastain Elite avidin-biotin complex kit and 0.1% diaminobenzidine (Thermo Scientific). The CD68—cells were counted using automated counting software (Image Pro 6.3; MadiusCybernetics, Bethesda, MD) in seven sections from each kidney (n = 4/group). The data are reported as the number of positive cells per ×200 field. The areas of VEGF staining in the glomerulus and tubular elements were identified in each section by a digitized image from five sections of each kidney (n = 5/group). The data are reported as the percentage of pixels stained per ×400 magnification using Image J software.

Masson’s trichrome staining. Kidney sections (5 μm) were stained with Masson’s trichrome to identify collagen fibers as described (61). The total area of collagen deposition was identified in four sections of the digitized images from each kidney and measured as the collagen-to-field ratio using Adobe Photoshop 7.0.

Statistics. All measurements are expressed as means ± SE. The effect of sex and STZ treatment were analyzed by two-way ANOVA with Bonferroni’s post hoc test (GraphPad Prism version 5.01, GraphPad Software, San Diego, CA). For angiotensinogen excretion, we performed a log transformation of the data before ANOVA. An unpaired Student’s t-test compared neprilysin, renin, and angiotensinogen protein expression between the control and diabetic groups.

RESULTS

Diabetes was induced in adult female and male hemizygous mRen2.Lewis rats at 11 wk of age with a single dose of STZ (65 mg/kg ip), and the rats were followed for 4 wk without insulin replacement. Induction of diabetes did not alter systolic blood pressure in either female or male mRen2.Lewis rats, although males exhibited higher pressure than females (Table 1). Body weight declined after 4 wk of diabetes in both female and male mRen2.Lewis rats by ∼10%. Blood glucose was markedly elevated to >300 mg/dl for both groups while circulating insulin tended to decline following STZ treatment (Table 1). Although ACE2 activity was barely detectable in the serum of control female mRen2.Lewis rats, the chromatograph reveals a markedly higher activity [peak of Ang-(1-7)] in serum of the diabetic females (Fig. 1A and B, respectively). Addition of the ACE2 inhibitor abolished the generation of Ang-(1-7) from ANG II in the diabetic serum (Fig. 1C). Kinetic studies on pooled serum from the females revealed a sevenfold increase in the apparent Vmax of ACE2 in the diabetic compared with control animals (650 vs. 96 nmol-min−1·ml serum−1), but comparable KM values (7 and 5 μM, respectively) (Fig. 1D). As shown in Fig. 2A, comparison of all groups revealed that serum ACE2 activity increased ninefold in the diabetic females and threefold in the diabetic males. Serum ACE activity also increased in both diabetic groups, and further analysis revealed a high correlation (r = 0.809, P < 0.0001) between circulating
ACE2 and ACE activities among all four groups (Fig. 2, C and D, respectively). Circulating neprilysin activity was not detectable using the HPLC-based assay (data not shown). Plasma angiotensinogen as determined by a specific rat ELISA was significantly lower in the male diabetic group compared with controls and tended to decline in the diabetic females (Table 1). Plasma levels of ANG II were higher in both female and male diabetic groups; however, the plasma content of Ang-(1-7) was significantly lower in the male diabetic group compared with controls and tended to decline in the diabetic females (Table 1). As shown in Fig. 3, aortic relaxation to 1 μM acetylcholine and 1 μM E_2 were significantly attenuated in the female diabetic rats (Fig. 3, A and B, respectively), but responses to both agents were similar in the male control and diabetic mRen2.Lewis rats. The constrictor response to phenylephrine was similar for control and diabetic groups; however, the males exhibited a greater degree of collagen deposition than females (Fig. 6).

