ACE2 and ANG-(1-7) in the rat uterus during early and late gestation

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[1]*Hypertension and Vascular Research Center and* [3]*Department of Obstetrics and Gynecology, Wake Forest University School of Medicine, Winston-Salem, North Carolina; and* [2]*Department of Nephrology, Pontificia Universidad Catolica de Chile, Santiago, Chile*

Neves LAA, Stovall K, Joyner J, Valdés G, Gallagher PE, Ferrario CM, Merrill DC, Brosnihan KB. ACE2 and ANG-(1-7) in the rat uterus during early and late gestation. *Am J Physiol Regul Integr Comp Physiol* 294: R151–R161, 2008. First published October 31, 2007; doi:10.1152/ajpregu.00514.2007.—The present study was designed to determine ANG peptide content [ANG I, ANG II, ANG-(1-7)], ACE2 mRNA, and the immunocytochemical distribution of ANG-(1-7) and ACE2 in the uteroembryonic unit during early and late gestation in Sprague-Dawley rats and in a rat model of pregnancy-induced hypertension, the reduced uterine perfusion pressure (RUPP) model. At early pregnancy ANG-(1-7) and ACE2 staining were localized in the primary and secondary decidual zone and luminal and glandular epithelial cells. During late gestation, ANG-(1-7) and ACE2 staining was visualized in the labyrinth placenta and amniotic and yolk sac epithelium. Uterine ANG II concentration at early pregnancy was significantly decreased by 21–55% in the implantation and interimplantation sites compared with virgin rats, whereas ANG-(1-7) levels were maintained at prepregnancy levels. At late gestation, uterine concentrations of ANG I and ANG II were significantly increased (30% and 25%, respectively). In RUPP animals, ANG-(1-7) concentration is significantly reduced in the uterus (181 ± 16 vs. 372 ± 74 fmol/g of tissue) and placenta (143 ± 26 vs. 197 ± 20 fmol/g of tissue). ACE2 mRNA increased in the uterus of early pregnant compared with virgin rats, yet within the implantation site it was downregulated. At late pregnancy, ACE2 mRNA is elevated by 58% in the uterus and decreased by 59% in RUPP animals. The regulation of ANG-(1-7) and ACE2 in early and late pregnancy supports the hypothesis that ANG-(1-7) and ACE2 may act as a local autocrine/paracrine regulator throughout pregnancy, participating in the early (angiogenesis, apoptosis, and growth) and late (uteroplacental blood flow) events of pregnancy.

renin-angiotensin system; implantation

PREGNANCY IS A CONDITION IN which there is a progressive increase in the activity of the renin-angiotensin system (RAS), evidenced by a marked increase in circulating concentration of angiotensinogen, renin activity, and ANG II (4, 9). With the discovery of ANG-(1-7) and ANG converting enzyme 2 (ACE2) (13), a novel homolog of ACE, studies have shown that these new components of the RAS are regulated during pregnancy. ANG-(1-7) is involved in mediating vasodilation and facilitating blood flow (5, 23, 31, 32, 39, 40), and pregnancy is associated with an increase in the vasodilator response to ANG-(1-7) in mesentery arteries (37). Recently, we showed that pregnancy is associated with increased circulating and renal ANG-(1-7) concentrations (6, 34, 49) at late gestation. In human pregnant subjects, Merrill et al. (34) and Valdés et al.

(49) demonstrated that plasma concentration and urinary excretion of ANG-(1-7) are increased during the third trimester. Merrill et al. (34) also demonstrated that plasma concentration of ANG-(1-7) was substantially reduced during preeclampsia. These results suggest that ANG-(1-7) may play a role in the vasodilatory adaptations of mid- and late pregnancy and a reduction in the formation of this peptide may be a key contributor to the hypertensive disorders of pregnancy.

ACE2 participates in ANG-(1-7) formation in rats (30), sheep (47), and humans (51) producing ANG-(1-7) from either ANG I or ANG II, (20, 42, 51) with a 400-fold higher catalytic activity for ANG II compared with ANG I. The preference for ANG II as substrate for ACE2 was clearly demonstrated in renal processing of ANG II to ANG-(1-7) but not the processing of ANG I to ANG-(1–9) in sheep (47). In pregnant rats, ACE2 was localized to similar regions as ANG-(1-7) in the kidney, and both ACE2 and ANG-(1-7) showed increased immunostaining, suggesting that ACE2 may participate in local renal ANG-(1-7) formation during pregnancy (7).

Based on the above findings, we raised the question of whether ANG-(1-7) and ACE2 were similarly expressed in the reproductive organs during early and late pregnancy and whether their levels were increased throughout pregnancy but were reduced during preeclampsia. Because the uterus undergoes major hemodynamic adaptations to meet the metabolic demands of the growing fetus, documentation of the presence and regulation of ANG-(1-7) and ACE2 in the uterus would provide information that may be important to counterbalance the activated RAS previously demonstrated in the uterus at late gestation. A number of studies implicate the RAS in the early events of pregnancy, including implantation and placentation (17, 28, 29, 48). ANG II increases uterine vascular permeability and decidualization (48), stimulates angiogenesis, and participates in regulation of growth processes during implantation and placentation (17, 28, 29). The present study was designed to determine the localization and temporal-spatial changes in the immunocytochemical distribution of ANG-(1-7) and ACE2, ANG peptide [ANG I, ANG II, and ANG-(1-7)] concentrations, and ACE2 mRNA in the uterus at early (*days 5* and *7*) and late (*day 19*) pregnancy. We also included an evaluation of ACE mRNA, since ACE also contributes to the ANG II/ANG-(1-7) balance. We used the reduced uterine perfusion pressure (RUPP) model as a model of pregnancy-induced hypertension (PIH). This is a well-characterized rat model that exemplifies features of PIH (1).

MATERIALS AND METHODS

Animals. Timed pregnant and age-matched virgin female Sprague-Dawley rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and housed individually under a 12:12-h light-dark cycle in an American Association for Accreditation of Laboratory Animal Care-approved facility. *Day 0* of pregnancy was designated as the day when sperm were found in the vagina. 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.

Experimental procedures. A vaginal smear was obtained from virgin rats, and vaginal cytology was used to determine the stage of the estrous cycle. On *day 5* of pregnancy (the day after implantation), implantation sites (ISs) were identified by increased uterine vascular permeability at the site of the blastocyst attachment through intracardiac injection of 0.2 ml of 0.5% Evans blue dye in briefly anesthetized animals. The animals were allowed to recover fully and were killed by decapitation 15 min later. In the next experimental group, on *day 7* of gestation, ISs were visually identified. The rats in the experimental group of *day 14* of gestation were anesthetized with isoflurane and were either sham operated or prepared for RUPP surgery by using a modification of a procedure previously described (1). Briefly, a silver clip (0.203 mm) was placed around the descending aorta just above the iliac bifurcation. Because compensation of blood flow to the placenta occurs in the pregnant rat through an adaptive increase in uterine blood flow, both right and left uterine arteries were clipped (0.221 mm), between the ovaries and the first bifurcation. Virgin female rats and normal and RUPP pregnant rats at *day 17* of gestation were placed in metabolic cages and 24-h urine was collected. Urine volume was measured and then kept at $-20°C$ until analyzed for total protein (BioRad, Hercules, CA) and creatinine (Caymen, Ann Arbor, MI) using colorimetric assays. After urine collection, pregnant animals were anesthetized with isoflurane and the carotid artery was cannulated with polyethylene tubing. On *day 19* of pregnancy, blood pressure was recorded (Biopac System; Santa Barbara, CA), and animals were killed immediately after.

