Uterine artery dysfunction in pregnant ACE2 knockout mice is associated with placental hypoxia and reduced umbilical blood flow velocity


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INTRAUTERINE GROWTH RESTRICTION (IUGR) is a leading cause of perinatal morbidity and mortality in humans (31). Maternal health factors such as chronic hypertension, preeclampsia, diabetes mellitus, chronic renal disease, poor nutrition, and smoking (57) and fetal genetic abnormalities increase the risk of IUGR. The uteroplacental vasculature undergoes tremendous adaptations during pregnancy, including vasculo/angiogenesis and remodeling of spiral arteries (44). These changes allow the expansion of blood volume in the uteroplacental unit and sufficient blood flow from the placenta to the fetus (44). Fetal metabolic demands progressively increase in the second half of pregnancy (37). Normal fetal growth is vastly dependent on adequate placental development, whereas increased vascular resistance and hypoxia are associated with reduced fetal weight (10a). However, the molecular basis of uteroplacental dysfunction in IUGR is not well understood.

The components of the renin-angiotensin system (RAS) are important mediators of uteroplacental vasculature. Angiotensin-converting enzyme 2 (ACE2) is a key enzyme of the RAS that degrades angiotensin II (Ang II) and forms Ang-(1–7). ACE2 plays a key regulatory role in the control of arterial pressure and vascular function (24) by balancing the vasoconstrictor Ang II/Ang II receptor 1 (AT1R) and vasodilator Ang-(1–7)/MAS (AT1–7R) arms of the renin-angiotensin system. ACE2 expression in human placenta was characterized by Valdes et al. (56) in the invading and intravascular trophoblasts, decidual cells, and umbilical cord, suggesting a paracrine influence of this enzyme in the uteroplacental unit. Moreover, human umbilical vein endothelial cells (HUVECs) isolated from pregnancies with small for gestational age (SGA) newborns had significantly lower levels of ACE2 mRNA compared with pregnancies associated with average for gestational age (AGA) newborns (48), suggesting that lower ACE2 levels in HUVECs are associated with UGR.

Several studies have investigated ACE2 expression in the uteroplacental unit of pregnant animals. ACE2 levels were higher in the uterus of normotensive pregnant rats compared with normotensive nonpregnant rats (39). ACE2 levels were lower in hypertensive pregnant rats that had reduced uterine perfusion pressure (RUPP) surgery compared with normotensive pregnant rats (39). RUPP surgery induces the reduction in uterine perfusion with subsequent placental ischemia by placing clips around the descending aorta and around both main uterine arteries (1, 39). It has been also shown that placenta generate threefold more ACE2 mRNA than gravid uterus or the kidneys, suggesting that the placenta is a significant contributor to the generation of ACE2 levels (systemic or uteroplacental) seen during rat pregnancy (27). In agreement with the hypothesis that ACE2 deficiency is associated with IUGR, a recent study from our group demonstrated that ACE2 knockout (KO) mice developed maternal and fetal growth restriction at late pregnancy (3). These changes were accompanied by increased levels of placental Ang II and decreased circulating Ang-(1–7), a result of ACE2 knockout and reduced degradation of Ang II (3). Mean blood pressure was elevated in virgin ACE2 KO...
mice and did not change with pregnancy (3). Since cardiac output was similarly increased in ACE2 KO and C57Bl/6 mice (3), the absence of blood pressure response in ACE2 KO mice during pregnancy implies a higher peripheral resistance. These findings suggest that the dysregulated RAS has a pathogenic role in the development of IUGR (3). Moreover, a recent study by Ibrahim et al. (21) showed that ACE2 activator, xanthone, decreased blood pressure in hypertensive pregnant rats, suggesting that ACE2 activation is protective against hypertension during pregnancy.

In male animals, ACE2 knockout increases endothelial dysfunction and the expression of inflammatory mediators in the aorta (55). ACE2 knockout also exacerbates Ang II-mediated aortic remodeling in mice due to increased oxidative stress and vascular smooth muscle cell apoptosis (46). On the other hand, ACE2 overexpression in hypertensive rats improves endothelial function (52, 53), suggesting a protective effect of ACE2 in vasculature.

Disturbances in the uteroplacental circulation have been implicated in the development of IUGR; however, the role of ACE2 in the regulation of the uteroplacental vasculature is unknown. The use of a pregnant ACE2 KO mouse model allowed us to test the hypothesis that ACE2 knockout induces uteroplacental dysfunction as early as day 14 of gestation before major growth of the fetus occurs (10a).

MATERIALS AND METHODS

Animals. The study was approved by the Institutional Animal Care and Use Committee of the Wake Forest School of Medicine (WFSM). ACE2 KO mice developed on a C57Bl/6 background were received from Susan B. Gurley MD and Thomas Coffman MD of Duke University; the colony was established at the Hypertension and Vascular Research Center at WFSM. As previously described (3), C57Bl/6 mice, purchased from Harlan Laboratories, were used in control experiments. Standard rodent Chow (Lab Diet 5000 - Prolab RMH 3000; PMI Nutrition International, Brentwood, MO) and water were available ad libitum throughout the experimental protocols.

