Differential development of umbilical and systemic arteries. I. ANG II receptor subtype expression

JEFFREY R. KAISER, BLAIR E. COX, TIMOTHY A. ROY, AND CHARLES R. ROSENFELD
Department of Pediatrics, Division of Neonatal-Perinatal Medicine, University of Texas Southwestern Medical School, Dallas, Texas 75235-9063

Kaiser, Jeffrey R., Blair E. Cox, Timothy A. Roy, and Charles R. Rosenfeld. Differential development of umbilical and systemic arteries. I. ANG II receptor subtype expression. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R797–R807, 1998.—In fetal sheep umbilical responses to angiotensin II (ANG II) exceed those by systemic vasculature. Two ANG II receptors (AT) exist, AT1 and AT2, but only AT1 mediates vasoconstriction in adult tissues. Thus differences in reactivity could reflect differences in subtype expression. Using competitive radioligand binding assays, we demonstrated AT1 predominance in umbilical arteries and AT2 in femoral arteries. Steady-state responses to intravenous ANG II (0.229–1.72 µg/min) were studied in 16 fetuses with umbilical and/or femoral artery flow probes without and with local AT1 (L-158,809) or AT2 (PD-123319) blockade. ANG II dose dependently (P < 0.001) increased umbilical resistance more than arterial pressure (MAP) while decreasing umbilical blood flow. Femoral vascular resistance also increased dose dependently (P = 0.02), but responses were less than umbilical (P = 0.0001) and paralleled increases in MAP; blood flow was unaffected. Cumulative local doses of L-158,809 (125 µg) inhibited all responses (P < 0.001); however, 1,000 µg of the AT2 antagonist had no effect. Plasma renin activity (PRA) was unaltered by local AT1 blockade, whereas PRA doubled (P = 0.001) after systemic infusion of only 50 µg of the AT2 antagonist and remained elevated. Differences in umbilical and femoral vascular responses to ANG II are in large part due to differences in AT subtype expression. Furthermore, in fetal sheep the ANG II negative feedback on PRA is mediated by AT1 receptors, and it is substantially more sensitive to receptor blockade than the vasculature.

The fetal sympathetic nervous system is not fully developed until late in gestation or after birth (3, 19). Thus the activity of the renin-angiotensin system (RAS) may play an important regulatory role in fetal cardiovascular homeostasis (2, 20–22, 26). This is supported by observations in fetal sheep that, although angiotensin II (ANG II) and α-adrenergic agonists cause dose-dependent increases in mean arterial pressure (MAP) and umbilical vascular resistance, there is greater sensitivity to the vasoconstricting effects of ANG II compared with catecholamines (1, 8, 47). Additionally, in studies using systemic administration of nonspecific ANG II receptor (AT) antagonists, the fetal RAS appears to modulate vasomotor tone in the resting state (20, 22) as well as during various “stress” states, e.g., hemorrhage (22, 37).

Although the activity of the RAS appears to be enhanced during development, fetal sheep were considered to be less responsive than pregnant ewes to infused ANG II (8, 47). However, the fetal metabolic clearance rate of infused ANG II is ∼10-fold greater than that of the adult (36); thus at similar rates of infusion (µg·min⁻¹·kg⁻¹) the fetus is actually exposed to significantly lower plasma concentrations of ANG II than the adult, thereby explaining in part the attenuated pressor responses previously reported (47). Although pressor responses were similar at equivalent plasma ANG II levels, the relative increases in umbilical vascular resistance exceeded those in maternal uteroplacental vascular resistance (36), suggesting enhanced umbilicoplacental sensitivity to exogenous ANG II. Furthermore, the umbilical circulation also demonstrated greater sensitivity to infused ANG II than the fetal systemic vasculature, confirming earlier observations by Iwamoto and Rudolph (21). These differences in vascular sensitivity and their underlying mechanisms have not been thoroughly investigated.

ANG II exerts its effects through activation of specific AT receptors. Two major AT subtypes have been identified and are distinguished pharmacologically by their differential affinities for specific selective antagonists (4, 7, 43). The AT1 is the primary subtype expressed in nearly all adult tissues, including the vascular smooth muscle (VSM), and its activation is responsible for most known biological actions of ANG II, including smooth muscle contraction (9, 16, 46). Except for uterine and cerebral arteries (10, 13, 41) and the myometrium of adult nonpregnant ewes (9) and women (13, 15), the AT2 subtype is primarily found in tissues of the developing fetus and neonate (16, 18, 41). Although the physiological function of the AT2 receptor is controversial, it has not been shown to mediate ANG II-induced contraction of smooth muscle (9, 16). This is noteworthy, because Cox et al. (11) recently reported that AT2 is the predominant receptor expressed in several systemic arteries of fetal sheep, whereas AT1 is the major subtype in the VSM of the umbilical artery.