Proteinuria and albuminuria were significantly higher in both female and male diabetic rats (Table 1). Excretion of angiotensinogen, an early marker of renal injury, also increased in both diabetic groups, although the magnitude of the change in protein was markedly higher in females (Table 1). As previously reported, basal levels of urinary angiotensinogen were significantly higher in the male mRen2.Lewis rat (10). Creatinine clearance, a measure of glomerular filtration rate (GFR), was similar between control and diabetic mRen2.Lewis rats of both sexes (Table 1). As shown in Fig. 4, there was significant correlation between the excretion of angiotensinogen and proteinuria in the four congenic groups \( (r = 0.881; P < 0.0001) \). The abundance of inflammatory cells (CD68+ macrophages and monocytes) in the tubulointerstitial area of the renal cortex was significantly higher in the female diabetic group but was not different among the diabetic and control males; males of either group exhibited a greater extent of inflammation than females (Fig. 5). Collagen staining (Masson’s trichrome) in the renal cortex was not different between the control and diabetic groups; however, the males exhibited a greater degree of collagen deposition than females (Fig. 6).

The staining for VEGF was significantly higher in the glomerulus of diabetic female and male mRen2.Lewis rats (Fig. 7).

Table 1. Physiological indices of diabetic female and male mRen2.Lewis rats

<table>
<thead>
<tr>
<th>Indices</th>
<th>Female Control</th>
<th>Female Diabetic</th>
<th>Male Control</th>
<th>Male Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>233 ± 6</td>
<td>203 ± 5*</td>
<td>350 ± 11</td>
<td>292 ± 4†</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>146 ± 8</td>
<td>164 ± 10</td>
<td>199 ± 14*</td>
<td>224 ± 10‡</td>
</tr>
<tr>
<td>Serum glucose, mg/dl</td>
<td>157 ± 7</td>
<td>373 ± 33*</td>
<td>142 ± 17</td>
<td>334 ± 16†</td>
</tr>
<tr>
<td>Plasma insulin, pg/ml</td>
<td>1.0 ± 0.3</td>
<td>0.2 ± 0.1</td>
<td>1.5 ± 0.5</td>
<td>1.5 ± 0.1‡</td>
</tr>
<tr>
<td>Serum CRP, ng/ml</td>
<td>270 ± 10</td>
<td>420 ± 17*</td>
<td>243 ± 25</td>
<td>268 ± 14‡</td>
</tr>
<tr>
<td>Plasma Aogen, μg/ml</td>
<td>2.1 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>2.5 ± 0.4</td>
<td>1.7 ± 0.1†</td>
</tr>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>0.85 ± 0.16</td>
<td>1.23 ± 0.37</td>
<td>0.75 ± 0.05</td>
<td>0.98 ± 0.19</td>
</tr>
<tr>
<td>Proteinuria, mg/mg Cr</td>
<td>0.23 ± 0.03</td>
<td>9.62 ± 1.83*</td>
<td>0.89 ± 0.18</td>
<td>7.60 ± 1.29†</td>
</tr>
<tr>
<td>Albuminuria, mg/mg Cr</td>
<td>0.09 ± 0.02</td>
<td>3.66 ± 0.69*</td>
<td>0.34 ± 0.08</td>
<td>3.81 ± 0.47†</td>
</tr>
<tr>
<td>Aogen excretion, ng/mg Cr</td>
<td>0.31 ± 0.06</td>
<td>343 ± 67*</td>
<td>81 ± 13§</td>
<td>293 ± 45†</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( (n = 5–7/group) \). SBP, systolic blood pressure; CRP, C-reactive protein; Aogen, angiotensinogen; Cr, creatinine. *\( P < 0.05 \) compared with female control. †\( P < 0.05 \) compared to male control. ‡\( P < 0.05 \) compared with female diabetic. §\( P < 0.05 \) compared with female control.