Timetable for the experiments. Homozygous female ACE2 KO mice between 12 and 18 wk of age were mated with male ACE2 KO mice. Day 0 of pregnancy was established by the presence of a vaginal plug or sperm in the vaginal smear. Mice received ultrasound scans at day 13 of gestation. In the evening of the same day, animals were placed in metabolic cages for 24-h urine collection (MMC 100; Hatteras Instruments, Cary, NC). Blood pressures were taken in the morning of the following day, and the animals were euthanized in the afternoon of day 14 of gestation (0.7 term) by decapitation. In addition, on the day of euthanasia, some animals were injected with hypoxyprobe-1 to assess placental hypoxia and were euthanized 1 h later according to the manufacturer’s instructions (25, 44). The main uterine artery was dissected and immediately placed in oxygenated ice-cold Krebs buffer for assessment of vascular reactivity. A 5-mm segment of the uterine artery and one uteroplacental unit per mouse were fixed in 10% formalin for 24 h followed by 70% ethanol. Maternal tibia length was determined in each animal. To account for differences in maternal body weights between animals within each study group, maternal and fetal characteristics were standardized to maternal tibia length (8, 65). All of the animals had blood pressure and ultrasound scans recorded. Additional animals were added for vascular experiments.

Blood pressure recordings. Systolic blood pressures (SBP) were recorded in trained conscious mice under restraint using an automated tail cuff system (SC-100, Hatteras Instruments) as described (28). We have experience with tail cuff blood pressure recordings, and our previous studies showed consistent and reproducible results (28, 62, 63). Metal holders specifically designed to restrain animals with minimal stress during blood pressure recordings were provided by Hatteras Instruments. Occluding (8 mm) and sensor (8 mm) cuffs were used for the measurements of SBP. Mice were acclimated to the system and to the warming-box exposure for 7 days before the actual recordings were performed. On the day of the experiment, mice were placed in warming boxes for 15 min prior to blood pressure recordings. SBPs were measured 10 times in each mouse; the total time for blood pressure recording was no more than 20 min. The data for each animal were averaged and reported as the mean ± SE for each experimental group.

Ultrasound of uterine and umbilical arteries. Mice were anesthetized with 1.5% isoflurane and placed on a heated platform for ultrasound imaging. Heart rate was determined using the ECG electrodes connected to the platform. All hair on the abdomen was removed by application of a shaving gel. Images were obtained using an MS550S transducer and Vevo 2100 ultrasound system (Visual Sonics, Toronto, ON, Canada) (10a). The angle between the ultrasound probe and the direction of the flow was kept at less than 50 degrees (33 degrees on average) during the recordings of the velocity waveforms. The velocities of the main uterine arteries were recorded below the bladder and at the level where the main uterine artery branches from the internal iliac artery. Umbilical arteries were visualized in the longitudinal plane of the fetoplacental unit using a two-dimensional mode. Color Doppler and pulsed wave (PW) Doppler modes were applied to establish a typical uterine or umbilical artery wave and to record peak systolic (Vmax) and minimum diastolic (Vmin) velocities. The resistance index was determined as (Vmax - Vmin)/Vmax (10a). The results were obtained in the arteries from two or three fetoplacental units in each animal and averaged.

Vascular reactivity. Uterine artery segments (maximum length 2 mm) were mounted between an isometric force transducer (Kistler Morce DSC 6, Seattle, WA) and a displacement device on a myograph (Multi Myograph, model 620M; Danish Myo Technologies, Aarhus, Denmark) using two stainless steel wires (diameter 25 µm), as previously described (29, 50). All isolated uterine arteries were first tested for stress-stretch responses during the normalization procedure, followed by the maximum responses to potassium chloride, phenylephrine, and acetylcholine. Then the contractile responses of uterine artery segments to Ang II were examined, Ang II response was also tested after preincubation with Afalls (1–7).

Experimental protocol. The myograph organ bath (5 ml) was filled with Krebs buffer maintained at 37°C and aerated with 95% O2-5% CO2. The vessels were washed and incubated for 30 min before the normalization procedure was performed. Each arterial segment was stretched in a stepwise manner. The internal circumference and corresponding wall tension at each stretch were calculated and plotted to produce a resting wall tension-internal circumference curve for that artery, using the DMT Normalization Module (ADInstruments). Arterial segments were normalized to 0.9·L100, where L100 is the internal circumference of the vessels at a transmural pressure of 100 mmHg (38). Optimal diameters (OD) were calculated as OD = 0.9·L100/π. After obtaining the OD, a 30-min equilibration period preceded the addition of test substances. Stress-stretch responses were obtained during the normalization procedure and were plotted and the data were adjusted to the exponential equation Y = Y0*exp(k*X), where k is the rate constant. Values of k were analyzed for each artery and then compared between groups. Our previous studies showed that the contractile response to KCl in the uterine artery attained maximal values at 75 mM (49). Therefore, after equilibration, uterine arteries were exposed to 75 mM KCl for 5 min and then washed with Krebs buffer. The procedure was repeated three times, and the last measurement was considered the maximal response to K+ ($K_{MAX}$). After washing and resting, uterine artery segments were exposed to a cumulative concentration-response curve of phenylephrine (PE) by exposing arteries to six (10^{-10} to 10^{-5} M) increasing concentrations in one-log steps, with each subsequent dose being introduced only after a steady response had been reached. The responses to PE were
expressed as the percentage of maximal contraction induced by KCl. Arteries were then washed again, and vasodilation to acetylcholine was tested in the uterine arteries preconstricted with a submaximal dose of PE (10⁻⁶ to 10⁻⁵ M). After attaining an equivalent level of contraction, a concentration response curve to acetylcholine (10⁻¹⁰ to 10⁻⁴ M) was calculated. Uterine artery segments were washed and, under resting tension, were exposed to a cumulative concentration-response curve of Ang II by exposing arteries to 10⁻¹⁰ to 10⁻⁷ M increasing concentrations in one-fourth log steps, with each subsequent dose being introduced after a steady response had been reached (every 2 min). The response to Ang II was expressed as the percent (%) of maximal contraction induced by KCl. Ang II response was also tested after preincubation with Ang-(1–7) (10⁶ M) alone or in the presence of MAS (AT1–7R) antagonist (10⁵ M) in some vessels.