The purpose of the present study, therefore, was to 1) determine AT subtype expression in umbilical and systemic (i.e., femoral) artery smooth muscle, 2) compare the responses to ANG II in the umbilical and femoral vascular beds, which express different AT subtypes, and 3) attempt to explain the differential vascular sensitivity to exogenous ANG II between the umbilical and fetal systemic vasculature. We hypothesized that differences in umbilical and systemic vascular responses to infused ANG II reflect AT1 expression in the former and AT2 in systemic arteries.

METHODS

Animal preparation. Sixteen chronically instrumented pregnant ewes of mixed Western breed bearing singleton gestations were studied between 120 and 140 days gestation (term...
Assessment of fetal responses to systemic ANG II infusions, with the use of techniques similar to those previously reported (47). Briefly, after an overnight fast, subcutaneous atropine (0.088 mg/kg) was administered to the ewe followed by intravenous pentobarbital sodium (7.5–10 mg/kg) and 1% ketamine hydrochloride (1–2 mg/kg) via a percutaneous jugular venous catheter. During surgery, 1% ketamine hydrochloride (1 mg/kg) was given as needed. The uterus was exteriorized through a midline abdominal incision. A fetal hindlimb was delivered through a small uterine incision, and two polyvinyl catheters containing heparinized saline (100 U/ml) were inserted into a fetal femoral artery and advanced 7.5 and 15 cm, with the tips lying just above the aortic bifurcation (for infusions) and below the level of the renal arteries (for cardiovascular monitoring), respectively. The ipsilateral femoral vein was cannulated for systemic infusion of drugs, with the catheter tip lying in the abdominal vena cava. An intraperitoneal incision was then made in the fetal abdomen lateral to the midline and below the insertion of the umbilical cord (47). One intra-abdominal umbilical artery was identified and fitted with a 4–5 mm (ID) electromagnetic flow probe (Micron Instruments, Los Angeles, CA). The fetal abdomen was closed over the flow probe head in two layers, and the leads were sutured to the skin. Through an inguinal incision the contralateral femoral artery was identified, and a 3–4 mm (ID) flow probe was implanted. A catheter was placed in the amniotic sac to monitor intra-amniotic pressure. The fetus was returned to the amniotic cavity, and the uterus was closed in two layers and returned to the peritoneal cavity. The catheters and flow probe leads were exteriorized through an incision in the fascia followed by closure of the maternal abdomen. Through a groin incision, catheters were inserted into a maternal femoral artery and vein to permit monitoring of maternal heart rate and blood pressure and for infusion of postoperative fluids. All catheters and flow probes were exteriorized via a subcutaneous tunnel and stored in a canvas pouch attached to the maternal flank.

After surgery ewes were maintained in individual stalls in the laboratory and given standard animal chow, hay, and water ad libitum. The ewes received intramuscular antibiotics (penicillin 600,000 U and gentamicin 60 mg) before surgery and on the first two postoperative days: fetal sheep received intravenous antibiotics (ampicillin 50 mg) on the day of surgery and first two postoperative days, then every other day thereafter. Catheters were flushed daily with sterile heparinized saline (250 U/ml) to maintain patency. Experiments were not initiated until after the fourth postoperative day. Experiments were divided into three components: 1) assessment of fetal responses to systemic ANG II infusions, 2) evaluation of fetal responses to ANG II after AT1 blockade in the umbilical and hindlimb circulations, and 3) evaluation of fetal responses to ANG II after AT2 blockade in the same vascular beds. This sequence was repeated to examine the effect of gestational age on these responses. At the completion of the studies, ewes were killed using an intravenous injection of phenobarbital (50 mg/kg). The fetuses were weighed and measured, and a necropsy was performed to confirm catheter and flow probe placements. Fetal weights were appropriate for gestation, and the low arterial catheter was always just above the aortic bifurcation. These studies were approved by the Institutional Review Board for Animal Research.

