Temporal-spatial expression of ANG-(1-7) and angiotensin-converting enzyme 2 in the kidney of normal and hypertensive pregnant rats

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Joey J. Neves, L. A. A. Granger, J. P. Alexander, B. T. Merrill, Chappell MC, Ferrario CM, Davis WP, Brosnihan KB. Temporal-spatial expression of ANG-(1-7) and angiotensin-converting enzyme 2 in the kidney of normal and hypertensive pregnant rats. Am J Physiol Regul Integr Comp Physiol 293: R169–R177, 2007. First published April 11, 2007; doi:10.1152/ajpregu.00387.2006.—We recently demonstrated that renin-angiotensin system (RAS) overactivity during late gestation in rats is associated with increased kidney and urine levels of ANG-(1-7) and enhanced kidney immunostaining of ANG-(1-7) and angiotensin-converting enzyme 2 (ACE2). To understand the temporal-spatial changes in normal and hypertensive pregnancies, the renal distribution of ANG-(1-7) and ACE2 in association with kidney angiotensin peptides and ACE2 activity was examined in virgin, normal pregnant (NP; gestational days 5, 15, and 19) and reduced uterine perfusion pressure (RUPP at day 19) pregnant Sprague-Dawley rats. ANG-(1-7) and ACE2 immunocytochemical staining increased 1.8- and 1.9-fold and 1.7- and 1.8-fold, respectively, at days 15 and 19 of NP, compared with virgin rats. ANG-(1-7) and ANG II concentrations were increased in the kidney at 19 days of gestation. ACE2 activity measured using a fluorescent substrate was increased 1.9- and 1.9-fold in the cortex and 1.9- and 1.8-fold in the medulla at days 15 and 19 of NP. In the RUPP animals, ANG-(1-7) immunostaining and concentration were significantly decreased compared with 19-day NP rats. ACE2 activity was unchanged in the cortex and medulla of RUPP rats. In conclusion, during NP, the concurrent changes of ACE2 and ANG-(1-7) suggest that ACE2 plays an important role in regulating the renal levels of ANG-(1-7) at mid to late gestation. However, the decrease in renal ANG-(1-7) content in the absence of a concomitant decrease in ACE2 implicates the participation of other ANG-(1-7) forming or degrading enzymes during hypertensive pregnancy.

renin-angiotensin system; pregnancy; reduced uterine perfusion pressure

PREGNANCY IS CHARACTERIZED by multiple changes within different components of the renin-angiotensin system (RAS). The physiological consequences of an altered RAS during pregnancy are unknown, and even less understood is how alterations in this system may contribute to hypertensive disorders during pregnancy. Previously, we demonstrated augmented renal concentration and urinary excretion of ANG-(1-7) during late (19 day) pregnancy in Sprague-Dawley rats (25). Renal immunocytochemical (ICC) distribution of both ANG-(1-7) and its associated enzyme angiotensin-converting enzyme 2 (ACE2), a carboxypeptidase that exhibits high catalytic efficiency to generate ANG-(1-7) from ANG II (8, 31, 33) showed a higher intensity of staining in the pregnant animals compared with virgin counterparts (5).

Human studies supported the potential importance of this heptapeptide in pregnancy, in that urinary ANG-(1-7) was found to increase throughout pregnancy (32). The initial increase in urinary ANG-(1-7) was found at 12 wk of gestation, but a tendency to increase was observed as early as 6 wk of gestation (32), suggesting that the early adaptive changes of the renal RAS system may contribute to the changes in fluid and electrolyte balance throughout the time course of pregnancy. Furthermore, in normal 3rd trimester pregnant human subjects, plasma ANG-(1-7) levels increased but were decreased in preeclamptic subjects (22), suggesting that a reduction in this peptide may be an important contributor to preeclampsia, a disease leading to fetal and maternal mortality in 3–5% of pregnancies. These studies suggest that temporal changes of the system over the time course of pregnancy may be disrupted in preeclampsia. Currently, it is not known if changes in ANG-(1-7) and ACE2 are functionally linked during pregnancy and preeclampsia.