**Immunohistochemistry.** After fixation in formalin and ethanol, one uterine artery segment and one placental section were embedded in separate paraffin blocks, and cut into 5-μm sections. Immunostaining was performed using the avidin biotin complex (ABC) method as described (62). Staining for all proteins required antigen retrieval treatment with sodium citrate buffer (pH 6.0) at 90–95°C for 30 min. Uterine arteries were incubated with the following primary antibodies: rabbit polyclonal affinity purified endothelial nitric oxide synthase, anti-eNOS (dilution: 1:100; BD Biosciences, San Diego, CA; cat. no. 610297), anti-AT1R (dilution: 1:400; Santa Cruz, cat. no. sc-1173), anti-AT2R (dilution: 1:400; Abcam, cat. no. ab19134), anti-MAS/ Ang-(1–7)R (dilution: 1:400, Alomone Labs, cat. no. AAR-013), monoclonal anti-α-smooth muscle (2.5 μg/ml; Sigma-Aldrich, St. Louis, MO; cat. no. A5228), monoclonal anti-skeletal myosin (dilution: 1:800; Sigma-Aldrich; cat. no. M4276), and secondary biotinylated goat anti-rabbit or anti-mouse antibody (dilution: 1:400; Vector Laboratories, Burlingame, CA). Uteroplacental unit sections were stained with anti-cytokeratin KRT7 rabbit polyclonal antibody (dilution: 1:400, Abcam, cat. no. ab19134), anti-endothelial nitric oxide synthase rabbit polyclonal antibody (dilution: 1:400, Santa Cruz, cat. no. sc-1173), anti-α-smooth muscle (2.5 μg/ml; Sigma-Aldrich, St. Louis, MO; cat. no. A5228), monoclonal anti-skeletal myosin (dilution: 1:800; Sigma-Aldrich; cat. no. M4276), and secondary biotinylated goat anti-rabbit or anti-mouse antibody (dilution: 1:400; Vector Laboratories, Burlingame, CA). One placenta

Table 1. Physiological characteristics of ACE2 KO and C57Bl/6 mice at day 14 of gestation

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>C57Bl/6 (n = 5)</th>
<th>ACE2 KO (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother’s BW/g/tibia length (cm)</td>
<td>17.90 ± 0.20</td>
<td>16.42 ± 0.37*</td>
</tr>
<tr>
<td>(Mother’s BW–total pup BW), g/tibia length (cm)</td>
<td>16.63 ± 0.30</td>
<td>15.30 ± 0.38*</td>
</tr>
<tr>
<td>Pup BW (g)/tibia length (cm)</td>
<td>0.15 ± 0.01</td>
<td>0.11 ± 0.01*</td>
</tr>
<tr>
<td>Pup BW, unadjusted (g)</td>
<td>0.21 ± 0.01</td>
<td>0.17 ± 0.01*</td>
</tr>
<tr>
<td>Pup number</td>
<td>8.70 ± 0.35</td>
<td>8.0 ± 0.28</td>
</tr>
<tr>
<td>Placental wt (g)/tibia length (cm)</td>
<td>0.05 ± 0.002</td>
<td>0.05 ± 0.001</td>
</tr>
<tr>
<td>Placental wt, unadjusted (g)</td>
<td>0.07 ± 0.003</td>
<td>0.08 ± 0.003*</td>
</tr>
<tr>
<td>Pup BW/placental weight ratio (per tibia length)</td>
<td>3.15 ± 0.28</td>
<td>2.44 ± 0.11*</td>
</tr>
<tr>
<td>Maternal Food Intake (g)</td>
<td>3.40 ± 0.24</td>
<td>4.0 ± 0.21</td>
</tr>
<tr>
<td>Maternal serum creatinine (mg/dl)</td>
<td>0.24 ± 0.01</td>
<td>0.17 ± 0.01*</td>
</tr>
<tr>
<td>Maternal heart rate (beats/min)</td>
<td>361.20 ± 28.9</td>
<td>349.20 ± 30.20</td>
</tr>
<tr>
<td>Mean kidney wt (g)/tibia length (cm)</td>
<td>0.12 ± 0.005</td>
<td>0.12 ± 0.004</td>
</tr>
<tr>
<td>Heart wt (g)/tibia length (cm)</td>
<td>85.10 ± 1.90</td>
<td>102.30 ± 5.10*</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>130.9 ± 1.90</td>
<td>143.3 ± 5.10*</td>
</tr>
<tr>
<td>Maternal Food Intake (g)</td>
<td>1.80 ± 0.01</td>
<td>1.70 ± 0.01*</td>
</tr>
<tr>
<td>Maternal serum creatinine (mg/dl)</td>
<td>0.29 ± 0.02</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>Maternal Food Intake (g)</td>
<td>0.003 ± 0.003</td>
<td>0.004 ± 0.003*</td>
</tr>
<tr>
<td>Pup number</td>
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</tbody>
</table>