ANG II dose-response experiments. In these studies we established a dose-response curve to intravenous infusions of ANG II to identify for each fetus the dose that would increase MAP ~50%. The doses examined (0.229, 0.573, 1.15, and 1.72 µg/min) were based on previous studies in our laboratory (36, 47). After a 30-min control period, a dose of ANG II (angiotensin amide, Sigma Chemical, St. Louis, MO) was infused through the femoral venous catheter for 7 min. This permits the establishment of steady-state responses in fetal MAP, heart rate (HR), and umbilical and femoral blood flows (36, 47). There was a 20-min interval between doses to allow all cardiovascular and arterial blood gas parameters to return to baseline. In all studies fetal and maternal MAP and HR and intra-amniotic fluid pressure were continuously monitored with pressure transducers (P23XL, Gould, Cleveland, OH) while monitoring blood flows with square-wave electromagnetic flowmeters (model RC2000, Micron Instruments). All parameters were recorded on an eight-channel pen recorder (model RS3800, Gould). Arterial blood was obtained for arterial blood gases and hematocrit (0.3 ml) before the initiation of each study.

Effects of receptor blockade. After identifying the dose of ANG II that increased fetal MAP ~50%, we determined the dose of L-158,809 (a gift from DuPont-Merck Pharmaceutical Research Division, Wilmington, DE), a highly selective AT1 antagonist (38), that would completely inhibit this response. To accomplish this, incremental doses (volume ~1.0 ml) of the antagonist diluted in isotonic saline were infused over 1 min through the lower femoral arterial catheter to preferentially block AT1 receptors in the umbilicoplacental and hindlimb vascular beds. Individual doses did not exceed 25 µg so as to prevent systemic overflow and receptor inhibition. Each dose of the antagonist was followed 5 min later by a 7-min intravenous infusion of the ANG II dose shown to increase MAP ~50%. This was repeated until complete inhibition of the vascular responses to infused ANG II was observed. In preliminary experiments we established that the effects of several doses of the AT1 antagonist were additive. Hemodynamic variables were continuously monitored as described above. Arterial blood samples (0.3 ml) were obtained before each study to assess blood gases and hematocrit. Arterial blood (3.0 ml each) was also obtained for measurements of plasma renin activity (PRA) before, during, and after receptor blockade (total = 25 ml, ~5% blood volume). Maternal red blood cells resuspended in sterile saline were used to replace fetal blood losses at the end of each study. To determine the duration of AT1 inhibition, systemic ANG II infusions were performed during succeeding days until hemodynamic responses returned to pretreatment values. At that time either additional experiments using the AT1 antagonist were performed or the same protocol was used to examine the effects of AT2 blockade.

To study the AT2 antagonist, increasing amounts (beginning at 1 µg; volume of 1.0 ml) of PD-123319 (a specific AT2 antagonist supplied by Parke-Davis Pharmaceutical Research Division, Ann Arbor, MI) were infused into the lower femoral artery catheter over 1 min followed 5 min later by a 7-min systemic infusion of ANG II. These experiments were continued until a total dose of 1,000 µg of the AT2 antagonist had been infused. The effects of AT2 inhibition were always studied last, because an effect was never observed and the duration of blockade, therefore, was less clear.