Therefore, the goal of the present study is to determine whether ANG-(1-7) and ACE2 are colocalized (spatial) and exhibit parallel changes (temporal) during normal and hypertensive pregnancies. Thus, the temporal spatial change in the immunocytochemical distribution of ANG-(1-7) and ACE2 in association with angiotensin peptide levels and ACE2 activity was assessed in the kidney of Sprague-Dawley rats during pregnancy (5, 15, and 19 days). In addition, we also sought to characterize and determine whether there was a reduction in the expression of ANG-(1-7) and ACE2 in a model of preeclampsia, the reduced uterine perfused pressure (RUPP) pregnant animals. We hypothesize that renal ANG-(1-7) is increased throughout the time course of normal pregnancy, and this increase is mediated by an upregulation of ACE2. During hypertensive pregnancy, we hypothesize that the concentration of ANG-(1-7) and ACE2 activity is downregulated.

METHODS

Animals. Timed pregnant and virgin female Sprague-Dawley rats between 12 and 14 wk of age were obtained from Harlan Laboratories (Indianapolis, IN) and housed individually under a 12:12-h light-dark cycle in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Day 0 of pregnancy was

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designated as the day when sperm were found in the vaginal smear. All protocols were approved by the Animal Care and Use Committee of Wake Forest University School of Medicine and are in compliance with National Institutes of Health guidelines.

Surgical procedures. Five groups of animals were used for analysis in this study: 1) virgin at diestrus, as determined by vaginal smear; 2) 5-day pregnant; 3) 15-day pregnant; 4) 19-day normal pregnant; and 5) 19-day RUPP. Conscious animals were killed by decapitation. Five-day pregnant animals were briefly anesthetized (less than 5 min) with isoflurane and were injected with 0.2 ml of 0.5% Evans blue dye (Sigma, St. Louis, MO) by heart puncture to confirm pregnancy by visualization of implantation sites after death. The animals quickly recovered from anesthesia and 15 min later were killed. Brief anesthesia did not change plasma levels of ANG II (33.5 ± 3.2 vs. 30.1 ± 3.0 pmol/l, P > 0.05 n = 16/group) and ANG-(1-7) (57.1 ± 7.0 vs. 68.6 ± 3.2 pmol/l, P > 0.05, n = 23/group) in unanesthetized virgin vs. 5-day pregnant rats, respectively. This unchanged level of plasma angiotensin peptides between virgin and 5-day pregnant rats indicates that the increase in tissue ANG-(1-7) at day 5 compared with virgin (Fig. 4) was likely attributable to pregnancy and not due to the short-term anesthetic effect. Animals assigned to the 19-day normal pregnant and RUPP groups were anesthetized with isoflurane and were either sham-operated or prepared for reduced uterine perfusion pressure surgery on the 14th day of pregnancy, as previously described (1, 14). Briefly, a silver clip (0.203 mm) was placed around the descending aorta just above the bifurcation, and two silver clips (0.221 mm) were placed around branches of both the right and left ovarian arteries. Metabolic studies, consisting of 24-h urine collection and enzymatic measurements.

Regulatory, Comparative and Integrative Physiology fetuses were excluded from the study.

on the 18th day of pregnancy for RUPP and sham animals. On day 19 of pregnancy, blood pressure was recorded (Biopac Systems, Goleta, CA) in conscious animals. A subgroup of 19-day unoperated pregnant animals was included to evaluate the effects of the surgery and anesthesia on components of the RAS. Comparison of the data from sham-operated and unoperated 5-day pregnant rats showed no differences in regard to kidney angiotensin peptides, plasma renin concentration, ACE2 activity, urine volume, or body weight (Supplemental data for this article is available online at the American Journal of Physiology—Regulatory, Comparative and Integrative Physiology website). Because of this finding, the data from the unoperated and sham-operated 19th day groups were combined. RUPP rats in which the clipping procedure resulted in maternal death or total reabsorption of the fetuses were excluded from the study.