Fig. 1. Angiotensin-converting enzyme 2 (ACE2) activity is higher in the serum of diabetic female mRen2.Lewis rats. The HPLC chromatograph reveals a small peak of Ang-(1-7), i.e., Ang 7, from ANG II in control serum (A), but a larger Ang-(1-7) peak in diabetic serum (B). Addition of an ACE2 inhibitor abolishes formation of Ang-(1-7) from ANG II in diabetic serum (C). Kinetic analysis (D) of serum ACE2 activity revealed higher \( V_{\text{max}} \) but similar \( K_m \) values in diabetic vs. control female rats (pooled serum; \( n = 5 \) and \( n = 6 \), respectively).
VEGF expression was also evident in the tubular elements of the renal cortex; however, the extent of staining was not different between the control and diabetic groups of either sex (Fig. 7). In regard to the renal RAS, cortical ACE2 activity was not different between female control and diabetic groups, while the male diabetics exhibited lower ACE2 activity (Fig. 8A). In contrast to circulating ACE, cortical ACE activity was significantly lower in both female (75%) and male (50%) diabetic groups compared with the controls (Fig. 8B). Other ANG II-forming activities from Ang I such as chymase were not detected in cortical homogenates of diabetic females or males (data not shown). Neprilysin activity was also significantly reduced in the female diabetic kidneys but unchanged in those of males (Fig. 8C). Immunoblotting revealed a marked reduction in neprilysin protein expression in the diabetic females consistent with reduced activity (see Fig. 8F). Cortical ANG II content tended to increase in both female and male diabetic groups, but this did not achieve statistical significance (Fig. 8D), while cortical Ang-(1-7) was similar between control and diabetic groups (Fig. 8E). Immunoblots of cortical tissue revealed higher angiotensinogen and renin protein expression in diabetic females but not the males while angiotensinogen excretion increased to a similar extent in both diabetic groups (Fig. 9, A–F).

**DISCUSSION**

The present studies compared the responses to early diabetes regarding changes in the circulating and renal RAS, as well as assessed the extent of renal injury in female and male hypertensive mRen2.Lewis rats. To our knowledge, the present study is the first to directly compare the circulating and renal RAS to induced diabetes in male and female hypertensive rats. Overall, the current results reveal that the adult females were not protected in regard to vascular dysfunction and certain indices of renal damage from early STZ-induced diabetes despite significant differences in systolic blood pressure. Indeed, the female mRen2.Lewis rats exhibited a marked increase in the inflammatory marker CRP that was not evident in the male diabetic animals, as well as a pronounced increase in proteinuria, albuminuria, and angiotensinogen excretion. Diabetic females also exhibited greater glomerular VEGF staining and higher levels of tubulointerstitial CD68⁺ cells, although the extent of inflammation was markedly less than in either the control or diabetic males. With respect to the circulating RAS, female diabetic mRen2.Lewis rats exhibited a similar response to males with significant increases in plasma levels of ANG II and no corresponding change in Ang-(1-7). Moreover, both diabetic males and females exhibited a similar increase in urinary excretion of angiotensinogen but a marked decline in ACE activity with no evidence of a non-ACE-dependent pathway within the kidney cortex. However, tissue levels of ANG II and Ang-(1-7) were similar between the control and diabetic groups for both females and males. These data suggest that early diabetes in the hypertensive females promotes an increase in the expression of renin and angiotensinogen in the kidney cortex.

![Fig. 2. Influence of diabetes on components of the circulating renin-angiotensin system in female and male mRen2.Lewis rats.](https://example.com/fig2.png)

![Fig. 3. Influence of diabetes on the vascular response to acetylcholine (Ach) and estradiol (E2) in the isolated aorta of female and male mRen2.Lewis rats.](https://example.com/fig3.png)
in circulating ANG II and CRP, as well as maintained expression of renal ANG II, which may contribute to the lack of vascular and renal protection in diabetic females.