Conscious animals (virgin and 5-, 7-, and 19-day normal pregnant and 19-day RUPP) were killed by decapitation in the morning, and trunk blood was collected for determination of serum 17β-estradiol by radioimmunoassays (RIA; Polymedco, Cortlandt Manor, NY). At *days 5* and *7* of pregnancy, the uterus was dissected and separated into either ISs or interimplantation sites (IISs) and was either placed in 4% paraformaldehyde or snap frozen on dry ice for analysis. At *day 19* of pregnancy, the uteroplacental unit was rapidly removed and either immediately placed in a solution of 4% paraformaldehyde or the uterus and placenta were dissected and frozen on dry ice. Frozen tissues were transferred to a $-80°C$ freezer until they were processed. Only uterus tissues from virgin animals at the diestrus and estrus phase of the estrous cycle were used. Uterus tissue from virgin at diestrus was used as the appropriate control for late gestation, since this is a stage where the levels of 17β-estradiol have been demonstrated to be the lowest. This was done to standardize the measure-ment of ANG peptides and components of the RAS, which are known to be influenced by estrogen (22, 45). Uterus tissue from virgin rats at estrus was used as the appropriate control for early gestation, as previously published (11) because this phase precedes ovulation and implantation. Late pregnant rats were excluded from data analysis when the clipping procedure left the mother with less than three pups due to reabsorption.

Immunohistochemistry. Tissues were fixed for 24 h and transferred to 70% ethanol with the exception of the uteroplacental unit at *day 19* of pregnancy. These tissues were sliced, the fetus was removed, and the tissue was left in 4% paraformaldehyde for an additional 12 h and then transferred to 70% ethanol. Immunohistochemistry distribution of ANG-(1-7) and ACE2 was obtained using the avidin-biotin method as previously published (7, 18, 25). All tissues from early (*days 5* and *7*) and late (19-day sham and RUPP) pregnant rats were prepared and analyzed side by side. The primary antibodies used were an affinity-purified rabbit polyclonal antibody to ANG-(1-7) and ACE2 produced by our laboratory at a dilution of 1:25 and 1:150, respectively, in 1% BSA. The secondary antibody was biotinylated goat anti-rabbit (Vector Laboratories, Burlingame, CA) diluted 1:400 in 1% BSA. The intensity of the expression of ANG-(1-7) and ACE2 was semiquantified by an observer blinded to the groups, according to a gradient range of 0 = absence, 1 = faint, 2 = moderate, 3 = intense, and 4 = very intense signal.

Tissue concentration of ANG peptides. Frozen tissues were rapidly weighed and homogenized as described by Allred et al. (2). Tissue homogenates were extracted using Sep-Pak columns as described previously (2, 35, 45). The eluate was divided for three RIAs [ANG I, ANG II, and ANG-(1-7)], and the solvent was evaporated. ANG I was measured by using a modification of commercially available Peninsula RIA kit (San Carlos, CA). ANG II was measured using a diagnostic kit, (Alpco, Windham, NH). ANG-(1-7) was measured using the antibody produced by our laboratory (2). The minimum detectable levels of the assays were 1.0 fmol/ml for ANG I, 0.8 fmol/ml for ANG II, and 2.8 fmol/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.

RNA isolation and reverse transcriptase/real-time polymerase chain reaction. Total RNA was isolated from tissue, using the Trizol reagent (GIBCO-Invitrogen, Carlsbad, CA), as directed by the manufacturer. The RNA concentration and integrity were assessed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano LabChip (Agilent Technologies, Palo Alto, CA). Approximately 1 μg of total RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase as previously described (19) The primer/probe sets for ACE were purchased from Applied Biosystems (Foster City, CA) and for ACE2 were our design (forward primer 5′-CCCAGAGAACAGT-GGACCAAAA-3′; reverse primer 5′-GCTCCACCACACCAAC-GAT-3′; and probe 5′-FAM-CTCCCGCTTCATCTCC-3′). All reactions were performed in triplicate and 18S ribosomal RNA, amplified using the TaqMan Ribosomal RNA Control Kit (Applied Biosys-

Table 1. *Maternal weight and serum 17β-estradiol levels of virgin, 5-days, 7-days, and 19-days pregnant, and RUPP Sprague-Dawley rats*

| | Virgin | | | | | |
	Estrus	Diestrus	5 Days PR	7 Days PR	19 Days PR	RUPP
No. of rats per group	13	18	25	29	15	12
Maternal body weight, g	239±4	251±4	223±4[a,b,c,d]	244±3[c,d,e]	312±6[a,b]	277±4[a,b,c]
17β-Estradiol, pmol/l	99±17[a]	37±3	23±2[b,c,d]	19±0.4[b,c,d]	159±22[a,b]	89±13[c]

Values are expressed as means ± SE. Differences between the means were evaluated using 1-way ANOVA and Bonferroni multicomparison test PR, pregnant; RUPP, reduced uterine perfusion pressure. [a]$P < 0.01$ vs. virgin diestrus rats; [b]$P < 0.05$ vs. virgin estrus rats; [c]$P < 0.01$ vs. 19-days PR; [d]$P < 0.01$ vs. RUPP, [e]$P < 0.01$ vs. 5 day.

Table 2. *Basal characterization of virgin and 19 days PR and RUPP Sprague-Dawley rats*

	Virgin	19 Days PR	RUPP
No. of rats per group	9	15	12
Litter size/implantation site, *n*		11.7±0.8	6.5±0.6†
Crown to rump length, cm		2.9±0.05	2.7±0.03†
Fetus weight, g		2.4±0.11	1.9±0.04†
Fetuses reabsorbed, *n*		0.3±0.1	6.0±0.9†
Mean arterial pressure, mmHg		105±1.8	122±1.8†
Urine volume, ml	15.2±1.4	29.5±1.3*	21.9±1.9*#
Urinary creatinine, mg/24 h	11±0.9	15±0.9*	9±0.7†
Urinary protein-to-creatinine ratio	0.4±0.03	0.5±0.05	0.8±0.11*†

Values are means ± SE. Differences between the means were evaluated using 1-way ANOVA or Student's *t*-test. *$P < 0.5$ vs. virgin rats; †$P < 0.05$ vs. 19 days PR.

tems), served as an internal control. The results were quantified as Ct values, where Ct was defined as the threshold cycle of PCR at which amplified product was first detected, and defined as relative gene expression (the ratio of target/control).

Statistical analysis. Comparisons between the groups were performed using one-way ANOVA followed by the Newman-Keuls post hoc test or unpaired Student's *t*-test (GraphPad Software, San Diego, CA). A *P* value <0.05 was considered statistically significant. All arithmetic means are ± SE.

RESULTS

Table 1 shows the body weight and serum estradiol levels of virgin and 5-days-, 7-days-, and 19-days-pregnant, and RUPP animals. Serum estradiol is increased seven- and eightfold at late gestation compared with *days 5* and *7* of gestation, respectively. Table 2 provides the comparison among the virgin,

19-days normal pregnant, and RUPP animals. RUPP animals showed a significant increase of 16% in blood pressure and had 14% fewer fetuses with smaller fetal weight (18%) and length (7%). There was also a significant increase of 72% in the urinary protein-to-creatinine ratio in RUPP animals compared with pregnant and virgin rats.

In virgin animals, faint ANG-(1-7) immunostaining was present in the luminal and glandular epithelium of the uterus (Fig. 1*B*); semiquantitative analysis revealed moderate ACE2 immunostaining in these areas (2.4 ± 0.3 and 2.1 ± 0.3, respectively) (Fig. 1*C*). Areas of immunostaining are indicated in the cross section of the uterus stained by hematoxylin and eosin (Fig. 1*A*). ACE2 immunostaining was visualized throughout the cytoplasm of the epithelial cells; however, ANG-(1-7) immunostaining was localized in the luminal surface of epithelial cells. The same distribution was observed during all phases of the estrus cycle (data not shown). Preabsorption of the ANG-(1-7) and ACE antibody with synthetic ANG-(1-7) or ACE2 peptide, respectively, yielded no staining (Fig. 1, *D* and *E*). Elimination of ANG-(1-7) and ACE2 primary antibody also resulted in absence of the staining (data not shown).