Determination of receptor subtype expression. The AT subtypes expressed in VSM of several fetal systemic arteries and the umbilicoplacental vasculature during ovine development have recently been characterized by Cox et al. (11). The AT2 receptor was observed in all systemic arteries, whereas only the umbilical artery expressed the AT1, which mediates vascular contractions (9, 17, 18). In the present study, we measured blood flow through the femoral artery, which was not included in those studies. We, therefore, determined the binding characteristics and the AT subtype in fetal femoral...
were performed with 10 femoral arteries, competitive radioligand binding studies added in increasing concentrations ranging from 10 concentrations from 0.3 to 0.4 nM. Unlabeled ANG II was done with umbilical arteries (5 nM, and the half-maximal inhibitory dose (IC50), and the percentages of placement curves for each AT antagonist and their respective density (fmol/mg protein), dissociation constants (nM), dis-
receptors without affecting the AT1 subtype (9). The same was for 125I of 83%.
the radioactivity was measured with a scintillation counter rinses of the filters with the buffer. After the filters were dry,
filtration through Whatman GF/C filters (Whatman Interna-tional, Maidstone, UK) under vacuum followed by three
which 100 µl of each of the protease inhibitors PMSF, leupeptin (5 µg/ml), and aprotinin (5 µg/ml) were added and prepared for AT binding assays as previously reported (9, 10, 13, 27, 35).
Receptor binding assays were performed on femoral artery samples with 100 µl of membrane preparation in a total volume of 150 µl of the 25 mM tris(hydroxymethyl)aminomethane (Tris) buffer with 0.2% bovine serum albumin (BSA). Tyrrosyl 125I-labeled [Sar1, Ile8]ANG II (129)-ANG II; 2,200 Ci/mmol; New England Nuclear, Boston, MA) was added in concentrations from 0.3 to 0.4 nM. Unlabeled ANG II was added in increasing concentrations ranging from 10^{-11} to 10^{-8} M, and the binding characteristics were determined from analysis of the displacement of labeled ligand. ANG II at 10^{-5} M concentration was used to determine nonspecific binding. After incubations for 90 min at 38°C, reactions were terminated by rapid addition of 4 ml of ice-cold 25 mM Tris buffer with 0.2% BSA. Bound and free ligand were separated by filtration through Whatman GF/C filters (Whatman Interna-tional, Maidstone, UK) under vacuum followed by three rinses of the filters with the buffer. After the filters were dry, the radioactivity was measured with a scintillation counter (Packard Instruments, Downers Grove, IL) with an efficiency for 125I of 83%.
To determine the AT subtypes present in the umbilical and femoral arteries, competitive radioligand binding studies were performed with 10^{11}-10^{-6} M of the peptide antagonist [Sar1, Ile8]ANG II, which has equal affinity for both subtypes, and 10^{-11}-10^{-5} M of the nonpeptide subtype-specific antago-nists losartan (specific for AT1; a gift from DuPont-Merck Pharmaceutical) and PD-123319. Specific binding was calculated by subtracting nonspecific binding, measured in the presence of 10^{-5} M [Sar1, Ile8]ANG II, from total 125I-ANG II binding. Additional plasma membrane preparations made from femoral arteries (n = 4) were preincubated with 10^{-6} M PD-123319, a concentration sufficient to saturate the AT2 receptors without affecting the AT1 subtype (9). The same was done with umbilical arteries (n = 4), but samples were preincubated with 10^{-6} M losartan. With the AT2 or AT1 blocked, competitive binding studies were performed to iden-
ify the presence of the other AT subtype. Total binding density (fmol/mg protein), dissociation constants (nM), dis-placement curves for each AT antagonist and their respective half-maximal inhibitory dose (IC50), and the percentages of AT subtypes were calculated from the specific binding data using a modification of the computer program LIGAND (30) adapted for microcomputers by McPherson (29) (Elsevier BIOSOFT, Cambridge, UK).
Radioimmunoassay. Measurements of PRA were obtained as the angiotensin I generated in vitro using a commercial kit (New England Nuclear, North Billerica, MA) and are expressed as nanograms ANG I formed per milliliter per hour.
Data analysis. Fetal MAP was corrected for variations in intrauterine pressure by subtracting simultaneous intra-amniotic pressure from MAP. Umbilical and femoral vascular resistance were computed by dividing the corrected fetal MAP by umbilical and femoral blood flow, respectively. Cardiovas-cular responses to each infusion of ANG II were assessed at 5–7 min, when steady-state responses were always achieved (36, 47). The magnitude of these responses was determined by comparing them with the hemodynamic measurements obtained just before each infusion of ANG II. Relative responses (%) are expressed as percent change from baseline values. To determine the presence of a dose response, the data were analyzed by one-way repeated-measures analysis of variance (ANOVA) followed by Newman-Keuls test for multiple com-parisons. Student’s t-test and two-way ANOVA were used where appropriate. Regression lines were obtained by the least-squares method and polynomial analysis of the data. Differences were considered statistically significant at P < 0.05. Values are presented as means ± SE.
RESULTS
Characterization of AT receptors. LIGAND analysis of the displacement of 125I-ANG II by unlabeled ANG II demonstrated a single class (r = 0.84, P < 0.05) of high-affinity saturable binding sites in femoral artery VSM (Fig. 1). The binding density averaged 238 ± 70 fmol/mg protein, and the dissociation constant was 2.6 ± 1.4 nM (n = 3), values consistent with those observed in other ovine fetal arteries studied during late gestation (35).
We determined the AT subtypes expressed in umbilical and femoral artery VSM by inhibition of 125I-ANG II binding with [Sar1, Ile8]ANG II and the subtype-specific antagonists losartan (AT1) and PD-123319 (AT2). The IC50 for these agents (n = 3) were 1.4 ± 0.4, 127 ± 18, and >100,000 nM (Fig. 2, top), respectively, for umbilical arteries and 2.2 ± 1.1, >100,000, and 6.2 ± 1.7 nM (Fig. 2, top), respectively, for femoral arteries.
![Fig. 1. Representative Scatchard plot of 125I-labeled ANG II binding to plasma membrane fractions from ovine fetal femoral artery. Each point was determined in duplicate, r = 0.84.](http://ajpregu.physiology.org/Downloadedfromhttp://ajpregu.physiology.org/Downloadedfromhttp://ajpregu.physiology.org/)
Thus AT1 expression was predominant in umbilical VSM and AT2 in femoral VSM. We then preincubated (see METHODS) plasma membranes with either 10^{-6} M losartan (umbilical artery) or PD-123319 (femoral artery) to determine if a small population of either the AT2 or AT1 existed (9). These studies (Fig. 2, bottom) revealed that only AT1 receptors were present in three of four umbilical arteries studied. In the fourth artery, AT2 receptors accounted for <5% of total binding. In contrast, only AT1 receptors were identified in three of the femoral arteries studied, whereas <5% AT1 receptors were found in the fourth.