To confirm that virgin animals were in diestrus and to observe hormonal changes throughout pregnancy, trunk blood was collected in tubes containing no inhibitors for measurement of serum 17β-estradiol by radioimmunoassay (Polymedco, Cortlandt Manor, NY). The kidneys were rapidly removed and fixed in 4% paraformaldehyde for 24 h for immunocytochemistry and histological examination by hematoxylin and eosin staining or quick frozen on dry ice for peptide and enzymatic measurements.

Immunochemistry. Kidney tissue was embedded in paraffin and 5-μm sections were obtained. Serial sections were obtained for cellular colocalization studies of ANG-(1-7) and ACE2. Immunocytochemical analysis was completed as described previously (5), using the avidin–biotin method. Kidneys from animals from different groups were processed in parallel. The primary antibodies used were an affinity-purified rabbit polyclonal antibody to ANG-(1-7) and a rabbit polyclonal antibody to ACE2 produced by our laboratory at dilutions of 1:25 and 1:150, respectively, in 1% BSA. The specificity of the ANG-(1-7) antibody for ICC was described previously (20) (4), but briefly, it does not cross react with either ANG I or ANG II. The sequence of the ACE2 immunogenic peptide was unique to ACE2 but not ACE or the ACE2 homolog collectrin. Sections of kidney from normal and hypertensive rats were stained with hematoxylin and eosin and examined by a board-certified veterinary pathologist (W. P. Davis, University of New Hampshire) to determine whether RUPP animals exhibit glomerular endotheliosis, a renal characteristic of preeclampsia.

Quantification of immunocytochemical staining. The ANG-(1-7) and ACE2 staining was assessed as previously described by Lehr et al. (21). Each kidney was photographed using constant settings for gain, filters, light intensity, diaphragm, and condenser aperture, as well as at a constant exposure of 105 ms and 108 ms for ANG-(1-7) and ACE2, respectively. All photographs were taken using a Zeiss microscope equipped with an AxioCam digital camera that transmits image data to the AxioVision software and were taken on the same day to ensure that the gain was constant (5). Using anatomical landmarks, we drew a grid composed of four regions on each kidney to ensure that photographs were taken from similar areas. Regions of the grid encompassed left, right, and two center inner cortex/outter medulla areas of the kidney. The immunostaining within the kidney tubules was determined by averaging the intensity from the left and right grid and one of the center grid regions (300–400 pixels) of the kidney of each animal. An average background level was measured based on the intensity within three glomeruli (300–400 pixels). Quantification completed on pictures taken at ×100 contained parts of 2–4 tubules or 1 glomerulus. The corrected tubule intensity was calculated as the difference between intensity of staining in the tubules vs. the glomeruli (background).

Table 1. Basal characterization of virgin, normal pregnant (5, 15, and 19 day), and RUPP SD rats