Figure 2, *A* and *I* show a macroscopic view of the IS and IIS at *days 5* and *7* of pregnancy. Cross-sections of the uterus at IS and IIS stained with hematoxylin and eosin are shown in Fig. 2, *B*, *J*, and *K*, and the areas immunostained are indicated. Analysis of ANG-(1-7) and ACE2 immunostaining revealed expression of the peptide and enzyme at the site of embryo implantation on gestation *days 5* and *7*. At *day 5* of gestation, faint-to-intense ANG-(1-7) and ACE2 staining was found in the primary decidual cells (1.3 ± 0.3 and 3.0 ± 0, respectively)
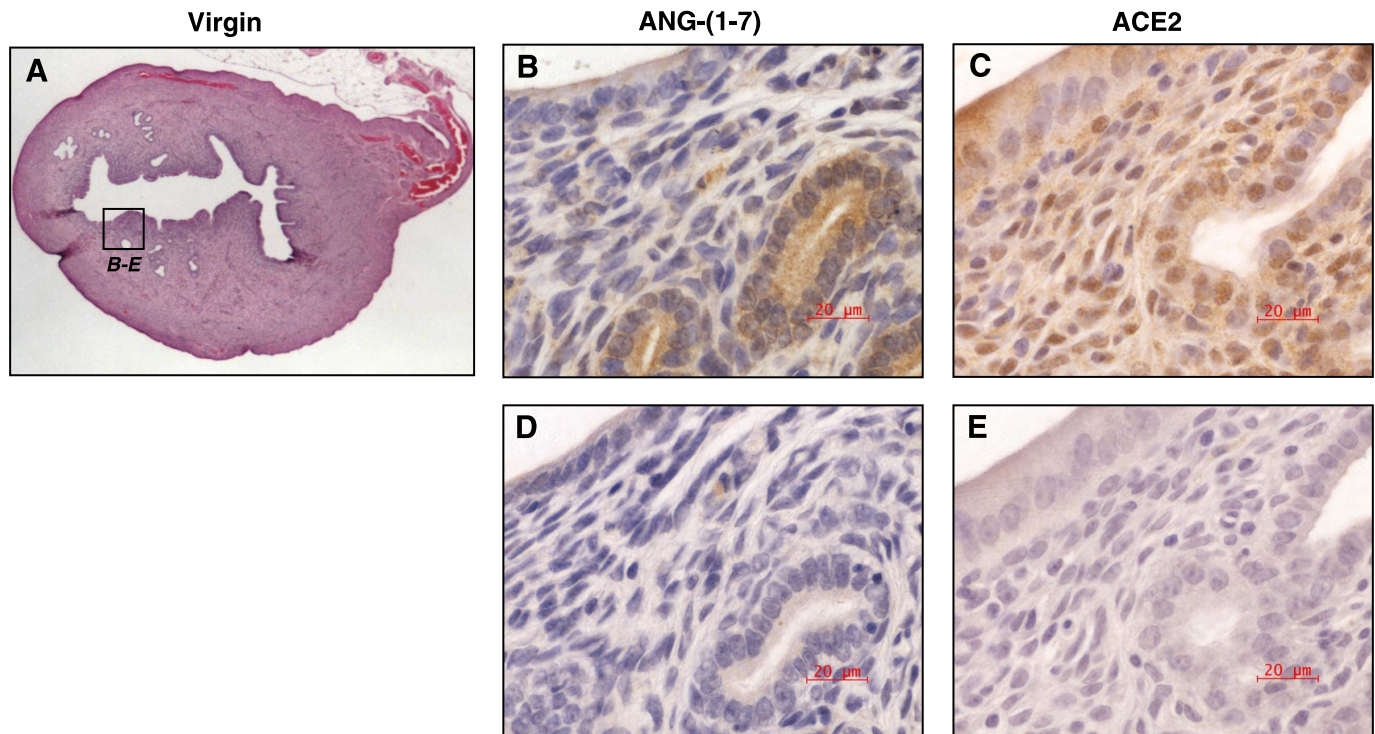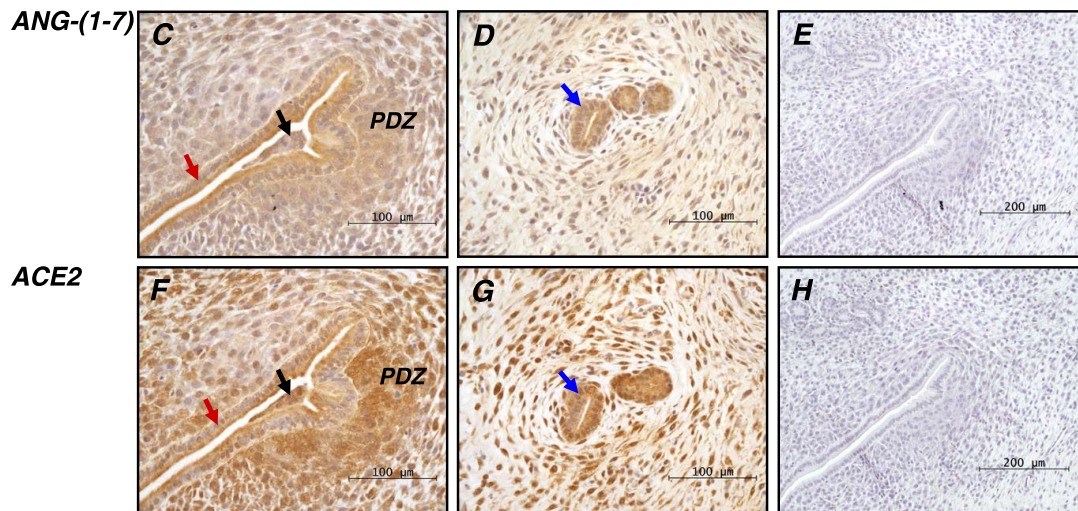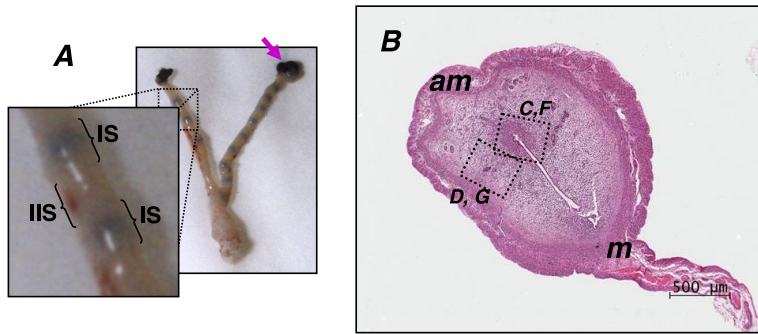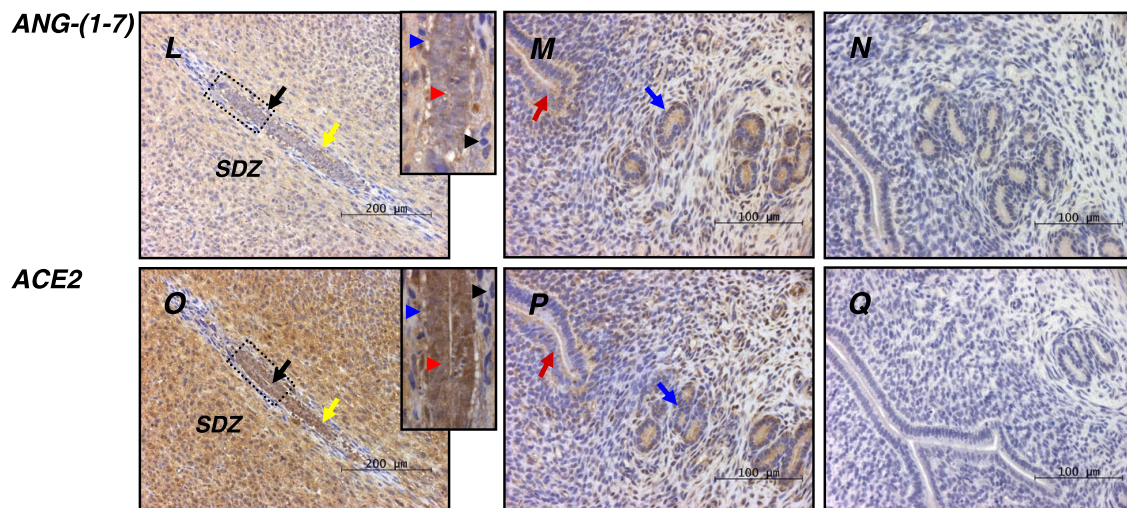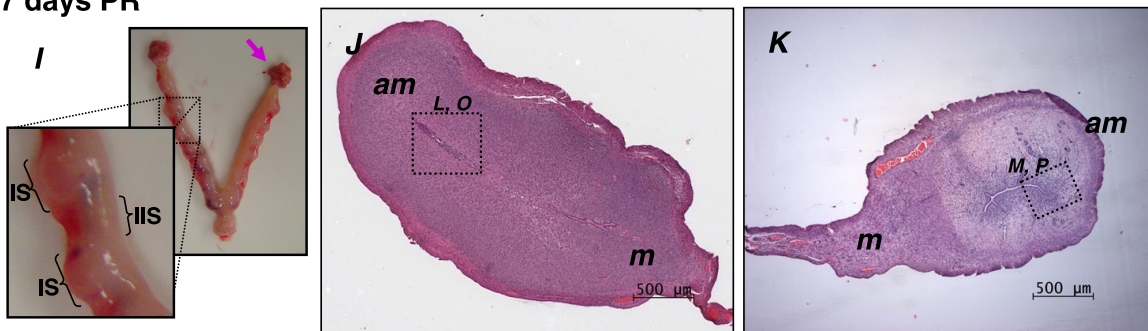