Umbilicalplacental and systemic responses to ANG II. Hemodynamic measurements obtained before and during steady-state responses to systemic infusions of ANG II (0.229–1.72 µg/min) are presented in Table 1. There was no gestational age-dependent effect of ANG II on the pressor responses or the increases in umbilical and femoral resistance (r^2 < 0.04, n = 31, P > 0.30); we, therefore, have included dose-response data as well as data obtained before AT receptor blockade with 1.15 and 1.72 µg/min. This accounts for the different number of studies for each dose noted in Table 1. Baseline values for all parameters obtained before each dose of ANG II do not differ and are consistent with those reported previously from our laboratory after correction for intra-amniotic pressure (36, 47). Arterial blood gases before ANG II infusions also were normal, PO2 averaging >18 mmHg, PCO2 ≤ 48 mmHg, pH 7.38, and hematocrit >33%. ANG II increased MAP at all doses in a dose-related fashion (P < 0.0001), the highest dose resulting in a 57.1 ± 2.0% increase from baseline (Table 1). HR decreased 8–15% with doses ≥ 0.573 µg/min, demonstrating an intact baroreflex. There also were dose-dependent decreases in umbilical blood flow and increases in umbilical vascular resistance, threshold responses occurring at 1.15 and 0.573 µg/min, (P < 0.01), respectively. Maternal MAP and HR were unaffected by fetal administration of ANG II.

To compare the effects of ANG II on umbilical and systemic responses, we examined the simultaneous “relative” changes in fetal MAP and umbilical resistance and blood flow, which permits an accurate com-

### Table 1. Fetal cardiovascular responses to continuous intravenous infusions of ANG II

<table>
<thead>
<tr>
<th>Rate of Infusion, µg/min</th>
<th>MAP, mmHg</th>
<th>HR, beats/min</th>
<th>Umbilical blood flow, ml/min</th>
<th>UmVR, mmHg·min^{-1}·l^{-1}</th>
<th>Femoral blood flow, ml/min</th>
<th>Femoral vascular resistance, mmHg·min^{-1}·l^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.229</td>
<td>Baseline</td>
<td>41.6 ± 0.9</td>
<td>168 ± 7</td>
<td>270 ± 35</td>
<td>51.3 ± 2.4</td>
<td>793 ± 38</td>
</tr>
<tr>
<td></td>
<td>Steady</td>
<td>47.2 ± 1.1†</td>
<td>164 ± 6</td>
<td>291 ± 41</td>
<td>51.9 ± 3.4</td>
<td>927 ± 85</td>
</tr>
<tr>
<td></td>
<td>state</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.573</td>
<td>Baseline</td>
<td>43.0 ± 0.9</td>
<td>165 ± 5</td>
<td>237 ± 32</td>
<td>50.7 ± 2.8</td>
<td>821 ± 48</td>
</tr>
<tr>
<td></td>
<td>Steady</td>
<td>43.4 ± 0.8</td>
<td>170 ± 3</td>
<td>226 ± 23</td>
<td>49.3 ± 3.1</td>
<td>853 ± 48</td>
</tr>
<tr>
<td></td>
<td>state</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.15</td>
<td>Baseline</td>
<td>43.8 ± 0.8</td>
<td>171 ± 3</td>
<td>213 ± 32</td>
<td>49.3 ± 3.1</td>
<td>43.0 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>Steady</td>
<td>43.3 ± 0.6</td>
<td>186 ± 3</td>
<td>242 ± 43</td>
<td>53.4 ± 6.3</td>
<td>549 ± 87</td>
</tr>
<tr>
<td>1.72</td>
<td>Baseline</td>
<td>43.3 ± 0.6</td>
<td>187 ± 3</td>
<td>226 ± 17</td>
<td>49.3 ± 3.1</td>
<td>50.0 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>Steady</td>
<td>43.4 ± 0.8</td>
<td>184 ± 3</td>
<td>216 ± 17</td>
<td>53.4 ± 6.3</td>
<td>549 ± 87</td>
</tr>
</tbody>
</table>