<table>
<thead>
<tr>
<th></th>
<th>Virgin</th>
<th>5-Day Pregnant</th>
<th>15-Day Pregnant</th>
<th>19-Day Pregnant</th>
<th>19-Day RUPP</th>
</tr>
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<tbody>
<tr>
<td>Blood pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
<td>106±3</td>
<td>120±3</td>
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<tr>
<td>Urine volume, ml</td>
<td>13.4±1</td>
<td>21.2±2.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.4±2.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28.4±1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.4±3.3&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Urinary creatinine, μmol/24 h</td>
<td>97.5±6.7</td>
<td>119.8±10.6</td>
<td>106.9±6.2</td>
<td>159.3±8.4&lt;sup&gt;g,d&lt;/sup&gt;</td>
<td>98.3±12.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Protein-creatinine ratio</td>
<td>0.99±0.04</td>
<td>0.54±0.12</td>
<td>0.77±0.17</td>
<td>0.37±0.05</td>
<td>0.75±0.15</td>
</tr>
<tr>
<td>Plasma 17β-estradiol, pmol/l</td>
<td>37.7±7.6</td>
<td>18.6±0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>122.0±10.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>320.8±2.8&lt;sup&gt;e&lt;/sup&gt;</td>
<td>105.0±27.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maternal body weight, g</td>
<td>257±7</td>
<td>237±5</td>
<td>286±5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>351.0±10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>270±6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fetus/implantation site number</td>
<td>15±1</td>
<td>14±1</td>
<td>11±2</td>
<td>5±1&lt;sup&gt;g&lt;/sup&gt;</td>
<td>2.0±0.15&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fetus weight, g</td>
<td>0.3±0.01</td>
<td>2.4±0.13&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.9±0.06</td>
<td>2.6±0.08&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.6±0.08&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM; n = 7/group (*P < 0.001 vs. virgin; *P < 0.01 vs. virgin; †P < 0.05 vs. virgin; ‡P < 0.01 vs. 5-day Pregnant; §P < 0.001 vs. 15-day Pregnant; ¶P < 0.001 vs. 15-day Pregnant). RUPP SD, reduced uterine perfusion pressure Sprague-Dawley rat 5. The 19-day RUPP group was only compared to the 19-day pregnant group using nonpaired r-test (*P < 0.0001 vs. 19-day Pregnant; †P < 0.01 vs. 19-day Pregnant; ¶P < 0.05 vs. 19-day Pregnant).
Angiotensin peptides. Frozen kidneys were rapidly weighed, homogenized, and extracted using Sep-Pak columns as previously described (2, 23, 30). The eluate was divided for three RIAs [ANG I, ANG II, ANG-(1-7)], and the solvent was evaporated. Recoveries of radiolabeled angiotensin added to the samples were determined during the extraction. Recovery of radiolabeled peptide averaged more than 65%, and results were corrected for recovery. ANG I was measured using a modification of commercially available Peninsula Laboratory RIA kit (Belmont, CA). ANG II was measured using the Alpco Diagnostics kit (Windham, NH). ANG-(1-7) was measured using the antibody produced by our laboratory (23, 30). The minimum detectable levels of the assays were 1.25 pg/ml for ANG I, 0.8 pg/ml for ANG II, and 2.5 pg/ml for ANG-(1-7). The intra- and interassay coefficients of variation for ANG I RIA are 18% and 22%, for ANG II are 12% and 22%, and for ANG-(1-7) are 8% and 20%, respectively.

ACE2 fluorescent enzymatic assay. Frozen kidney cortex and medulla were homogenized in reaction buffer (25 mM HEPES buffer, 125 mM NaCl, 10 μM ZnCl₂; pH 7.4) using a TissueLyser. Homogenates were placed in a centrifuge and spun at 2,500 g for 5 min. The supernatant was spun at 28,000 g for 10 min. The pellet was resuspended in reaction buffer. The resultant pellet was resuspended in reaction buffer and placed in a centrifuge and spun again at 28,000 g for 10 min. This tissue pellet was incubated with 0.5% Triton X-100 overnight on ice at 4°C. Samples were centrifuged at 28,000 g for 5 min, and the protein concentration (bicinchoninic acid (BCA) protein kit; Pierce, Rockford IL) for the Triton-solubilized supernatant was determined. The ACE2 enzymatic assay was conducted using assay buffer (0.05 M 2-Morpholinoethane-sulfonic acid, 0.3M NaCl, 10 μM ZnCl₂; pH 6.8) and the synthetic substrate (7-methoxycoumarin-4-yI)acetyl-ala-pro-lys(2,4-dinitrophenyl-OH) (AnaSpec, San Jose, CA). Twenty micrograms of protein was incubated with ACE2 assay.