Fig. 1. Immunohistochemical identification of ANG-(1-7) (*B*) and ACE2 (*C*) in the luminal and glandular epithelium of the uterus in virgin Sprague-Dawley rats. *A*: photograph of the uterus stained with hematoxylin and eosin; framed areas are shown at higher magnification on panels *B–E*. *D* and *E*: immunoreactivity for ANG-(1-7) and ACE2 was absent after preincubation of the primary antibody with 10 μmol/l of ANG-(1-7) or ACE2 peptide, respectively.

5 days PR

ANG-(1-7)
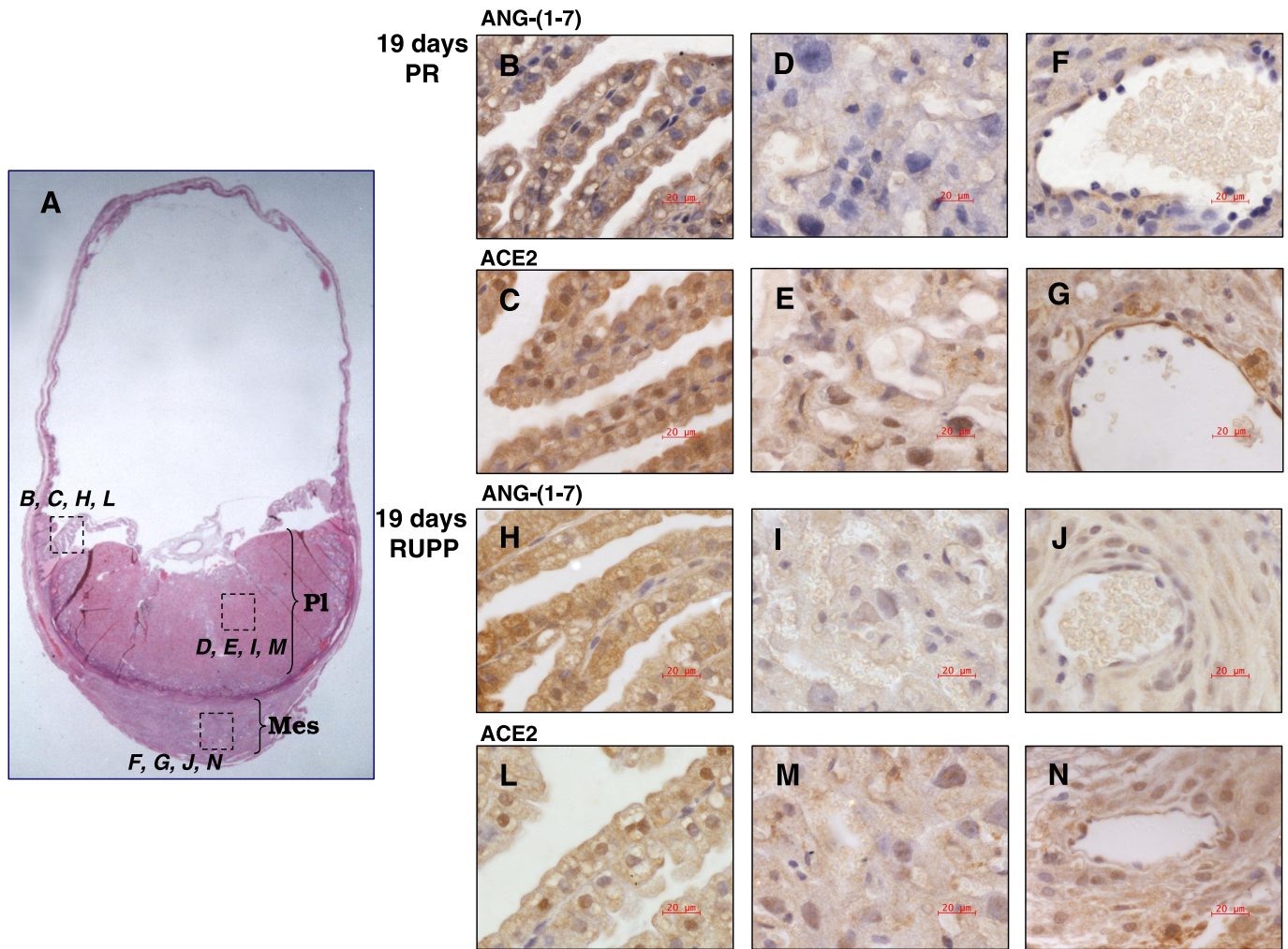

ACE2

7 days PR

ANG-(1-7)

ACE2

Fig. 3. Immunohistochemical identification of ANG-(1-7) and ACE2 in the yolk sac epithelium (*B, C, H,* and *L*), labyrinth region of the placenta (*D, E, I,* and *M*), and vessels of the mesometrial triangle (*F, G, J,* and *N*) of the uterus of 19-days-pregnant and -RUPP Sprague-Dawley rats. RUPP, reduced uterine perfusion pressure. *A*: photograph of the uteroplacental unit stained with hematoxylin and eosin; framed areas are shown at higher magnification on *B–N*. Pl, placenta; Mes, mesometrium triangle.

(Fig. 2, *C* and *F*) of the IS. Moderate ANG-(1-7) and ACE2 staining were present in the luminal and glandular epithelial cells of the IS (Fig. 2, *D* and *G*) and IIS (data not shown). At *day 7* of gestation faint-to-moderate ANG-(1-7) and ACE2 staining (0.8 ± 0.2 and 1.8 ± 0.2, respectively) were present at the secondary decidual zone (Fig. 2, *L* and *O*). ACE2 and ANG-(1-7) staining were also present on endoderm (Fig. 2, *L* and *O*). ACE2 staining was also present in ectoderm and ectoplacental cone (Fig. 2*O*); only very faint staining was observed for ANG-(1-7) in those areas. No ANG-(1-7) or ACE2 staining was visualized in the trophoblast giant cells. At *day 7*, moderate ANG-(1-7) and ACE2 staining were also observed in the luminal and glandular epithelial cells of the IIS

(Fig. 2, *M* and *P*). In addition, faint ANG-(1-7) and ACE2 staining were present at the myometrium of the IIS and IS at *days 5* and *7* (data not shown). Preabsorption of the ANG-(1-7) and ACE antibody with synthetic ANG-(1-7) or ACE2 peptide, respectively, yielded no staining (Fig. 2, *E, H, N,* and *Q*). Elimination of ANG-(1-7) and ACE2 primary antibody also resulted in absence of the staining (data not shown).