We employed a sensitive HPLC-based method to measure the conversion of the endogenous substrates Ang I and ANG II to the enzymatic products ANG II and Ang-(1-7) for the determination of ACE and ACE2 activities (34, 35, 44, 45). Early-onset diabetes increased ACE2 activity ninefold in female and threefold in male mRen2.Lewis rats, the greater magnitude of change in ACE2 likely due to the lower circulating activity in control females. Although one would expect higher ACE2 activity in female mRen2.Lewis rats, the greater activity in the control males may reflect a compensatory increase in response to the higher pressure and likely greater vascular damage. Tikellis and colleagues (52) reported a two-fold increase in circulating ACE2 in STZ-induced diabetes of normotensive male mice using a synthetic substrate for ACE2. Utilizing the same synthetic assay, Soro-Paavonen et al. (49) reported a significant increase in serum ACE2 activity (~20%) in male type 1 diabetic patients with micro- or macroalbuminuria compared with controls or a diabetic cohort without albuminuria (49). These investigators also found higher serum ACE2 activity (~30%) in males than females (49). Our kinetic studies on pooled serum revealed a sevenfold higher \( V_{\text{max}} \) value in the diabetic group with similar \( K_m \) constants, implying that an increase in circulating ACE2 protein accounts for the higher activity. Although the source for circulating ACE2 is not currently known, ACE2 may be actively shed from the
vascular surface through metallosecretases such as ADAM 10 and ADAM 17, previously shown to release the enzyme from renal and pulmonary membranes (1, 18). Despite the increased ACE2 activity and reduced levels of angiotensinogen in males, circulating ANG II was significantly higher in both diabetic groups. The Tikellis study (52) did not assess circulating ANG II or Ang-(1-7), but Zimpelmann and colleagues (67) reported an increase in plasma ANG II levels in normotensive male rats following 2 wk of diabetes. Although we did not measure plasma renin, the higher levels of ANG II may reflect a coordinate increase in circulating ACE activity in early diabetes. The magnitude of change in ACE was less than that of ACE2 in diabetic rats; however, the overall activity level for ACE was markedly higher. Nevertheless, the increase in circulating ACE2 may attenuate a greater increase in ANG II in diabetic hypertension. Indeed, the exacerbation of diabetic injury following treatment with an ACE2 inhibitor or in ACE2 knockout mice may reflect, in part, the loss of the circulating enzyme and the ability to effectively buffer circulating ANG II (47, 48, 60).

In contrast to the marked increase in circulating ACE2, cortical ACE2 activity slightly increased in females, but was significantly reduced in the male diabetic kidneys. These data clearly reveal a marked difference in the regulation of circulating and renal ACE2 in the diabetic rat. Moreover, our preliminary study reveals lower ACE2 activity in the diabetic heart of the female mRen2.Lewis rat, further indicative of tissue-selective regulation of ACE2 (43). Tikellis et al. (52, 53) reported lower ACE2 expression in both STZ-induced diabetic rats and mice; the latter study in mice was associated with a marked reduction in the renal content of Ang-(1-7). Lo et al. (26) also reported lower cortical ACE2 protein in the male Akita type 1 diabetic mouse, and the enzyme was primarily expressed in the apical region of the proximal tubules with little or no expression in the glomerulus. The Akita mice also exhibited higher ANG II excretion but reduced Ang-(1-7) in the urine compared with the wild-type controls, consistent with lower tubular ACE2 (26). However, higher ACE2 expression and activity were evident in STZ-treated female mice, as well as in the female db/db mouse, a model of type II diabetes (59, 63, 64). Renal ACE activity was markedly reduced in both female and male diabetic mRen2.Lewis rats, consistent with previous studies in STZ-treated mice and rats (52, 53, 63, 64). Moreover, the Tikellis and Zimplemann studies (53, 67) found no significant change in renal ANG II, although similar to current results, ANG II content tended to increase. It is quite...
possible that lack of change in renal or cortical ANG II may reflect alterations in the peptide and its processing enzymes in different renal compartments (5). Ye et al. (63) reported reduced ACE2 expression in the glomerulus of the \textit{db/db} mouse kidney but enhanced immunostaining for ACE. These investigators found the opposite pattern of staining in the proximal tubules (63). Leehey et al. (21) also found reduced ACE2 activity in isolated glomeruli from STZ diabetic male rats, associated with higher ANG II content, but ACE activity was not changed. We did not detect other ANG II-forming activities in the diabetic kidney that would potentially compensate for the marked reduction in ACE activity, and this may portend an additional mechanism for ANG II expression in the kidney distinct from intrarenal synthesis. Our preliminary binding data suggest that the AT\textsubscript{1} receptor is not downregulated in the diabetic female kidney, and we speculate that the sustained levels of cortical ANG II may also reflect uptake and intracellular sequestration of the circulating peptide (3, 57). Indeed, we show that chronic administration of exogenous ANG II restores the renal levels of the peptide in tissue ACE knockout mice (27, 28). Moreover, Li and colleagues (22, 23) find an AT\textsubscript{1} receptor-dependent mechanism for internalization of ANG II in proximal tubule cells and the intact kidney. Lower ACE2 activity may also contribute to intrarenal ANG II in the male diabetic kidney; reduced nephrilysin activity would attenuate metabolism of ANG II in the proximal tubules of the female diabetics and lead to higher tissue levels.

