Enhanced Renal Immunocytochemical Expression of ANG-(1-7) and ACE2 During Pregnancy

K. Bridget Brosnihan, Liomar A.A. Neves, JaNae Joyner, David B. Averill, Mark C. Chappell, Renu Sarao, Josef Penninger, Carlos M. Ferrario

Abstract—Previously we demonstrated that kidney concentration and urinary excretion of angiotensin-(1-7) are increased during normal pregnancy in rats. Since this finding may reflect local kidney production of angiotensin-(1-7), we determined the immunocytochemical distribution of angiotensin-(1-7) and its newly described processing enzyme, ACE2, in kidneys of virgin and 19-day-pregnant Sprague-Dawley rats. Sprague-Dawley rats were killed at the 19th day of pregnancy, and tissues were prepared for immunocytochemical by using a polyclonal antibody to angiotensin-(1-7) or a monoclonal antibody to ACE2. Angiotensin-(1-7) immunostaining was predominantly localized to the renal tubules traversing both the inner cortex and outer medulla. ACE2 immunostaining was visualized in the renal tubules of both virgin and pregnant rats. The quantification of angiotensin-(1-7) and ACE2 immunocytochemical staining showed that in pregnant animals, the intensity of the staining increased by 56% and 117%, respectively (P<0.05). This first demonstration of the immunocytochemical distribution of angiotensin-(1-7) and ACE2 in kidneys of pregnant rats shows that pregnancy increases angiotensin-(1-7) immunocytochemical expression in association with increased ACE2 intensity of staining. The findings suggest that ACE2 may contribute to the local production and overexpression of angiotensin-(1-7) in the kidney during pregnancy. (Hypertension. 2003;42[part 2]:749-753.)

Key Words: angiotensin ■ renin-angiotensin system ■ pregnancy ■ kidney ■ angiotensin-converting enzyme 2

Previously we demonstrated for the first time that activation of the renin-angiotensin system (RAS) during pregnancy is associated with augmented kidney concentration and urinary excretion of angiotensin-(1-7) [Ang-(1-7)].1 Ang-(1-7), formed from either Ang I or Ang II and shown to exert vasodilatory, antiproliferative and natriuretic effects,1–9 was the predominate angiotensin peptide found in the kidney and urine of pregnant rats, reaching levels that were 3- and 2.5-fold greater than Ang II in the kidney and urine, respectively. There was an increase in the Ang-(1-7)/Ang II, indicating that there may be increased enzymatic conversion of Ang II into Ang-(1-7). At the same time, there was no change in Ang-(1-7) in the circulation. These findings suggest that there may be an enhanced renal local production and overexpression of Ang-(1-7) in pregnancy without a systemic contribution.

The profile of peptides in the kidney with increased content of Ang-(1-7) without a buildup in the levels of Ang II is consistent with an enzymatic pathway of a recently described enzyme of the RAS, ACE2. ACE2 is a carboxypeptidase that converts Ang I into Ang-(1–9), but it also exhibits high catalytic efficiency to generate Ang-(1-7) from Ang II.10–12 The ACE2 catalytic activity for Ang II as compared with Ang I is 400-fold higher, making Ang II a central player in the metabolic pathway for the formation of Ang-(1-7). Chappell et al13 provided the first direct evidence for the presence of ACE2 staining in the renal tubular system of the rat. The high kidney concentrations of Ang-(1-7) during pregnancy raised the question of whether ACE2 may be localized in association with Ang-(1-7) in the kidney such that it could account for the increased production of Ang-(1-7). In these studies, we sought to determine if pregnancy changed the intensity or distribution of immunocytochemical (ICC) staining of Ang-(1-7) and ACE2 in the kidney.

Experimental Procedures

Animals
Timed pregnant and age-matched virgin female Sprague-Dawley rats were obtained from Charles River Laboratory (Wilmington, Mass) and housed individually under a 12-hour light/dark cycle in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Charles River Laboratories calculates day 1 of pregnancy as the day when sperm are 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 NIH guidelines.

Surgical Procedure
At the 19th day of pregnancy or an equivalent age in virgin female rats, the animals were killed by decapitation. The 19th day of pregnancy was selected in accordance with our previous publication describing the kidney content and urinary excretion of angiotensin peptides. The kidneys were rapidly removed and immediately placed in a solution of 4% formalin acetate or frozen on dry ice. Frozen tissues were transferred to a −80°C freezer until processed.