At *day 19* of pregnancy, faint ANG-(1-7) and ACE2 immunostaining was found in the labyrinth region of the placenta (Fig. 3, *D* and *E*, respectively); no staining was observed in the placenta spongiosa (data not shown). A cross section of the uterus, indicating the areas stained, is shown in Fig. 3*A*. ACE2 immunostaining was also found in the vessels and cells of the

mesometrial triangle (0.6 ± 0.2 and 0.9 ± 0.1, respectively) (Fig. 3G); however, very faint-to-absent staining was found for ANG-(1-7) in these regions (Fig. 3F). ANG-(1-7) and ACE2 immunostaining was also present in the endodermal epithelium of the inverted yolk sac (1 ± 0 and 2.8 ± 0.3, respectively; Fig. 3, B and C), and faint staining was present in the amnion membrane (data not shown). The same pattern of distribution was found in RUPP animals (Fig. 3, H–N). Faint ANG-(1-7) and ACE2 staining was visualized in the myometrium of virgin, pregnant, and RUPP rats. No staining for ANG-(1-7) was observed in the umbilical cord of pregnant and RUPP animals; however, ACE2 staining was found in the endothelium and smooth muscle cells of the umbilical cord (data not shown).

Figure 4 shows the concentration of ANG peptides in the uterus of virgin rats and in the IS and IIS of pregnant rats at days 5 and 7 of gestation. ANG I concentration in the IS at day 7 was decreased by 15% compared with the virgin uterus. ANG II concentration was decreased in both the IS and IIS at days 5 (21% and 40%, respectively) and 7 (55% and 30%, respectively) of gestation compared with virgin rats, whereas ANG-(1-7) did not change. The ratio of ANG II/ANG-(1-7) was significantly reduced by an average of 53% in all sites, whereas the ratio of ANG I/ANG II was significantly increased by an average of 43% in all sites compared with virgin rats. Temporal-spatial analysis of ANG concentration at early pregnancy is also shown in Fig. 4. Spatial comparison (IIS vs. IS) of ANG peptide content revealed a 32% increase in ANG II concentration in the IS at day 5 but a 26% decrease at day 7. ANG-(1-7) concentrations was significantly decreased by 31% at day 7 in the IS. The only temporal change (day 5 vs. 7) in peptide content was a decrease of 42% in ANG II at day 7 in the IS. Spatial comparison of the ratio of ANG II/ANG-(1-7) showed an increase in the IS vs. IIS at day 5 of gestation, whereas temporal comparison revealed a decrease in the IS at day 7 compared with day 5. The ANG I-to-ANG II ratio showed an increase in the IS at day 7 when compared with day 5.

Uterine and placental concentration of ANG peptides in virgin rats and 19-days-pregnant and -RUPP animals is shown in Fig. 5. ANG-(1-7) was the predominant peptide in the virgin uterus [ANG-(1-7)>ANG II>ANG I, $P < 0.05$]. In the pregnant uterus, a similar pattern was found, but ANG-(1-7) concentration was not significantly different from ANG II [ANG-(1-7) = ANG II>ANG I]. Uterine ANG I and ANG II concentrations were significantly increased by 30% and 25%, respectively, in pregnant animals compared with virgin female rats ($P < 0.05$, Fig. 5). In uteruses of RUPP animals there was a significant decrease in ANG-(1-7) concentration compared with pregnant rats (181 ± 16 vs. 372 ± 74 fmol/g of tissue, respectively $P < 0.05$); the concentration was also significantly lower than in uteruses of virgin animals (286 ± 22 fmol/g of tissue; Fig. 5). The ANG II-to-ANG-(1-7) ratio, used as an index of ACE2 activity, did not change in the uteruses of normal pregnant compared with virgin rats, but it increased significantly in the uterus of RUPP animals (Fig. 5A). On the other hand, the ANG I-to-ANG II ratio, used as an index of ACE activity, did not change in the uterus when comparing virgin and normal pregnancy, but decreased in the uterus of RUPP animals compared with the 19-day pregnant group (Fig. 5A). Placental ANG II was the predominate peptide ($P < 0.05$). ANG II and ANG-(1-7) concentrations were significantly reduced by 22% and 27% in RUPP animals (Fig. 5); however, there was no change in ANG II-to-ANG-(1-7) and ANG I-to-ANG II ratios (Fig. 5B).

At early pregnancy, both ACE2 and ACE mRNAs were significantly elevated compared with virgin rats, regardless of the day of gestation or site (one exception was the lack of change in ACE mRNA in the IS at day 7; Fig. 6). In all cases, the IS levels of ACE2 and ACE mRNAs were significantly lower than IIS, showing a 25% and 72% decrease in ACE2 mRNA and a 58% and 69% decrease in ACE mRNA at days 5 and 7, respectively. Temporal analysis (day 5 vs. 7) of ACE2 mRNA expression shows a 1.5-fold increase in ACE2 mRNA in the IIS at day 7 compared with day 5, but a 1.8-fold decrease
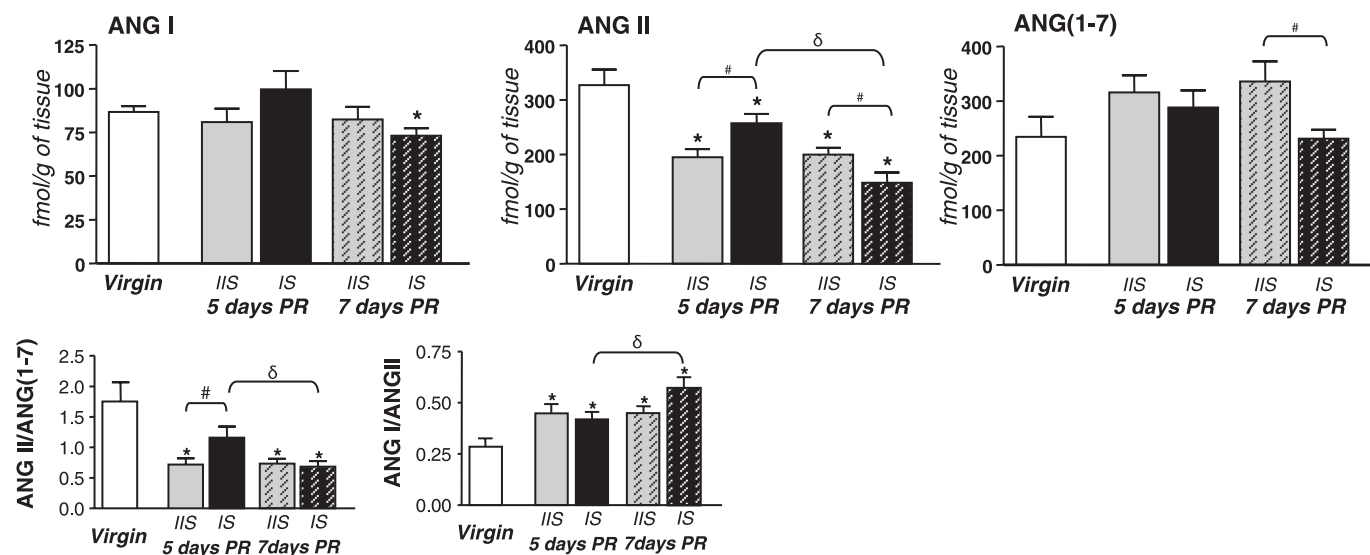