Fig. 1. A: Renal distribution of ANG-(1-7) immunostaining in virgin, pregnant (5, 15, and 19 days), and reduced uterine perfusion pressure (RUPP) (19 days) Sprague-Dawley rats at ×100 magnification. Insets: note lack of glomeruli staining at each time point. No stain (control) was visible when sections were incubated with a primary antibody preabsorbed with 10 μmol/l ANG-(1-7). B: quantification of the staining revealed that ANG-(1-7) increased significantly throughout pregnancy reaching the highest level at days 15 and 19 and decreased in RUPP animals. Values are expressed as means ± SE. Differences between the means [virgin and pregnant (5, 15, 19 days)] were evaluated using a one-way ANOVA with Newman-Keuls post hoc test, whereas 19-day-pregnant and 19-day RUPP were compared using a Student’s t-test; n = 5 or 6 per group. *P < 0.001 vs. Virgin; μP < 0.05 vs. 5d-Preg; #P = 0.01 vs. 19d-Preg.
buffer containing inhibitors (10 μM lisinopril, 10 μM SCH 39 370 (Schering Plough, Kenilworth, NJ), 2 μM amastatin, 10 μM bestatin, 10 μM benzyl succinate), and 1 mM of substrate at 37°C for 1 h. Parallel samples were incubated with the above-mentioned reaction mixture in the presence of 1 mM MLN4760 (Millennium, Cambridge, MA), a specific ACE2 inhibitor to determine specific ACE2 activity. At the end of the incubation, all samples were treated with 667 μM EDTA. Fluorescence was measured using a Perkin-Elmer LS50 fluorometer (excitation 320 nm, emission 405 nm). For comparison, ACE2 activity was also analyzed using an HPLC method, which measures the conversion of 125I ANG II to 125I ANG-(1-7), as previously described (10). A highly significant correlation (r = 0.77, P < 0.01) was found between the two methods evaluating ACE2 activity in virgin (n = 4) and pregnant (n = 5) kidney cortex samples.

Statistical analysis. Comparisons between virgin and pregnant animals were analyzed by one-way ANOVA followed by Newman-Keuls or Dunnett post hoc test. Comparisons of 19-day pregnant and 19-day RUPP animals were completed by unpaired Student’s t-test. A logarithmic transformation of the angiotensin peptide data was performed before analysis to normalize the group variances. A probability of <0.05 was considered statistically significant. All values were expressed as means ± SE.

RESULTS

Basal characteristics of body weight, urine volume, urinary protein/creatinine, and serum 17-β estradiol of virgin and pregnant rats are shown in Table 1, together with the baseline characteristics of 19-day RUPP animals. Maternal body weight increased at days 15 and 19 of pregnancy and was significantly reduced in 19-day RUPP animals compared with normal 19-day pregnant animals. Urine volume increased throughout

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![Fig. 2.](image-url)
pregnancy reaching the highest level at 19 days of pregnancy and did not change in RUPP animals. Urinary creatinine was significantly increased in 19-day normal pregnant animals and significantly reduced in 19-day RUPP animals, and the protein/creatinine ratio used as an index of proteinuria was significantly increased in RUPP animals compared with normal 19-day pregnant rats. RUPP animals showed higher levels of mean blood pressure compared with normal pregnant animals. Serum 17β-estradiol was increased at days 15 and 19 of normal gestation and was significantly reduced in 19-day RUPP animals. RUPP animals had a fewer number of fetuses, and those fetuses had lower fetal weight and smaller size. On histological examination, mild glomerular lesions, including hypercellularity and variable lobulation, were observed in several of the RUPP rats.

ANG-(1-7) immunostaining was present in the proximal and distal tubules of the inner cortex and outer medulla region of the kidney in virgin, pregnant, and RUPP animals, but it was not found in the glomeruli of any of the groups (Fig. 1A). The intensity of the ANG-(1-7) staining in the tubules increased on the 15th and 19th days of pregnancy compared with the virgin and day 5 pregnant rats (Fig. 1B). ANG-(1-7) staining was significantly decreased in RUPP animals by 38% compared with the 19th day normal pregnant animals (Fig. 1B). ACE2 immunoreactivity was present in the proximal and distal tubules of the inner cortex/outer medulla of the kidney of virgin, pregnant, and RUPP animals but absent in the glomerulus (Fig. 2A). Both ANG-(1-7) and ACE2 immunostaining were present in the cytoplasm of the renal tubular cells. There was no difference in the distribution of staining between the groups. ACE2 immunoreactivity showed a similar profile to ANG-(1-7), reaching statistically increased levels at the 15th and 19th day of pregnancy. In the RUPP animals, ACE2 immunoreactivity was less than that in normal pregnant animals but did not reach statistical significance (Fig. 2B). Immunostaining of immediately adjacent serial sections showed that ANG-(1-7) and ACE2 are present in the same proximal and distal tubular cells in the inner cortex/outer medulla with minimal staining in the glomerulus (Fig. 3), showing 95–98% colocalization of ANG-(1-7) and ACE2. As we previously published (5), ACE2 immunoreactivity showed a wider kidney outer cortical distribution than ANG-(1-7) with some ACE2-containing cells not being positive for ANG-(1-7). On the other hand, ANG-(1-7)-staining cells without ACE2 were not observed. Preabsorption with ANG-(1-7) or the immunogenic peptide for ACE2 abolished the staining (Figs. 1A and 2A controls, respectively). The absence of staining was also observed when the primary antibodies for ANG-(1-7) and ACE2 were not added (data not shown).