Values are means ± SE; n is no. of experiments. HR, heart rate; UmVR, umbilical vascular resistance. Statistical significance by paired t-test compared with baseline: *P < 0.01, †P < 0.001, ‡P < 0.0001. Data for mean arterial pressure (MAP) are corrected for intra-amniotic pressure.
parison of changes in different vascular parameters. At doses of ANG II ≤0.573 µg/min, the relative rise in MAP was similar to that of umbilical resistance, thus umbilical blood flow remained unchanged (Fig. 3A). However, at higher doses the relative rise in umbilical vascular resistance exceeded (P < 0.0001) that of MAP and umbilical blood flow fell.

Femoral responses to ANG II. Continuous measurements of femoral blood flow were available for analysis in six fetal sheep. Baseline femoral blood flow was similar to that reported by others (5, 44), averaging 50.6 ± 1.5 ml/min (Table 1), and was unchanged at all doses of ANG II studied, averaging 52.0 ± 2.2 ml/min (n = 38). In contrast, femoral vascular resistance (Table 1, Fig. 3B) increased at each dose of ANG II in a dose-dependent manner (ANOVA, P = 0.016). When we compared the simultaneous relative changes in MAP and femoral resistance and blood flow (Fig. 3B), the pattern of response differed from that observed for the umbilical vasculature. The rise in resistance paralleled and equaled that of MAP, whereas blood flow was unchanged. However, at 1.72 µg/min, the rise in MAP modestly exceeded that of resistance; thus blood flow rose modestly. The relationship between the percent rise in MAP and femoral resistance was linear (r² = 0.55, n = 38, P < 0.001) and did not differ from the line of identity.

When we compared the relative responses by the femoral and umbilical placental vascular beds (Fig. 3), which take into account differences in baseline resistance, increases in umbilical resistance exceeded those in femoral resistance at all but the lowest dose of ANG II; thus the dose-response curves were significantly different (P = 0.001, 2-way ANOVA), and increases in umbilical resistance were two- to threefold greater at 1.15 and 1.72 µg ANG II/min.

Effects of local AT₁ receptor blockade. Eight cumulative dose-response experiments were performed in five animals using the AT₁ antagonist, which was infused into both the umbilical and hindlimb circulations simultaneously as described in METHODS. These experiments were performed with a dose of ANG II that increased MAP ~50% (1.36 ± 0.10 µg/min). When we examined the responses by the umbilical circulation, hemodynamic measurements obtained before and immediately after the infusion of each dose of the AT₁ antagonist did not differ (P ≥ 0.9). Cumulative doses of the AT₁ antagonist (Fig. 4) resulted in parallel inhibition (P < 0.001, n = 60) of ANG II-induced increases in umbilical resistance (r² = 0.81) and MAP (r² = 0.76) and decreases in umbilical blood flow (r² = 0.77). Complete inhibition was observed with cumulative total dose of ~125 µg of the AT₁ antagonist; 50% inhibition occurred with 20–25 µg.