Fig. 4. ANG peptide concentration in the IS and IIS at days 5 and 7 of pregnancy and the uteruses of virgin female Sprague-Dawley rats at estrus. Values are expressed as means ± SE. Differences between the means were evaluated using 1-way ANOVA or Student's t-test. *$P < 0.05$ vs. virgin; #$P < 0.05$ vs. IIS; δ$P < 0.05$ vs. 5 days; n = 9–20.
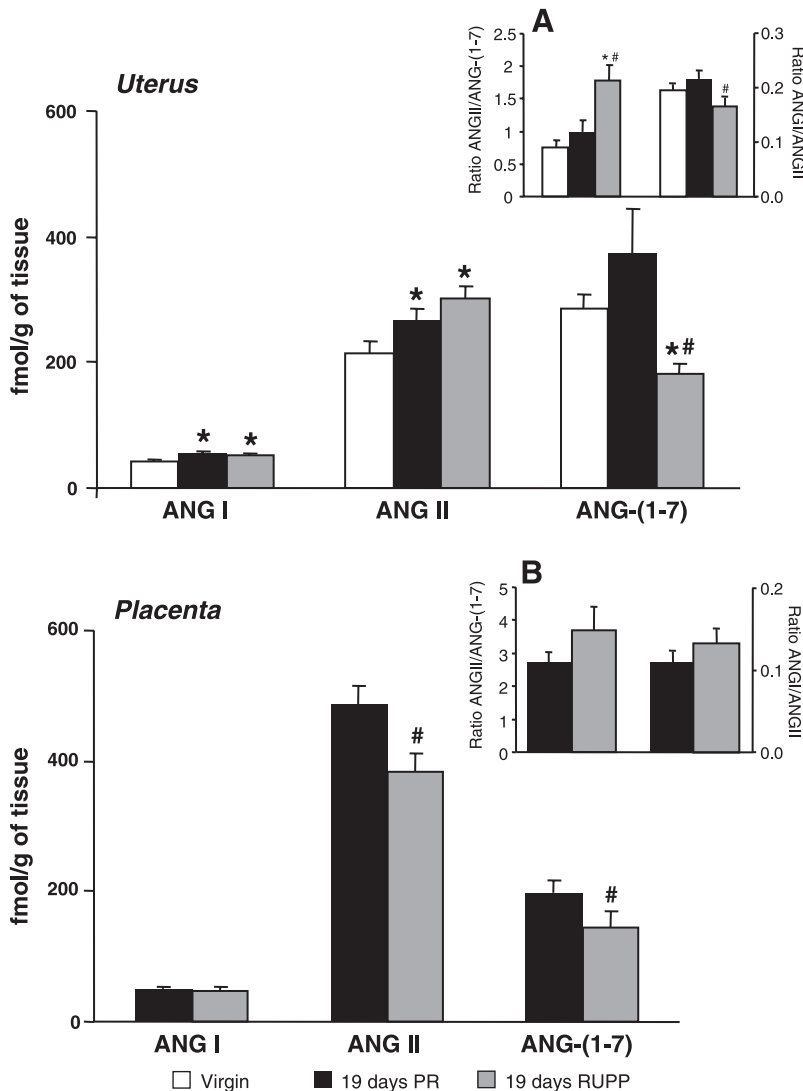
Fig. 5. Uterus and placenta ANG peptide concentrations in virgin rats at diestrus and 19-days-pregnant and -RUPP Sprague-Dawley rats. *A* and *B*: ANG II-to-ANG-(1-7) (values are on the *left y*-axis) and the ANG I-to-ANG II (values are on the *right y*-axis) ratios in the uterus and placenta, respectively. Values are expressed as means ± SE. Differences between the means were evaluated using 1-way ANOVA or Student's *t*-test. *$P < 0.05$ vs. virgin rats; #$P < 0.05$ vs. pregnant rats; $n = 8$–16.

in the IS at *day 7*. ACE mRNA in the IS was significantly less at *day 7*.

At *day 19* of pregnancy, ACE2 mRNA was elevated (58%, $P < 0.05$) in the uterus compared with virgin rats (Fig. 7), but ACE mRNA tended to decrease, not reaching statistical significance. Reduction in uterine perfusion pressure caused a decrease of 59% in ACE2 mRNA and 36% increase in ACE mRNA compared with normal pregnant uteruses ($P < 0.05$, Fig. 7). Placental ACE2 mRNA (Fig. 7) did not change in RUPP animals, but there was a significant decrease in ACE mRNA.

DISCUSSION

This is the first report to demonstrate the distribution and expression of ANG-(1-7) and ACE2 in the uterus during early and late gestation. At early gestation, ANG-(1-7) and ACE2 immunostaining was found in implantation and IISs (decidua, luminal, and glandular epithelium, embryo, and ectoplacental cone). During late pregnancy ANG-(1-7) and ACE2 staining was found on epithelial cells of the yolk sac and amnion. The same pattern of distribution was reported for other vasodilators, such as prostaglandins (15, 41) and bradykinin (5, 23, 31,

32, 39, 40). Several actions of ANG-(1-7) are mediated by prostaglandins, bradykinin, and nitric oxide (5, 23, 31). The similarity of the expression sites for these mediators is suggestive of a possible autocrine/paracrine interaction; ANG-(1-7) could directly or indirectly influence endometrial function and fluid transport through prostaglandins and bradykinin.

The presence of ANG-(1-7) and ACE2 in epithelial cells surrounding the implanting embryo suggests luminal secretion of the peptide. ANG-(1-7) and ACE2 staining was also found in the primary and secondary decidual zone. During this time point in pregnancy, angiogenic events take place in concert with antiangiogenic events (24), indicating that regulation of factors that permit a fine tuning of these events is important for the outcome of pregnancy. ANG-(1-7) could act by inhibiting the angiogenic and proliferative processes, as previous studies have demonstrated (21, 33). Machado et. al. (33) showed that ANG-(1-7) inhibits angiogenesis and fibrovascular tissue growth in a mouse sponge model of angiogenesis. Others also showed that ANG-(1-7) inhibits mitogen-stimulated growth of cultured rat thoracic aortic vascular smooth muscle cells (21).

The expression of ANG-(1-7) and ACE2 in decidual tissue surrounding the trophoblast cells is suggestive of a possible
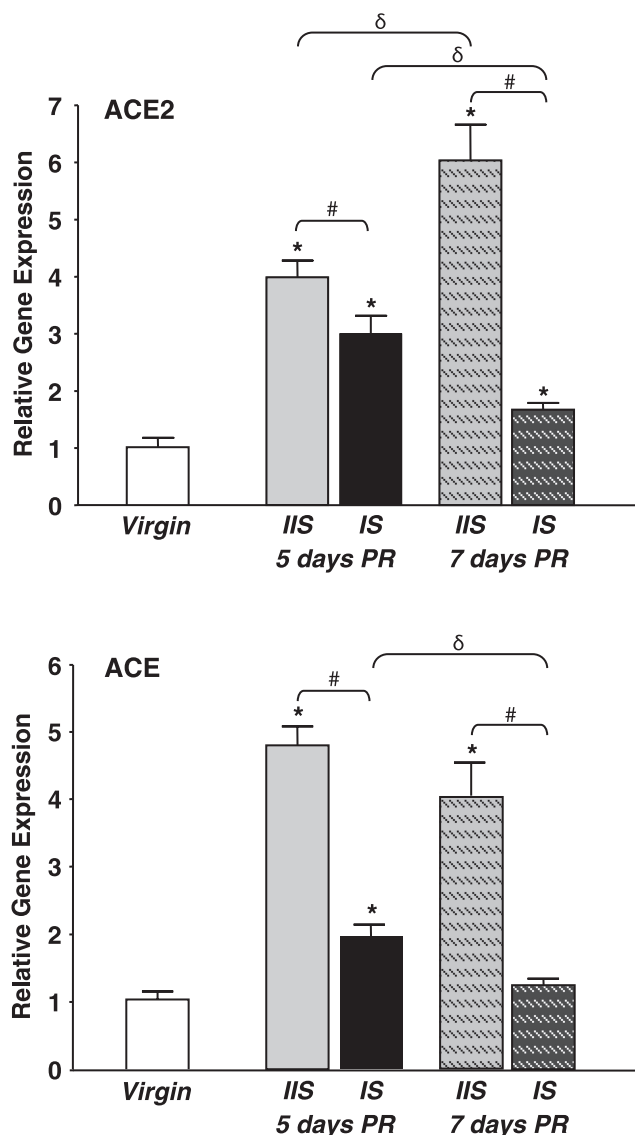
Fig. 6. ACE2 and ACE mRNA in the IS and IIS at *days 5* and *7* of pregnancy and uterus of virgin female Sprague-Dawley rat at estrus. ACE2 and ACE mRNA from IS and IIS at *days 5* and *7* are expressed relative to the virgin rat uterus. Differences between the means were evaluated using 1-way ANOVA or Student's *t*-test. *$P < 0.05$ vs. virgin rats; #$P < 0.05$ vs. IIS; δ$P < 0.05$ vs. 5 days; $n = 5$–9.