Renin and angiotensinogen protein expression were higher in the female but not the male diabetic rats while angiotensinogen excretion increased to a similar level in both diabetic groups. Basal levels of urinary angiotensinogen were markedly lower in the female mRen2.Lewis rats than the males before the induction of diabetes. We reported similar findings in a previous study on salt-dependent changes in blood pressure and renal injury in the mRen2.Lewis rat (10). Renal angiotensinogen expression is stimulated by testosterone, which may contribute to sex differences in hypertension and renal damage (13, 62). The higher basal levels of urinary angiotensinogen in

---

**Fig. 7. VEGF immunostaining in the renal cortex of control and diabetic mRen2.Lewis rats. Top:** representative sections from female control (A), female diabetic (B), male control (C), and male diabetic (D) rats. Colorimetric images (5 sections/kidney) were analyzed by Image J software, and data are expressed as percentage of pixels stained. Values are means ± SE. Magnification ×400. *P < 0.05 vs. control (n = 5/group).**
the male mRen2.Lewis rat may also reflect increased filtration of circulating angiotensinogen due to greater glomerular damage in the male congenics (10, 15). Uptake of filtered angiotensinogen by the proximal tubules may also be reduced in the males as a result of more pronounced tubular damage and a reduced capacity of transporters such as megalin and cubulin to internalize angiotensinogen, as well as other proteins (25, 37, 55). Utilizing the male (mRen2)27.SD transgenic rats, the founder strain for the mRen2.Lewis rat, Habibi and colleagues (17) find evidence for both glomerular and tubular damage in the hypertensive strain. In fact, we show a high correlation between urinary excretion of angiotensinogen and proteinuria among the control and diabetic mRen2.Lewis rats. Similarly, a high correlation between urinary levels of angiotensinogen and proteinuria was observed in the mRen2.Lewis rats maintained on normal- and high-salt diets (10). Although urinary angiotensinogen markedly increased in both high salt female and male mRen2.Lewis rats, we found no coordinate increase in cortical protein expression, mRNA content, or release of angiotensinogen from cortical slices that would contribute to higher tubular fluid levels of angiotensinogen (10). Moreover, the molecular forms of circulating angiotensinogen (55 and 60 kDa) were similar to that in the urine as opposed to only the 60-kDa isoform in cortical tissue of the congenic kidney (10). Angiotensinogen protein increased in the cortex of the diabetic female congenics; however, it would appear unlikely that a twofold higher expression of cortical angiotensinogen would completely account for the marked increase in the urinary levels of the precursor in the diabetic females. Nonetheless, the high levels of angiotensinogen, regardless of their origin from the circulation and/or the renal tubules, may contribute to an activated ANG II-AT1 receptor axis within the nephron compartment of the diabetic kidney.

The extent of albuminuria, proteinuria, and angiotensinogen excretion was similar between the female and male diabetic mRen2.Lewis rats despite the significant difference in systolic blood pressure. Kelly et al. (20) previously utilized STZ-treated female (mRen2)27.SD transgenic rats as a model of renal injury and diabetic nephropathy following 12 wk of diabetes. Similar to the present results, renal renin was elevated after 4 wk and continued to increase by 12 wk in the diabetic (mRen2)27.SD rats; however, renal ANG II content was not determined (20). In contrast to the Kelly study, Hartner et al. (16) concluded that renal injury in the diabetic (mRen2)27.SD strain reflected the underlying hypertension (and activated RAS) rather than aggravated diabetic damage. The latter study

Fig. 8. Influence of diabetes on enzyme and peptide components of the renin-angiotensinogen system in the renal cortex of female and male mRen2.Lewis rats. Enzyme activities are represented as fmol-min⁻¹·mg protein⁻¹ and peptide content as fmol/mg protein. inset: immunoblot of cortical neprilysin (NEP) protein expression (90 kDa) normalized to actin in female control and diabetic rats. Values are means ± SE; n = 5–8/group. *P < 0.05 vs. respective controls. †P < 0.05 vs. female controls. ‡P < 0.05 vs. female diabetic rats.