While renal ANG I concentration did not change during pregnancy (Fig. 4), ANG II was increased at the 19th day of pregnancy.
pregnancy, and ANG-(1-7) was increased at the 5th and 19th day of gestation compared with virgin rats. In RUPP animals, ANG I and ANG II concentrations did not change, while ANG-(1-7) concentration was significantly decreased compared with normal 19-day pregnant rats. The ratio of angiotensin peptides, used as an index of enzymatic activities, showed that there was no difference in the ANG I/ANG II and the ANG II/ANG-(1-7) ratios during pregnancy and only the ANG-(1-7)/ANG I ratio was significantly decreased in RUPP animals compared with normal 19-day pregnant rats.

During normal pregnancy, ACE2 activity increased in both the renal cortex and the medulla at the 15th and 19th day of pregnancy compared with virgin counterparts (Fig. 5), a profile consistent with ACE2 immunostaining. In RUPP animals, ACE2 activity was not different compared with 19-day normal pregnant animals in the renal cortex or the medulla.

DISCUSSION

The present study is the first demonstration of the enhanced renal ANG-(1-7) staining in association with ACE2 immunostaining and enzymatic activity throughout the time course of pregnancy. In normal pregnancy, there is a progressive increase in the intensity of staining in the renal proximal and distal tubules for ANG-(1-7) and ACE2, increasing significantly at mid (15 day) and late (19 day) gestation. As early as the 5th day of pregnancy, there is a tendency for an increase in the intensity of staining of ANG-(1-7). Kidney ANG-(1-7) concentration as measured by RIA was significantly increased at days 5 and 19 of pregnancy. The increased renal ACE2 activity in the cortex and medulla at the 15th and 19th days of gestation suggests that ACE2 may contribute to the renal expression of ANG-(1-7) and that subsequent alterations in ANG-(1-7) levels may function in regulation of blood pressure and/or hydromineral balance. On the other hand, RUPP ani-
mals, illustrating characteristics that are reflective of pre-eclampsia, such as increased blood pressure, increased proteinuria (as evidenced by an increased protein-creatinine ratio), and decreased pup size, number, and weight (1), showed a significant decrease in inner cortex/outer medulla ANG-(1-7) staining and a decrease in kidney ANG-(1-7) peptide levels with no change in ACE2 activity. The reduction in ANG-(1-7) without a concomitant decrease in ACE2 suggests that ACE2 may not be the primary enzyme involved in ANG-(1-7) formation in the kidney during hypertensive pregnancy and that additional ANG-(1-7)-forming or -degrading enzymes may come into play (Fig. 6). The reduction of ANG-(1-7) in the RUPP kidney suggests its contribution to the regulation of blood pressure and/or hydromineral balance may be disrupted.

The condition of increased ANG-(1-7) and ACE2 together with increased kidney ANG-(1-7) peptide concentration and ACE2 activity in pregnancy compared with the virgin level is consistent with our earlier findings that both urinary excretion and kidney content of ANG-(1-7) were increased at the 19th day of pregnancy (5, 25). The finding that both ANG II and ANG-(1-7) increase at the 19th day of pregnancy suggests that an increase in substrate (ANG II) may be involved in the increase of ANG-(1-7). These findings support our hypothesis that the ANG-(1-7) increase is mediated by upregulation of ACE2. However, the ratio of ANG II/ANG-(1-7), which is sometimes used as an index of ACE2 enzymatic activity, remained unchanged during pregnancy, revealing its limitation in assessing enzymatic activity throughout pregnancy.