Immunocytochemistry
Tissues were left in fixative for 24 to 48 hours before being transferred to 70% ethanol. Kidney tissues were then imbedded in paraffin, and 5-μm sections were obtained. Immunocytochemistry distribution of Ang-(1-7) and ACE2 were obtained through the use of the avidin-biotin method as previously published. All tissues from pregnant and virgin rats were prepared and analyzed in paired sections. The primary antibodies used were an affinity-purified rabbit polyclonal antibody to Ang-(1-7) produced by our laboratory at a dilution of 1:25 in 1% BSA and a mouse monoclonal antibody to ACE2 diluted 1:300 in 1% BSA provided by J. Penninger. Ang-(1-7) antibody purification and characterization have been described previously. The secondary antibody used was biotinylated goat anti-rabbit for Ang-(1-7) staining or biotinylated horse anti-mouse for ACE2 staining (Vector Laboratories), diluted 1:400 in 1% BSA. Sections were stained brown with 3,3′-diaminobenzidine (DAB, Sigma Chemical Co) in Tris-buffered saline (0.05 mol/L, pH 7.6 to 7.7) and counterstained with hematoxylin before being dehydrated and mounted. Some sections were not counterstained with hematoxylin and were used for quantitative analysis. To validate the staining procedure, kidney sections were incubated with the secondary antibody alone without the primary antibody. In addition, the specificity of the staining obtained with the Ang-(1-7) antibody was assessed by preabsorption of the antibody. In addition, the specificity of the staining obtained with the Ang-(1-7) antibody was assessed by preabsorption of the antibody with synthetic Ang-(1-7) and ACE2 were not influenced by preabsorption of the Ang-(1-7) antibody with synthetic Ang-(1-7) yielded no staining (Figure 1H).

Discussion
Pregnancy increased the intensity of staining for both Ang-(1-7) and ACE2 in the renal tubules as compared with the virgin animals. Overall, the distribution of staining for Ang-(1-7) and ACE2 in the kidney was similar, with intense staining present in the tubules in the inner cortex and outer medulla and almost no staining in the glomeruli and inner medulla; however, ACE2 showed a broader distribution throughout the cortex. The patterns of distribution of staining for both Ang-(1-7) and ACE2 were not influenced by pregnancy. The renal distribution of Ang-(1-7) shown in virgin and pregnant rats is in agreement with previous studies by Ferrario et al., who provided the first direct evidence for the presence of Ang-(1-7) staining in the renal tubular system of the rat. The renal distribution of Ang-(1-7) immunostaining is in accordance with functional studies, which demonstrated that Ang-(1-7) has an important role in renal homeostasis.

Quantification of Immunocytochemical Staining
The Ang-(1-7) and ACE2 staining was quantified as previously published. The digitization of images was performed with a Zeiss microscope equipped with an AxioCam digital camera that transmits the image data to the AxioVision software (Zeiss). All images were obtained with the use of a ×20 objective. Three ×20 fields of the inner cortex/outer medulla region containing at least one glomerulus were chosen from each animal to best reflect the overall immunostaining contained on the entire section. For the entire study, the image intensity was kept at an identical level. The corrected quantitative intensity was calculated as the difference between intensity of the immunostaining in tubules versus glomeruli (background). The results were averaged from 4 to 5 rats in each group.

Statistical Analysis
Comparisons between the groups were performed with the use of a 2-tailed, unpaired Student t test (GraphPad Software). A probability value of <0.05 was considered statistically significant. All values are presented as mean±SEM.

Results
The Ang-(1-7) immunostaining was present in the renal tubules of the inner cortex and outer medulla region of virgin and pregnant rats (Figures 1A and 1B), but there was little or no staining in the inner medulla region. Similar observations were made for ACE2, except that it showed a wider distribution over the entire cortex in the virgin and pregnant rats (Figures 2A and 2B). There was no immunostaining within the glomeruli for either Ang-(1-7) or ACE2 in virgin and pregnant Sprague-Dawley rats (Figures 1C and 1D, 2C and 2D). The Ang-(1-7) and ACE2 immunostaining was visualized in the cytoplasm of the renal tubules cells (Figures 1E and 1F, 2E and 2F). In the collecting tubules of the inner medullary region, the immunostaining for Ang-(1-7) and ACE2 was less dense (data not shown). The intensity of the Ang-(1-7) and ACE2 staining in the tubules of pregnant rats appeared to be greater as compared with the virgin animals (Figures 1C and 1D and Figures 2C and 2D). The quantification of Ang-(1-7) and ACE2 immunostaining intensity showed that in pregnant animals, the intensity of the staining increased by 56% and 117%, respectively (P<0.05, Figure 3). Figures 1G and 2G show the absence of staining when the primary Ang-(1-7) and ACE2 antibodies were not added. Preabsorption of the Ang-(1-7) antibody with synthetic Ang-(1-7) yielded no staining (Figure 1H).

Pregnancy increased the intensity of staining for both Ang-(1-7) and ACE2 in the renal tubules as compared with the virgin animals. Overall, the distribution of staining for Ang-(1-7) and ACE2 in the kidney was similar, with intense staining present in the tubules in the inner cortex and outer medulla and almost no staining in the glomeruli and inner medulla; however, ACE2 showed a broader distribution throughout the cortex. The patterns of distribution of staining for both Ang-(1-7) and ACE2 were not influenced by pregnancy. The renal distribution of Ang-(1-7) shown in virgin and pregnant rats is in agreement with previous studies by Ferrario et al., who provided the first direct evidence for the presence of Ang-(1-7) staining in the renal tubular system of the rat. The renal distribution of Ang-(1-7) immunostaining is in accordance with functional studies, which demonstrated that Ang-(1-7) has an important role in renal homeostasis.