Data are means ± SE. ACE2 KO, angiotensin-converting enzyme 2 knockout mice; BW, body weight. *P < 0.05 vs. C57Bl/6 mice.
and one uterine artery per stain were examined under a light microscope and photographed at \( \times 50 \) or \( \times 200 \) magnifications with a QImaging Retiga 1300R CCD digital camera and SimplePCI (version 6) software: 6 fields per uterine artery section and 12 sections per one placenta (4 sections per each: labyrinth, junctional zone, and mesometrium). Images were analyzed using Adobe Photoshop 7.0 by the investigator blinded to the experimental groups. Staining for eNOS, actin, or myosin was normalized to the intensity of the background and to the maximal value of the red, green, and blue intensity (RGB) component and reported as relative intensity units (41, 62). The following stains were performed to assess morphological features of uterine arteries: periodic acid Schiff stain (PAS, Sigma-Aldrich; cat. no. 365-544-8) to assess connective tissue components (collagen and muscle cells). The area of cells stained by PAS and Picrosirius red stain (Sigma-Aldrich; assay components cat. no. 197378-100G; P6744-16A; 36-554-8) to assess fibrinoid content, Verhoeff’s stain (American Registry of Pathology) to detect fibrinoid content, and the extent of decidualization. Decidual and mesometrial areas of the placenta (4 sections per each: labyrinth, junctional zone, and mesometrium). The decidual and mesometrial areas of the placenta were evaluated for the depth of extravillous (EVC) and arterial trophoblasts (ATB) and the extent of decidualization. Decidual and mesometrial areas of the placenta were evaluated for the depth of extravillous (EVC) and arterial trophoblasts (ATB).

### Table 2. Vascular reactivity in C57Bl/6 and ACE2 KO mice at day 14 of gestation

<table>
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<tr>
<th>Characteristics</th>
<th>C57Bl/6</th>
<th>ACE2 KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_{MAX} )</td>
<td>1.13 ± 0.12</td>
<td>1.90 ± 0.15*</td>
</tr>
<tr>
<td>PheMAX (% ( K_{MAX} ))</td>
<td>139.0 ± 7.0</td>
<td>169.0 ± 9.0 *</td>
</tr>
<tr>
<td>( pD2 ) (−log EC50)</td>
<td>6.76 ± 0.47</td>
<td>6.48 ± 0.13</td>
</tr>
<tr>
<td>ACh MAX</td>
<td>73 ± 7</td>
<td>76 ± 7</td>
</tr>
<tr>
<td>( pD2 ) (−log EC50)</td>
<td>6.43 ± 0.44</td>
<td>6.19 ± 0.57</td>
</tr>
<tr>
<td>Ang II MAX (% ( K_{MAX} ))</td>
<td>91.0 ± 5.0</td>
<td>93.0 ± 13.0</td>
</tr>
<tr>
<td>( pD2 ) (−log EC50)</td>
<td>8.5 ± 0.03</td>
<td>8.64 ± 0.04*</td>
</tr>
</tbody>
</table>

Data are means ± SE. \( K_{MAX} \), maximal response to potassium chloride (K+); PheMAX, maximal response to phenylephrine; ACh, acetylcholine; \( pD2 \), negative logarithm of the molar concentration producing the half-maximum response (EC50).

Trophoblast invasion. Decidual and mesometrial areas of the placenta of ACE2 KO and C57Bl/6 mice were examined for the depth of the trophoblast invasion and for the presence of trophoblast cells in the arterial walls (47). Trophoblast cells were identified by positive staining for cytokeratin KRT7, as described above. The distribution of extravillous (EVC) and arterial trophoblasts (ATB) and the extent of their invasion into mesometrium were analyzed using semiquantitative analysis as described by Pijnenborg et al. (47) as follows: 1 = high numbers evenly spread; 2 = high numbers focally spread; 3 = low numbers evenly spread; 4 = low numbers focally spread.

Assessments of placental hypoxia. 1) Hypoxyprobe-1 was injected intraperitoneally in the ACE2 KO and C57Bl/6 mice at day 14 of gestation (60 mg/kg maternal body wt). One hour later, the mother was euthanized, and placentas were fixed in 10% formalin for 24 h followed by 70% ethanol. After the fixation, placentas were embedded in paraffin, cut into 5-μm sections, and immunostained for hypoxyprobe-1 following the manufacturer’s protocol (25, 44). Hypoxic areas were identified in sections of the placenta using a Hypoxyprobe-1TM kit (Chemicon, Temecula, CA; cat. no. HP1-100kit) that contains pimonidazole hydrochloride and a primary antibody for the detection of pimonidazole. Pimonidazole binds to thiol-containing proteins in hypoxic cells; its level in the cells has been correlated with oxygen electrode measurements (51). 2) HIF-2α is a transcription factor that induces the expression of hypoxia-regulated genes. Levels of HIF-2α are increased under hypoxic conditions (11, 19). In addition, HIF-2α protein, but not HIF-1α, was induced in cytotrophoblast cells by hypoxia (18). Immunostaining for HIF-2α (1:100 antibody dilution, Abcam, San Francisco, CA; cat. no. ab199) was performed on paraffin-embedded sections using the ABC-DAB protocol described above. The relative intensities of pimonidazole and HIF-2α staining were analyzed in the whole placenta section as described above.