Because ACE2 is colocalized with ANG-(1-7) in tubules of the inner cortex/outer medulla and there is a similar increase in the profile of the two throughout pregnancy, our findings suggest that ACE2 may be critically involved in the formation of ANG-(1-7) in this region during pregnancy. The broader distribution of ACE2 in the outer cortex without ANG-(1-7), as first shown in our earlier publication (5) and confirmed in these studies, suggests an ACE2 activity that may be independent of ANG-(1-7) in the outer cortex. Possible candidates to consider for ACE2 substrate are ANG I to ANG-

![Fig. 6. Schematic of the renin-angiotensin system demonstrating ANG-(1-7) generating and degrading enzymes. NEP, nephrilysin; PE, prolyl endopeptidase; PCP, prolyl carboxypeptidase.](http://ajpregu.physiology.org/)

Because pregnancy is well documented to be a condition of hypervolemia, the antidiuretic actions of ANG-(1-7) may come into play in mid- to late pregnancy and elicit responses similar to those observed in the state of water loading.

ANG-(1-7) increases the glomerular filtration rate (18) and dilates renal afferent arterioles through nitric oxide release (26). Although the systemic hemodynamic role of ANG-(1-7) in pregnancy has not been determined, pregnancy increases the vasodilator response of ANG-(1-7) in mesenteric resistance vessels (25), which is consistent with ANG-(1-7) contributing to the decreased total peripheral resistance and counterbalancing the actions of ANG II.

The increase in intensity of staining of ANG-(1-7) and ACE2 together with increased kidney ANG-(1-7) peptide concentration and ACE2 activity in pregnancy compared with the virgin level is consistent with our earlier findings that both urinary excretion and kidney content of ANG-(1-7) were increased at the 19th day of pregnancy (5, 25). The finding that both ANG II and ANG-(1-7) increase at the 19th day of pregnancy suggests that an increase in substrate (ANG II) may be involved in the increase of ANG-(1-7). These findings support our hypothesis that the ANG-(1-7) increase is mediated by upregulation of ACE2. However, the ratio of ANG II/ANG-(1-7), which is sometimes used as an index of ACE2 enzymatic activity, remained unchanged during pregnancy, revealing its limitation in assessing enzymatic activity throughout pregnancy.

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![Fig. 5. Kidney cortex (A) and medulla (B) ACE2 enzyme activity in virgin, pregnant (5, 15, and 19 day), and RUPP (19 day) Sprague-Dawley rats. Values are expressed as means ± SE. Differences between the means were evaluated as previously described in Fig. 1; n = 13–15 per group. *P < 0.001 vs. Virgin; αP < 0.01 vs. virgin; βP < 0.01 vs. 5d-Preg; μP < 0.05 vs. 5d-Preg.](http://ajpregu.physiology.org/)
(1-9) without a subsequent conversion to ANG-(1-7) and/or apelin and dynelin A.

This is the first study that demonstrates that ACE2 activity is upregulated in the kidney in pregnancy and would be consistent with estrogen having a role in the upregulation of the ACE2 gene. The ACE2 gene has two putative estrogen response elements, (17), but few studies have evaluated the functionality of this regulation. Recent studies consistent with the upregulation of ACE2 were described by Grigore et al. (15) showing that in female offspring of intrauterine growth restricted mothers, ACE2 activity increased sixfold, while in male offspring, ACE2 activity decreased sixfold. Also, estrogen treatment was found to be associated with upregulation of renal ACE2 and protection from renal damage in the renal wrap model of hypertension (19). The gradual increase in estrogen found in the present study (Table 1), while correlative, supports the idea that the increased ACE2 is mediated by increased estrogen. Our current study suggests that ACE2 activity may be responsive to estrogen, which increases over the time course of pregnancy. These studies do not eliminate the possibility that progesterone could also be involved in ACE2 gene regulation, as it also increases during the time course of pregnancy. Studies evaluating the role of specific hormones on ACE2 mRNA regulation are warranted in normal and hypertensive pregnancy.