The increased ICC expression of Ang-(1-7) during late pregnancy is in agreement with our previous study showing increased kidney concentration of Ang-(1-7). In pregnancy, the kidney ratio of Ang-(1-7)/Ang II increased to 2.48±0.97, as compared with 0.89±0.36 in the virgin rats. These ratios have been used to provide evidence of enzymatic conversion
of angiotensins, and the pattern observed in pregnancy is consistent with an efficient conversion of Ang II to Ang-(1-7). ACE2, which has only recently been recognized to be a participant in the RAS, is a metallopeptidase with carboxypeptidase properties that participates in the formation of Ang-(1-7) from Ang II or Ang I. The catalytic efficiency of ACE2 for generating Ang-(1-7) from Ang II is almost 400-fold greater than that for the conversion of Ang I to Ang-(1-9) and 10- to 600-fold higher than that of two other Ang-(1-7) forming enzymes, prolyl oligopeptidase and prolylcarboxypeptidase, respectively.\(^\text{11}\) ACE2 appeared to be colocalized with Ang-(1-7) in the tubules of the inner cortex and outer medulla, and its increased ICC expression in the kidneys of pregnant rats would suggest that ACE2 is a primary candidate for Ang-(1-7) formation.

Our previous findings of increased kidney levels of Ang-(1-7) with increased urinary excretion of Ang-(1-7) without a change in circulating levels of Ang-(1-7) during pregnancy is important because it supports the local renal production of Ang-(1-7). In pregnancy, the percent increase in the urinary excretion rates of Ang I (93%) and Ang-(1-7) (60%) were greater than the increase in urine flow (46%) in the virgin rats, suggesting that there is active secretion of Ang I and Ang-(1-7) from the kidney that is greater than the diuresis associated with pregnancy. Since plasma levels of the peptides were unchanged at this stage of pregnancy (19th day), the increase is unlikely to be due to uptake from the circulation. The site of Ang-(1-7) production in the kidney has not been characterized. To assess this, it will be important to determine the kidney localization of Ang II in association with ACE2. Ang II staining has been localized to juxtaglomerular cells and proximal tubules.\(^\text{25}\) In addition to an intracellular localization within the kidney,\(^\text{25,26}\) Ang II has been shown to be in tubular fluid,\(^\text{27}\) interstitial fluid compartments, and urine,\(^\text{1,28}\) making it available as a substrate for either intracellular or tubular formation of Ang-(1-7). Because ACE2 has been shown to be present in the urine in addition to the tubular location described in these studies (unpublished data from Dr Mark Chappell, 2003), it is likely that it also can be secreted. Thus both renal intracellular and interstitial formation of Ang-(1-7) may occur. Future studies are required to address this issue.

Other enzymes that have been demonstrated to participate in the formation of Ang-(1-7) include neprilysin and thimet
oligopeptidase, with the contribution of each enzyme dependent on tissue source and vascular compartment. In the kidney, neprilysin has been shown to be a major contributor to Ang-(1-7) formation. However, we have shown previously that renal neprilysin activity is not changed with estrogen replacement, making it unlikely to be responsible for the increase in Ang-(1-7) formation because of the increase in estrogen that occurs in pregnancy. On the other hand, ACE has also been shown to participate in the metabolism of Ang-(1-7) by metabolizing Ang-(1-9) or by degrading Ang-(1-7) to inactive metabolites. Since we have previously shown that estrogen downregulates ACE activity and mRNA expression in kidney cortex and medulla and that serum ACE is reduced in pregnancy, the contribution of ACE to the elevated Ang-(1-7) levels with pregnancy is a likely possibility, more by preventing its degradation than enhancing its formation. Although our study provides evidence that indicates that ACE2 may play a critical role on the Ang-(1-7) formation, in vivo studies evaluating the participation of the different metabolic pathways for Ang-(1-7) formation during pregnancy need to be investigated.

The present study is the first demonstration of the immunocytochemical distribution of Ang-(1-7) and its processing enzyme ACE2 in the kidney of pregnant rats. The increased ACE2 intensity in association with an increased Ang-(1-7) immunostaining in pregnancy suggests that ACE2 may act to contribute to the overexpression of Ang-(1-7) in the kidney during pregnancy and shift the Ang II/Ang-(1-7) balance away from Ang II.

**Perspectives**

Recent studies by Crackower et al on ACE2 expression and blood pressure regulation, taken together with the information available on the Ang-(1-7) vasodilator and blood pressure-lowering effects, suggest that ACE2 may play a critical role in blood pressure regulation by modulating the balance of the vasoconstrictor and vasodilator components of the RAS. The present study indicates that ACE2 may contribute to a shift in the Ang II/Ang-(1-7) balance during pregnancy by contributing to increased Ang-(1-7) formation. Because pregnancy is a physiological condition, where there is an activation of the RAS but blood pressure is normal or decreased, the enhanced expression of Ang-(1-7) may play a critical role in the maintenance of blood pressure. Critical areas for further investigation concern the identification of the role ACE2 plays in Ang-(1-7) formation in
vivo in late pregnancy, as well as assessing ACE2 expression in reproductive organs during pregnancy. More important will be the evaluation of ACE2 expression and its role in Ang-(1-7) formation during preeclampsia.

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