sion in the fetal placenta/labyrinth, but no in situ labeling of ACE2 on the maternal side of the rat placenta. Additional studies are necessary to provide an understanding of the functional role of ANG-(1-7) on the fetal side of the placenta. Reduction in uterine perfusion pressure did not change the pattern of ANG-(1-7) and ACE2 distribution in the uteroplacental unit. The absence of ANG-(1-7) immunostaining in some areas of ACE2 staining suggests the presence of other ACE2 substrates, such as ANG I, apelin, and dynorphin, within the uteroplacental unit. The mismatch of ANG-(1-7) and ACE2 staining in some regions with a broader distribution of ACE2 has been previously described in the kidney (7).

The presence of ANG-(1-7) and ACE2 in the endoderm and ectoplacental cone is also in accordance with distribution at *day 19* of pregnancy where we found positive staining for ANG-(1-7) and ACE2 in the yolk sac membranes and labyrinth placenta (36). The endoderm cells are active in absorption, digestion, and transport of material from the yolk sac cavity, and around *day 7* of gestation the visceral endoderm becomes vascularized (16). By *day 7*, fetal blood vessels also begin to develop within the ectoplacental cone (16).

To our knowledge, this is the first study to report the ANG peptide profile in the uterus during early and late gestation in normal pregnant rats and in hypertensive pregnancy. Early pregnancy was associated with a decrease in uterine ANG II levels, and no change in ANG-(1-7) as compare to virgin rats at the estrus phase of estrous cycle. The increase in ACE2 mRNA in uterine IS and IIS and the associated decrease in the ANG II-to-ANG-(1-7) ratio are consistent with an action of ACE2 resulting in the degradation of ANG II. However, one would have anticipated that there would be an associated increase in ANG-(1-7) under these circumstances. The lack of change in uterine ANG-(1-7) indicates that there must be additional enzymes that may be degrading ANG-(1-7). The increase of ACE expression is consistent with its acting to degrade ANG-(1-7); however, the increase in ACE does not explain the decrease in ANG II. The data are consistent with ACE2 being more significant than ACE in predicting the levels of ANG II in the uterus at early gestation. The data also show that the ratio of ANG II/ANG-(1-7) may not always reflect ACE2 activity, especially if ACE is regulated independently of ACE2, which may cause the ratio to change in an ACE2-independent manner.

The RAS expression is spatially and temporally regulated during implantation and decidualization. We showed an increase in ANG II levels at the site of implantation at *day 5* of pregnancy, which is consistent with ANG II playing a role in the decidualization process. Previous studies demonstrated that ANG II increases uterine decidualization, endometrial vascular permeability, and stimulates angiogenesis and growth during implantation and placentation (17, 28, 29, 48). At *day 5* of gestation, the ANG-(1-7) levels were unchanged in the IS, but the ratio of ANG II/ANG-(1-7) in the IS was elevated indicating a change in the balance favoring ANG II actions. *Day 7* of gestation was marked by decreases in both ANG-(1-7) (31%) and ANG II (26%) levels at the IS. The decrease in ANG-(1-7) is consistent with the striking decrease in ACE2 mRNA observed by *day 7* of gestation; and the decrease in ANG II is consistent with the marked decrease in ACE mRNA. These findings suggest that dampening of ANG II's and ANG-(1-7)'s actions in the IS is required for a successful pregnancy.

paracrine action of ANG-(1-7). ANG-(1-7) and ACE2 staining were absent in the trophoblast cells, which agrees with the patterns of distribution of ANG-(1-7) and ACE2 in the placenta at *day 19* of pregnancy. At late pregnancy, prominent ANG-(1-7) and ACE2 staining was found in the labyrinth region of the placenta consistent with our previous study in humans (50) where we found specific staining for ANG-(1-7) and ACE2 in cytotrophoblasts, syncyciotrophoblasts, and the endothelium of the blood vessels of the primary and secondary villi, regions similar to the labyrinth placenta in rodents. However, in human placenta, we also reported ACE2 and ANG-(1-7) expression in the maternal stroma, invading and intravascular trophoblasts, and the decidual cells. In the rat placenta, no staining was found in the trophospongium region, confirming the findings of Riviere et al. (43) who observed a high level of ACE2 expres-
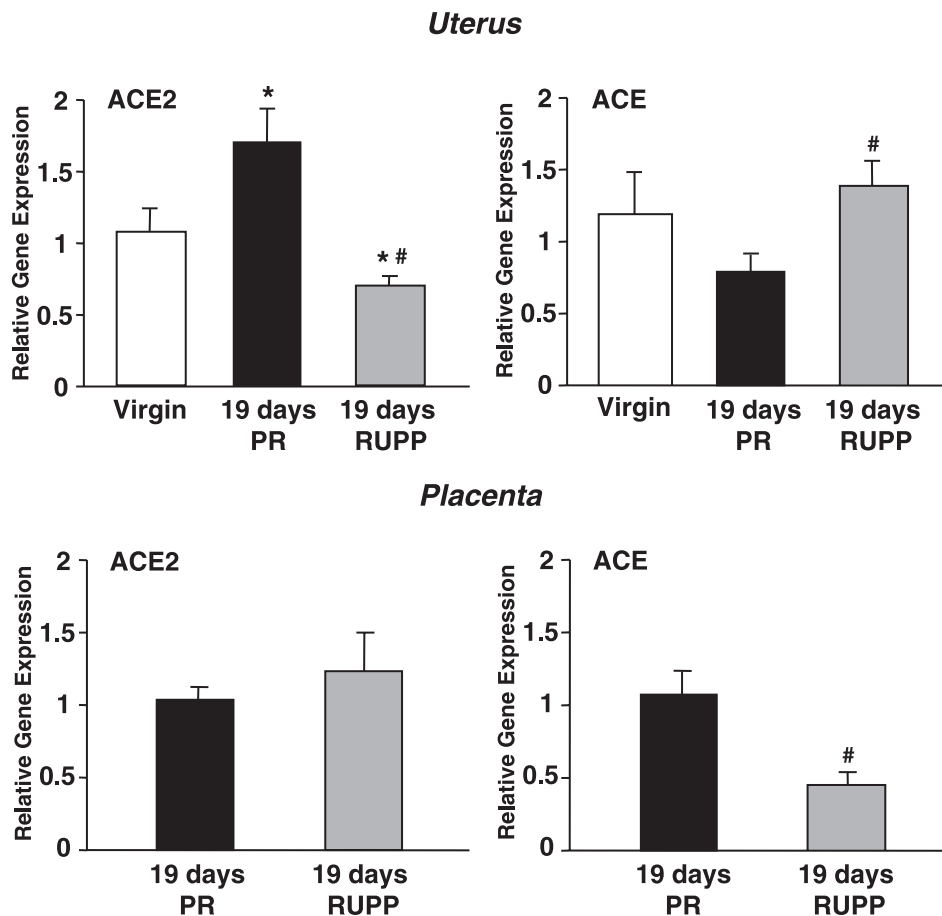
Uterus

Placenta

Fig. 7. Uterine and placental ACE2 and ACE mRNA in virgin rats at diestrus and 19-days-pregnant and -RUPP Sprague-Dawley rats. ACE2 and ACE mRNA from pregnant and RUPP uteruses is expressed relative to the virgin control. ACE2 and ACE mRNA from RUPP placenta is expressed relative to the pregnant control. Values are means ± SE. Differences between the means were evaluated using 1-way ANOVA or Student's t-test. $*P < 0.05$ vs. virgin rats; $\#P < 0.05$ vs. pregnant rats; $n = 7$–8.