Our studies provide evidence for the first time that the RUPP preeclampsia model is characterized by the failure to increase ANG-(1-7) in the kidney. Previous reports from our laboratory also demonstrated reduced circulating levels of ANG-(1-7) in human preeclamptic pregnant subjects (22). The increase in blood pressure in the RUPP with the reduction in intrauterine growth restriction is consistent with the model as described by Granger et al. (13). The renal lesions demonstrated by RUPP animals are similar to glomerular endotheliosis, which is a characteristic of the kidney in preeclamptic women. In addition, this model is associated with reductions in renal plasma flow, glomerular filtration rate, and a hypertensive shift in the pressure natriuresis relationship (13). Our finding of an increase in urinary protein/creatinine is consistent with altered renal function associated with pregnancy-induced hypertension. Our studies offer the possibility that a reduction in ANG-(1-7) may be an important endogenous mediator of renal function and hemodynamics in the RUPP model and in pregnancy-induced hypertension.

In RUPP animals, ANG-(1-7) immunostaining and concentration, as determined by RIA, were decreased with no change in ACE2 immunostaining or activity. This mismatch in regulation of ACE2 activity and peptide product suggests that there may be other ANG-(1-7)-forming enzymes that may be downregulated or ANG-(1-7)-degrading enzymes that may be upregulated in the RUPP animals, and these may be responsible for the decreased ANG-(1-7) content (Fig. 6). A candidate ANG-(1-7)-generating enzyme for consideration is neprilysin (NEP), since the ANG (1-7)/ANG I ratio was decreased in RUPP animals, suggesting that decreased ANG-(1-7) in the RUPP could result from decreased NEP. NEP has also been shown to be present in the kidney and to be involved in the generation of ANG-(1-7) in the brush border (3). Recent studies (24) have demonstrated that kidney NEP is unresponsive to estrogen replacement. No studies, however, have been conducted evaluating the regulation of NEP during pregnancy or in RUPP animals. A candidate for ANG-(1-7)-degrading enzyme for consideration is ACE, which has been demonstrated to be present in the kidney and to be downregulated by estrogen (12). A downregulation of ACE would lead to increased ANG-(1-7), while an upregulation of ACE would lead to decreased ANG-(1-7). In the present study, RUPP animals exhibited lower levels of estradiol, suggesting that the decreased renal ANG-(1-7) could result from an upregulation of ACE. Either of these enzymes, NEP or ACE, could be regulated in the RUPP and participate in reduction of ANG-(1-7) content.

In the present study, RUPP animals exhibited lower estradiol levels compared with normal 19-day pregnant rats. Studies in humans demonstrate that serum estradiol values after 34-wk gestation were significantly lower in preeclamptic women compared with normal controls (27). Salas et al. (27) suggest that the relative fall in estradiol concentration may be due to altered metabolic function of the fetoplacental unit mainly through the reduction in uteroplacental blood flow. Changes in estradiol in RUPP rats are consistent with data in preeclampsia.

The current studies have provided novel insights into the temporal and spatial expression of ANG-(1-7) and the angiotensin-converting enzyme homolog, ACE2, in the kidney during pregnancy. The coincident location of ANG-(1-7) and ACE2 in the inner cortex/outer medulla suggests local tissue generation of ANG-(1-7). Our data support the concept that during normal pregnancy, enhanced ACE2 may contribute to an increasing rate of conversion to ANG-(1-7). In pregnancy-induced hypertension, as elicited with the RUPP model, ANG-(1-7) peptide showed decreased expression. This finding is consistent with reduced circulating levels of ANG-(1-7) in human preeclamptic subjects and suggests that the reduced level of the peptide may contribute to the hypertension. The lack of correlation between ACE2 activity and ANG-(1-7) in the RUPP suggests that other enzymes may come into play in determining the levels of ANG-(1-7) in pregnancy-induced hypertensive states.

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