Fig. 9. Influence of diabetes on renin and angiotensinogen expression in the renal cortex of female and male mRen2.Lewis rats. Immunoblots of renin (55 kDa) and angiotensinogen (Aogen; 60 kDa) protein expression normalized to actin from control and diabetic female and male rats are shown. Aogen excretion is expressed as ng/mg creatinine. Values are means ± SE; n = 5–6/group. *P < 0.05 vs. controls.
revealed no differences in renal injury between the control and STZ-treated transgenics (16). It should be noted that the Kelly study (20) used female transgenics made diabetic at 6 wk of age while male transgenic rats were utilized by Hartner (16) (diabetes induced at 11 wk). Clearly, age and sex differences may well contribute to the conflicting results following STZ-induced diabetes in the (mRen2)27.SD strain. Comparison of the female and male diabetic mRen2.Lewis rats revealed essentially a similar phenotype with no vascular and variable renal protection in the female group. Circulating CRP was significantly higher in the female diabetic rats but did not change in the male diabetic group. Although serum CRP is considered a marker of inflammation, CRP may directly contribute to vascular dysfunction through reduced nitric oxide or increased oxidative stress (17, 38, 44). Vascular relaxation to acetylcholine and estrogen were significantly reduced in the diabetic mRen2.Lewis females, which would support a role for CRP; however, the mechanism for the attenuated relaxation response awaits further study. Renal inflammation (presence of CD68+ cells) increased in the female diabetic group and did not change in the males, although the control males exhibited a greater degree of inflammation than females. In this regard, the Harter study (16) also reported that the extent of inflammation was similar in control and diabetic male (mRen2)27.SD rats. In contrast, glomerular VEGF immunostaining increased to a similar extent in both female and male diabetic animals. The increase in glomerular VEGF may promote glomerular permeability, which would contribute to the increased proteinuria and angiotensinogen excretion in the female and male diabetic animals (36, 42). The extent of collagen staining was higher in the males, but was not different between the control and diabetic groups of either sex. Finally, the neprilysin response to early diabetes was strikingly different between the female and males mRen2.Lewis rats. As reported previously, the female congeneric exhibits significantly higher neprilysin activity and expression than the males (35). Early diabetes markedly reduced neprilysin activity in females compared with that in males while neprilysin was unchanged in control and diabetic males. Whether this reduction in renal neprilysin contributes to diabetic injury or is a protective mechanism in the female congenics is not known. Neprilysin has multiple substrates, including the natriuretic peptides, and inhibitors against the enzyme were considered as potential antihypertensive agents at one time; however, their effectiveness may be limited since neprilysin is the primary endopeptidase in the kidney that converts ANG I to Ang-(1-7), as well as metabolizes ANG II [to Ang-(1-4)] and endothelin (8, 40). To our knowledge, experimental studies with neprilysin inhibitors have only been assessed in males, including a study in STZ-treated spontaneously hypertensive rats, which showed cardiac hypertrophy despite a smaller increase in ANG II as well as reduced levels of Ang-(1-7). The present results in the diabetic hypertensive animals reveal that higher circulating ACE2 was not associated with lower plasma levels of ANG II. The presence of high circulating and vascular ACE activity may mitigate against increases in ACE2 in our model since higher ACE activity would still generate ANG II and degrade Ang-(1-7) (8). Moreover, the altered ACE2/ACE response coupled with an increase in CRP may contribute to vascular damage in the diabetic females. Provided the administration of soluble ACE2 achieves comparable or higher levels of ACE, this approach would support the rationale for ACE2 therapy in both female and male diabetic hypertensive subjects. Interestingly, the marked decline in ACE activity in the diabetic kidney may attenuate an increase in ANG II as well as reduced levels of Ang-(1-7). Thus the therapeutic increase in ACE2 in the setting of reduced ACE such as the diabetic kidney may achieve a more optimal ratio of Ang-(1-7) to ANG II in this tissue to reduce renal damage.

ACKNOWLEDGMENTS

Portions of this work were presented at the 2011 Experimental Biology and 2011 Council for High Blood Pressure meetings.

GRANTS

This work was supported in part by National Heart, Lung, and Blood Institute Grants HL56973, HL51952, HL112237, and HL103592 and an American Heart Association Fellowship.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