The temporal-spatial differences in ANG II/ANG-(1-7) and ACE2/ACE mRNAs between the implantation and IISs suggest that the implanting blastocyst regulates local ANG levels and ACE2/ACE expression. However, the influence of local steroid hormonal environment needs to be taken into consideration, although other factors must be also involved, because the IISs, which are exposed to the same hormonal milieu as the ISs, have a different expression of ANG peptides and ACE2 and ACE gene regulation. These studies for the first time describe that within the pregnant uterus of early gestation there is upregulation of ACE2 and ACE gene expression that is temporally and spatially downregulated within the ISs. The dynamic regulation of the ANG peptides at early gestation could reflect an effect of estrogen and/or progesterone. Studies using mice lacking estrogen receptor-α have emphasized that implantation requires the action of estrogen, while progesterone is essential for both implantation and decidualization (14, 27). Previously, we showed that estrogen increases ANG I and ANG-(1-7) and decreases ANG II in the circulation (8). In the ovary, higher estrogen levels found during the estrous cycle increased ANG-(1-7) but did not change ANG II (12). Estrogen is known to downregulate ACE mRNA (22); thus its increase compared with values in virgin rats is consistent with the reduced serum estradiol levels. The role of progesterone has resulted in conflicting studies, but, in general, progesterone was shown to increase renin and ACE (44, 46) and not to change ANG I and ANG II concentration (10, 38). There is no report regarding the effect of progesterone on ACE2 and/or

ANG-(1-7) levels. Most likely, the regulation of the RAS during the implantation and decidualization period reflects a combined influence of steroid hormones, the embryo-uterine interactions, and other regulatory factors.

Our findings of an increase in the uterine concentration of ANG I and ANG II and a tendency to increase ANG-(1-7) at *day 19* of pregnancy agrees with previous reports showing an activation of the uterine RAS during late gestation. We demonstrated previously that pregnancy is associated with an increased renal and urinary concentration of ANG I, ANG II, and ANG-(1-7) in Sprague-Dawley rats. Elevated plasma and urinary levels of ANG I, ANG II, and ANG-(1-7) have also been reported in pregnant women at the third trimester (34, 49). On the other hand, we showed previously that plasma levels of ANGs did not change in rats at *day 19* of pregnancy compared with virgin rats (37). These findings are suggestive of tissue-specific regulation of the RAS during late pregnancy in rats.

The present study showed that in the RUPP model of hypertensive pregnancy there is a marked decrease in the uterine and placental concentration of ANG-(1-7). ANG II levels were also significantly decreased in the placenta of RUPP animals but remained elevated in the uterus compared with virgin rats. Studies done by Kalenga et al. (26) reported no change in ANG II levels in placenta of full-term, preeclamptic subjects; however, recent studies from our laboratory showed elevated levels of ANG II in the placenta of preeclamptic chorionic villi (3). Our previous studies showed that ANG-

(1-7) immunostaining remained unchanged in the placenta of preeclamptic compared with normal pregnant subjects. Species difference could account for the discrepancy found between the present study and previous ones in humans. Another consideration is that the RUPP model, although it has many features that closely resemble preeclampsia in women (1), may not completely mimic preeclampsia in its entirety.

Our results strongly suggest that ACE2 is involved in the processing of ANG-(1-7) in the uteroplacental unit of early and late pregnancy in normal and hypertensive pregnancy. The earliest report of ACE2 expression in the embryo was that of Crackower et al. (13) who found ACE2 mRNA at *day 15* of embryonic development with no expression at *day 7*. However, no detailed information was provided regarding how embryonic tissue was obtained at *day 7* of gestation, nor was there information whether this included the whole IS (uterus and embryo tissue) or embryo alone. Our study clearly demonstrates the presence of ACE2 and ANG-(1-7) using immunohistochemistry in the implanting blastocyst. Although the function of ACE2 and ANG-(1-7) in implantation remains to be determined, Crackower et al. (13) did suggest that ACE2 did not appear to be necessary for establishment and maintenance of pregnancy, since female ACE2 knockout mice were fertile. However, no detailed examination of the reproductive function in ACE2-deficient animals was provided; moreover, based on the findings of the present study, we would anticipate that overexpression of ACE2 would impair decidualization and placentation since ANG-(1-7) and ACE2 mRNA were markedly decreased at *day 7* of pregnancy in the ISs. Studies showed that ACE2 catalyzes the formation of ANG-(1-7) from ANG II, indicating that ACE2 may be an important regulator of the balance of ANG II/ANG-(1-7). The marked uterine increase in the ANG II-to-ANG-(1-7) ratio in the 19-day RUPP animals (Fig. 5*A*) reflects the limited conversion of ANG II to ANG-(1-7), due to the decreased uterine ACE2 in RUPP animals. In RUPP uterus, ACE mRNA upregulation compared with that of normal pregnant rats is consistent with both the increase in ANG II and the reduction in the ANG I-to-ANG II ratio. The downregulation of ACE2 and the upregulation of ACE mRNA in the RUPP are consistent with the counterregulatory regulation of ACE and ACE2, contributing to the balance of ANG II and ANG-(1-7).

Although in the present study ANG-(1-7) levels were decreased in the placenta of the RUPP, no change in ACE2 mRNA was found. The decreased ANG-(1-7) levels could result from the decreased formation of ANG II due to reduced placental ACE expression. Alternately, a finding of no change in ACE2 mRNA does not eliminate the possibility that there could be translational or posttranslational mechanisms that would result in decreased ACE2 activity or protein without a change in ACE2 message levels. The finding of no change in placenta ACE2 mRNA agrees with our previous study that showed no change in ACE2 immunoreactivity in human placenta of preeclamptic pregnancy. These results suggest that ACE2 may regulate ANG peptide balance differentially within the uteroplacental unit, although the participation of other enzymes important to ANG-(1-7) formation, such as neprilysin and prolyendopeptidase, should also be taken into consideration.

Perspectives and Significance

In the present study, we demonstrated that ANG-(1-7) and ACE2 are regulated temporally and spatially in the developing rat uteroembryonic unit during early gestation, suggesting that ANG-(1-7) may be a local mediator of embryo-uterine interactions during implantation and decidualization. The present data also support the concept that successful implantation and decidualization are obtained by blunting ANG-(1-7) effects in a paracrine and autocrine mode. On the other hand, normal late gestation is associated with an enhanced expression of the RAS as demonstrated by increased levels of ANG I and ANG II and a tendency to increase ANG-(1-7) in the uterus, whereas hypertensive pregnancy is associated with a marked decrease in uterine ANG-(1-7) concentration in the presence of sustained elevated ANG II. To our knowledge, the physiological role of ANG-(1-7) in the uterus and placenta during early and late gestation has not been established. In the uterus and placenta at late gestation, the location and increased presence of ANG-(1-7) support the hypothesis that this peptide and its forming enzyme ACE2 may participate in the maintenance of uterine and placenta blood flow. However, studies are still warranted to evaluate its contribution to the regulation of cell proliferation, angiogenesis, fetal nutrition, and trophoblast invasion at the different stages of pregnancy.

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