Statistical analysis. Comparisons between ACE2 KO and C57Bl/6 groups were evaluated using unpaired \( t \)-tests. Data obtained from the concentration-dependent responses of each arterial segment in each group were fitted to a logistic curve to determine maximal response and sensitivity. The curve had the form \( Y = \frac{1}{1 + 10^{{b} \cdot \logEC50 - X}} \times \text{Hill Slope} \) where \( X \) represents the logarithm of the drug concentration and \( Y \) the response. Values of

![Fig. 2. Uterine arteries responses to Ang II (A and B) and semiquantitative analysis of angiotensin receptor (ATR) expression (C) in uterine arteries of ACE2 KO and C57Bl/6 mice at day 14 of gestation. A: concentration-dependent response to Ang II. B: maximal response to Ang II (10⁻⁵ M): Ang II alone, after preincubation with Ang-(1–7) (10⁻⁵M) or a combination of Ang-(1–7) with AT₁R/MAS receptor antagonist [n-Ala]-Ang-(1–7) (10⁻⁵ M) for 20 min. Data are means ± SE. * \( P < 0.05 \) vs. C57Bl/6; # \( P < 0.05 \) vs. ACE2 KO control (Ang II contraction); \( n = 5–7 \) for vascular responses; \( n = 4 \) in each group for immunostaining analysis.]
sensitivity were expressed as pD₂ (pD₂ = -Log EC₅₀), with EC₅₀ values obtained from the curve fittings. GraphPad Prism IV (San Diego, CA) plotting and statistical software were used. All measurements were expressed as means ± SE. The criterion for statistical significance was P < 0.05.

RESULTS

Physiological indexes. SBP was higher in pregnant ACE2 KO than in WT C57Bl/6 mice (Table 1) as previously observed at late gestation in the ACE2 KO pregnant mouse (3). The magnitude of difference in SBP in pregnant mice was similar to the difference observed in nonpregnant mice (ACE2 KO: 106.8 ± 3.2 vs. C57Bl/6: 94.2 ± 2.2 mmHg, n = 4). Moreover, maternal urinary protein excretion, serum creatinine, and kidney and heart weights were similar between groups (Table 1). Maternal weight was significantly lower in ACE2 KO mice, and this difference was sustained when total fetal weight was subtracted from the maternal weight. Maternal weight was independent of maternal food intake (Table 1). Fetal weight was lower in the ACE2 KO mice than in WT mice. There were no differences in placental weights; however, pup-to-placental weight ratio, an indirect measure of placental insufficiency, was significantly lower in the ACE2 KO vs. WT mice (Table 1).

Uterine artery reactivity and immunohistochemistry. ODs of uterine artery segments were not different between groups (236.0 ± 12.0 vs. 240.0 ± 7.0 μm, n = 4 in each group). Resting tension also was similar in both groups (1.75 ± 0.09 vs. 1.77 ± 0.09 mN/mm²). Similarly, the stress-stretch response and the rate constant were not statistically different between studied groups (Fig. 1, A and B). No difference in vasodilation in response to acetylcholine was observed in C57Bl/6 vs. ACE2 KO mice (Fig. 1C and Table 2). In addition, the intensity of the uterine artery eNOS immunostaining was similar between C57Bl/6 and ACE2 KO mice (Fig. 1D).

In contrast to the acetylcholine response, active tension, assessed as a response to 75 mM KCl, was significantly greater in uterine arteries of ACE2 KO (Table 2). In addition, as shown in Fig. 1E and Table 2, uterine arteries from ACE2 KO mice displayed greater maximal response to phenylephrine, with no difference in sensitivity. Since the sensitivity to Ang II decreases during pregnancy, we compared the reactivity of uterine arteries to Ang II. Uterine arteries from ACE2 KO mice were more sensitive to Ang II compared with C57Bl/6 mice (n = 5–6; Fig. 2A and Table 2). Preincubation with Ang-(1–7) at the concentration of 1 μM for 30 min decreased maximal contraction to Ang II in ACE2 KO but not in WT mice (Fig. 2B). A preincubation with Ang-(1–7) antagonist, [D-Ala⁷]-Ang-(1–7) increased the Ang II contraction in the uterine artery of ACE2 KO but not in WT mice (Fig. 2B). There were no differences in the expression of AT₁R or AT₁₇/MAS receptors in the uterine arteries of ACE2 KO vs. C57Bl/6 mice (Fig. 2C). However, the expression of AT₂R determined by the immunostaining was lower in the uterine arteries of ACE2 KO vs. C57Bl/6 mice (Fig. 2C).

Fig. 3. Images (A) and semiquantitative analysis (B) of morphological markers of arterial stiffness (PSR), elasticity (VH), fibrinoid content (PAS), and structural proteins (actin, myosin) of uterine arteries in ACE2 KO and C57Bl/6 mice. PAS, periodic acid Schiff stain; PSR, Picrosirius red stain; VH, Verhoeff’s stain. Data are means ± SE. *P < 0.05 vs. C57Bl/6. Magnification ×40.
Greater responses to phenylephrine and Ang II in ACE2 KO mice were associated with lower fibrinoid content as assessed by PAS staining ($n = 4$ in each group) and a tendency for a higher collagen expression in the media of uterine artery as assessed by Picrosirius red staining ($P = 0.05$, $n = 4 – 5$; Fig. 3B). There were no differences observed in the intensity of actin or myosin between ACE2 KO and C57Bl/6 mice ($n = 3 – 4$). The area of Verhoeff staining indicative of elastin distribution in the uterine arteries was also similar in the two strains (Fig. 3B; $n = 3 – 4$).

Uterine artery ultrasound data. There were no differences in the uterine artery peak systolic or minimum diastolic velocities or resistance index of ACE2 KO compared with C57Bl/6 mice ($n = 4$ in each group; Fig. 4A).

Trophoblast invasion. The extent of extravillous (EVTB) and arterial trophoblast (ATB) invasion in the decidua and mesometrium was compared between ACE2 KO and C57Bl/6 mice. No differences in EVT or ATB distribution were found between ACE2 KO and C57Bl/6 mice ($3.7 \pm 0.1$ vs. $3.4 \pm 0.4$ number of EVT and AT combined). In both strains, the EVTBs evenly invaded into the decidua, reaching the upper limit of the decidua (Fig. 4B). Both strains showed variable signs of ATB invasion into the decidual arteries, going from no invasion in some arteries to complete invasion (Fig. 4B, inset). There were low numbers of ATBs and EVTBs in arterial walls within the mesometrium in both strains.

Placental hypoxia. Cross-sections of the placenta were immunostained for the hypoxia marker pimonidazole and HIF-2α. As shown in Fig. 5, A and B, pimonidazole staining was higher in the trophospongium and the labyrinth of the ACE2 KO vs. C57Bl/6 placenta (1.9-fold, $n = 4 – 5$). In addition, HIF-2α immunostaining (Fig. 5, C and D) was also greater in decidual and labyrinth areas of the ACE2 KO mice compared with C57Bl/6 ($n = 4 – 5$).

Umbilical artery ultrasound data. Peak systolic velocities of umbilical arteries were significantly lower in the ACE2 KO mice (Fig. 6; $n = 8 – 9$). Minimum diastolic velocities of the umbilical arteries were similar in ACE2 KO and C57Bl/6 mice (Fig. 6; $n = 8 – 9$). The resistance index of umbilical arteries in ACE2 KO was lower than that of C57Bl/6 mice ($0.90 \pm 0.01$ vs. $0.95 \pm 0.01$, $n = 8 – 9$; Fig. 6).

DISCUSSION

We recently reported that ACE2 knockout is associated with reduced fetal weight at late pregnancy (3); however, the mechanisms underlying this growth-restricted phenotype are not known. In the present study, we found that the reduced fetal weight in ACE2 KO mice at day 14 of gestation is associated with uteroplacental dysfunction evidenced by greater uterine artery reactivity to vasoconstrictors, abnormal uterine artery remodeling, and placental hypoxia.

Most fetal growth in mice occurs during the last trimester of pregnancy (30). Intrauterine growth restriction is characterized by an abnormal vascular resistance and compromised blood flow (22). During normal pregnancy, blood flow progressively increases in the uteroplacental circulation, including the uterine artery and its branches and the umbilical artery (10a). The main
uterine arteries provide ~80% of total uteroplacental flow (44). As a result of proper remodeling, uteroplacental vessels become more resistant to vasoactive substances compared with nonremodeled vessels (44). In addition, local mediators play a prominent role in the regulation of uteroplacental vascular reactivity (44). The relaxation of uterine arteries during normal pregnancy is mediated by nitric oxide- and endothelial-derived hyperpolarizing factors and prostacyclin (10, 42). Contrary to the responses of uterine artery isolated from pregnant eNOS knockout IUGR mice or small myometrial arteries isolated from human IUGR pregnancies (26, 61), we found similar relaxation responses to acetylcholine in ACE2 KO and WT mice, suggesting that ACE2 may not interfere with endothelial-dependent relaxation at day 14 of gestation in mice. Moreover, global gene knockout may result in a series of compensatory events that may mask the effects of ACE2 knockout in pregnancy. For example the activation of nephrilysin or other enzymes that can generate Ang-(1–7) (13) may be upregulated in the placenta of ACE2 KO mice. The levels of placental Ang-(1–7) were similar in ACE2 KO and WT mice at late gestation (3). Since Ang-(1–7) increases the release of nitric oxide, it may contribute to the acetylcholine-induced relaxation of uteroplacental vessels.

Here, we demonstrate that ACE2 KO mice have a higher response of the uterine artery to potassium chloride than WT mice, suggesting increased hypertrophy or hyperplasia of vascular smooth muscle cells (43). However, immunostaining for contractile proteins such as α-smooth muscle cell actin showed no difference and myosin staining only tended to increase. It is possible that our study was underpowered to detect significant differences in contractile proteins between groups. Optimal diameters and the stress-stretch responses are directly related to the elastic properties of the arterial tissue; no difference in these parameters suggests similar elastic properties of uterine arteries in ACE2 KO and WT mice. Similarly, equivalent values of resting tension between studied groups would suggest that the vascular hypertrophic and hyperplastic processes are unaffected by ACE2 at least in midpregnancy at the level of the uterine artery. The arterial response to potassium chloride is dependent on the influx of extracellular calcium and the activity of voltage-gated calcium channels (23). It is possible that higher expression of these channels or increased calcium signaling may augment the response to potassium chloride in the uterine artery of ACE2 KO mice. We demonstrate that uterine arteries of ACE2 KO mice were more responsive to phenylephrine than WT controls. As an α1-adrenergic agonist, phenylephrine can constrict blood vessels through the opening of voltage-gated calcium channels and through G protein-coupled receptor signaling (9, 34, 66). The even higher KMAX of phenylephrine exceeding that of high potassium suggests that
the latter pathway may be accentuated in the uterine artery of ACE2 KO mice. Increased contraction of the main uterine artery to phenylephrine late in pregnancy was also seen in eNOS knockout mice, which develop fetal growth restriction at late pregnancy (26). Furthermore, compared with normal pregnant women, higher contraction to arginine vasopressin was reported in small myometrial arteries of pregnant women who had fetal growth restriction, suggesting that greater uterine artery contraction is associated with reduced fetal growth (61).

Reduced sensitivity of pressor response to Ang II is a characteristic feature of normotensive pregnancy (2, 15, 16). The concentration-dependent vasoconstriction to Ang II was greater in the uterine arteries from pregnant ACE2 KO mice vs. WT mice. Upregulation of AT1R or increased infiltration of neutrophils in the uterine artery has been suggested to play a role in the increased sensitivity to Ang II during pregnancy (2, 5, 15, 16). The concentration-dependent vasoconstriction to Ang II was greater in the uterine arteries from pregnant ACE2 KO mice vs. WT mice. Upregulation of AT1R or increased infiltration of neutrophils in the uterine artery has been suggested to play a role in the increased sensitivity to Ang II during pregnancy (2, 5, 15, 16). The concentration-dependent vasoconstriction to Ang II was greater in the uterine arteries from pregnant ACE2 KO mice vs. WT mice. Upregulation of AT1R or increased infiltration of neutrophils in the uterine artery has been suggested to play a role in the increased sensitivity to Ang II during pregnancy (2, 5, 15, 16). The concentration-dependent vasoconstriction to Ang II was greater in the uterine arteries from pregnant ACE2 KO mice vs. WT mice. Upregulation of AT1R or increased infiltration of neutrophils in the uterine artery has been suggested to play a role in the increased sensitivity to Ang II during pregnancy (2, 5, 15, 16). The concentration-dependent vasoconstriction to Ang II was greater in the uterine arteries from pregnant ACE2 KO mice vs. WT mice. Upregulation of AT1R or increased infiltration of neutrophils in the uterine artery has been suggested to play a role in the increased sensitivity to Ang II during pregnancy (2, 5, 15, 16). The concentration-dependent vasoconstriction to Ang II was greater in the uterine arteries from pregnant ACE2 KO mice vs. WT mice. Upregulation of AT1R or increased infiltration of neutrophils in the uterine artery has been suggested to play a role in the increased sensitivity to Ang II during pregnancy (2, 5, 15, 16). The concentration-dependent vasoconstriction to Ang II was greater in the uterine arteries from pregnant ACE2 KO mice vs. WT mice. Upregulation of AT1R or increased infiltration of neutrophils in the uterine artery has been suggested to play a role in the increased sensitivity to Ang II during pregnancy (2, 5, 15, 16). The concentration-dependent vasoconstriction to Ang II was greater in the uterine arteries from pregnant ACE2 KO mice vs. WT mice. Upregulation of AT1R or increased infiltration of neutrophils in the uterine artery has been suggested to play a role in the increased sensitivity to Ang II during pregnancy (2, 5, 15, 16). The concentration-dependent vasoconstriction to Ang II was greater in the uterine arteries from pregnant ACE2 KO mice vs. WT mice. Upregulation of AT1R or increased infiltration of neutrophils in the uterine artery has been suggested to play a role in the increased sensitivity to Ang II during pregnancy (2, 5, 15, 16). The concentration-dependent vasoconstriction to Ang II was greater in the uterine arteries from pregnant ACE2 KO mice vs. WT mice. Upregulation of AT1R or increased infiltration of neutrophils in the uterine artery has been suggested to play a role in the increased sensitivity to Ang II during pregnancy (2, 5, 15, 16). The concentration-dependent vasoconstriction to Ang II was greater in the uterine arteries from pregnant ACE2 KO mice vs. WT mice. Upregulation of AT1R or increased infiltration of neutrophils in the uterine artery has been suggested to play a role in the increased sensitivity to Ang II during pregnancy (2, 5, 15, 16). The concentration-dependent vasoconstriction to Ang II was greater in the uterine arteries from pregnant ACE2 KO mice vs. WT mice. Upregulation of AT1R or increased infiltration of neutrophils in the uterine artery has been suggested to play a role in the increased sensitivity to Ang II during pregnancy (2, 5, 15, 16). The concentration-dependent vasoconstriction to Ang II was greater in the uterine arteries from pregnant ACE2 KO mice vs. WT mice. Upregulation of AT1R or increased infiltration of neutrophils in the uterine artery has been suggested to play a role in the increased sensitivity to Ang II during pregnancy (2, 5, 15, 16).

Our data also show Ang-(1–7) induced attenuation of Ang II constriction in the uterine artery of ACE2 KO mice. This effect was achieved, at least in part, via Ang-(1–7) acting on its own receptor, because the Ang-(1–7) antagonist increased the Ang-(1–7) attenuation of the vasoconstriction to Ang II. Tallant et al. (54) demonstrated that Ang-(1–7) can bind to AT1R in vitro when used at higher micromolar concentrations. Therefore, the possibility of Ang-(1–7) binding to AT1R and thereby limiting AT1R bioavailability for Ang II is feasible. In addition, Ang-(1–7) can activate AT2R so the lower density of AT2R might limit actions of Ang-(1–7) in vivo (58). Neves (40) reported that Ang-(1–7) induces vasodilation of mesentery artery in normotensive pregnant rats, suggesting a regulatory role for this peptide in regional blood flow adaptations in pregnancy. Moreover, we (64) recently reported that Ang-(1–7) increases skin blood flow in normotensive pregnant women, suggesting a protective role of Ang-(1–7) in skin microcirculation. Nevertheless, a beneficial role of Ang-(1–7) in the regulation of uterine vasculature during hypertensive pregnancy, particularly in one associated with the activation of the RAS, needs further investigation.

Increased uterine blood flow during normal pregnancy is likely due to remodeling of the uteroplacental vasculature. The predictive and diagnostic value of reduced uterine artery flow in pregnancies with IUGR is controversial. Absent or reversed diastolic flow has been shown in such pregnancies (17), but not consistently (45). Abnormal uterine artery resistance can be influenced by incomplete systemic and local adaptations to pregnancy, including endothelial dysfunction and stiffness of maternal vasculature and placental pathology alterations (12). In our study, we found no differences in uterine artery veloc-

![Fig. 6. Analysis of umbilical artery velocities and the resistance index of C57Bl/6 and ACE2 KO mice at midgestation. A: representative wave forms of Vmax and Vmin points of umbilical artery velocity measurement. Arrows indicate Vmax and Vmin of the umbilical artery velocity wave. B: average measurements of Vmax and Vmin and the calculated resistance index. Data are means ± SE; n=8–9. *P<0.05 vs. C57Bl/6.](http://ajpendo.physiology.org/doi/10.1152/ajpendo.00596.2014/pdf)
placentation abnormalities or resistance in pregnant ACE2 KO vs. WT mice at day 14 of gestation, suggesting that modest fetal growth restriction in ACE2 KO mouse is not accompanied by alterations in uterine artery hemodynamics despite morphological changes. Nor did we find differences in trophoblast invasion between strains. In contrast to human pregnancy, trophoblast invasion in mice is limited to the decidual compartment (14) and continues into late stages of gestation. Variable degrees of arterial trophoblast invasion into the decidual arteries may be more consistent with day 14 of gestation rather than a difference due to the ACE2 knockout. Differences in trophoblast invasion or uterine artery flow between ACE2 KO and WT mice may develop as pregnancy progresses and the fetus grows. In addition, an increased sensitivity of uterine artery to vasoconstrictors may be an early event that does not reflect alterations in uterine artery flow at this stage of gestation. Moreover, sustained uterine flow may prevent further decreases in fetal weight in ACE2 KO mice.

Umbilical artery ultrasound can be used to identify placental insufficiency complications and fetal growth restriction. Clinically, alterations in umbilical flow velocity are more reliable in detecting early-onset fetal growth restriction before 34 wk of gestation than later in gestation (30). During normal pregnancy, progressive increase in flow velocity of umbilical artery from mid to late gestation is essential for the expansion of the fetoplacental vasculature in the labyrinth as an adaptation to the growing fetus (10a). Reduced umbilical artery flow velocity in ACE2 knockout may suggest an incomplete adaptation of the labyrinth. Our finding of a lower umbilical arterial resistance in association with increased hypoxia was unexpected. However, it is known that abnormalities in the umbilical artery resistance are strongly related to IUGR (30). The reduction in resistance index was associated with a reduction in peak systolic velocity, an unusual pattern that may reflect impaired placental or suboptimal fetal vascular development that could lead to hypoxia. Moreover, abnormal placenta and placental vascular disease in addition to hypoxia of the uteroplacental unit have been associated with the development of fetal growth restriction (20). Increased hypoxia of the placenta results in the presence of less vascular labyrinth with abnormalities in the fetotrophoblast capillary bed such as the reduced number of vascular segments, vascular volume, and the total length of arterial tree (20). Moreover, hypoxia increases maximal responses to potassium chloride and the thromboxane A2 mimetic U46619 in human isolated chorionic plate arteries (59, 60), suggesting that reduced oxygen tension may augment vascular reactivity of placental vessels. Attenuated umbilical flow velocity, frequently associated with reduced fetal weight, may be a compensatory adaptation in response to placental hypoxia observed in ACE2 KO mice.

As shown in our study, the ACE2 KO model allows the investigation of the role of ACE2 in hemodynamic and vascular alterations during IUGR pregnancy. Since this model exhibits no major organ damage, it can be particularly useful for the investigation of the hemodynamic and placental abnormalities that occur during pregnancy, including the early stages of pregnancy that may be critical to the development of IUGR. As stated above, one of the limitations of this model is global ACE2 gene knockout that may result in a series of compensatory events that may mask the contribution of ACE2 deletion to pregnancy and IUGR. Future studies will focus on the direct targeting of ACE2 in the placenta to assess the contribution of placental ACE2 to hypoxia as well as on distinguishing the role of the fetal vs. maternal ACE2 in IUGR.

In summary, IUGR is a leading cause of perinatal morbidity and mortality in humans; however, the molecular mechanisms of this pregnancy-induced complication are not well understood. Using mice with total ACE2 knockout allowed us to investigate the contribution of ACE2 to the development of IUGR. Our study revealed that uterine artery dysfunction in ACE2 KO mice is associated with placental hypoxia and greater uterine artery reactivity to vasoconstrictors, suggesting a protective role of ACE2 in the uteroplacental unit. Increased contractility of uterine artery in ACE2 KO mice may represent an early event that as pregnancy progresses may translate into hemodynamic changes in the uterine artery. Placental hypoxia and uterine artery dysfunction develop before the major growth of the fetus occurs and may explain the growth-restricted phenotype. Our findings also emphasize the similarities of the ACE2 KO model of fetal growth restriction to human pregnancy associated with fetal growth restriction.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s). The authors have no relationships to disclose.

AUTHOR CONTRIBUTIONS


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