To evaluate the relationship between ANG II-induced rises in fetal MAP and umbilical vascular resistance, we examined the simultaneous relative rise in MAP and umbilical resistance during ANG II dose-response experiments. Before AT₁ blockade, this relationship was best described by a second-order regression (r² = 0.79, P < 0.0001), with the rise in MAP plateauing after

Fig. 3. Effects of systemic infusions of ANG II on the simultaneous relative changes in umbilical vascular resistance (UmVR), fetal mean arterial pressure (MAP), and umbilical blood flow (A) and femoral vascular resistance (FemVR) and blood flow (FemBF; B). Number of experiments at each dose of ANG II is noted in Table 1. Data are means ± SE. Dose-response curves for resistance differ at P = 0.0001 by 2-way ANOVA.

Fig. 4. Effects of increasing doses (n = 60) of the AT₁ receptor antagonist L-158,809 on steady-state ANG II-induced changes in UmVR, MAP, and umbilical blood flow (UmBF). ○, Responses to ANG II alone; horizontal lines, mean values; ●, responses to ANG II after a local dose of L-158,809. Procedure used is described in METHODS.
umbilical resistance increased 125–150%. Further increases in MAP were attenuated by baroreceptor mechanisms, as evidenced by decreases in HR (Table 1). This relationship was unaffected by local AT1 blockade. When we examined the relationship between the inhibitory effects of local AT1 blockade on umbilical resistance and the rise in MAP (Fig. 5), there was a highly significant linear relationship, suggesting that ANG II-induced increases in MAP are related to the simultaneous increases in umbilical vascular resistance.

The duration of AT1 blockade was assessed by examiningpressor responses to intravenous ANG II over the succeeding days. Responses to ANG II gradually returned to pretreatment values by 72–96 h (Fig. 6), resulting in an estimated half-life of ~31 h. Although umbilical artery AT1 blockade was complete immediately after treatment with the AT1 antagonist, basal MAP, HR, and umbilical vascular resistance did not differ from baseline values at any time during the period of inhibition (Table 2).

Five cumulative dose-response experiments with the AT1 antagonist were available from four animals to assess the effects on the femoral vascular responses to intravenous ANG II infusions. Increasing doses of the AT1 antagonist infused directly into the umbilical and femoral circulations did not alter basal MAP or femoral blood flow. The AT1 antagonist (1–125 µg, n = 28) caused dose-dependent inhibition (Fig. 7) of ANG II-induced rises in femoral resistance and MAP, whereas blood flow was unchanged; thus the linear relationship \( r^2 = 0.76 \) between the relative changes in femoral vascular resistance and MAP observed with ANG II alone was maintained and did not differ from the line of identity.

Effects of local AT2 blockade. Three cumulative dose-response studies were performed with the AT2 antagonist using the protocol described for the AT1 antagonist. The total cumulative dose of the AT2 antagonist was ~10-fold greater than that for the AT1 antagonist, i.e., 1,000 µg vs. 125 µg. The AT2 blockade did not alter basal MAP (49 ± 1.2 vs. 49 ± 1.2 mmHg), umbilical resistance (274 ± 89 vs. 265 ± 91 mmHg·min\(^{-1}\)·l\(^{-1}\)), or HR (150 vs. 153 ± 3 beats/min). Moreover, responses to intravenous ANG II were unaffected \( (r^2 < 0.04, P > 0.3) \); Fig. 8). Local AT2 blockade also did not alter ANG II-mediated changes in femoral vascular resistance or blood flow \( (r^2 < 0.2, P > 0.1); \) data not shown).

PRA. To determine if the AT antagonists spilled over into the systemic circulation, we measured PRA in arterial blood obtained before, during, and after local and systemic doses of the AT antagonists. During the dose response, samples were taken 20 min after completing a pressor response to permit clearance of infused ANG II and return of PRA to basal values (36). In studies of local infusion of the AT1 antagonist (Fig. 9A) basal PRA was 11.4 ± 2.5 ng·ml\(^{-1}\)·h\(^{-1}\), a value similar to that previously reported for late-gestation fetal sheep (20, 22, 26, 37), and was unchanged across the entire dose response \( (n = 5, P = 0.51) \), averaging 11.8 ± 2.4 and 12.5 ± 2.2 ng·ml\(^{-1}\)·h\(^{-1}\) during and after completion of the study (total dose 125 µg), respectively.

Local infusion of cumulative doses of the AT2 antago-

### Table 2. Measurements of MAP, UmVR, and HR before and after treatment of the umbilical vascular bed with L-158,809

<table>
<thead>
<tr>
<th></th>
<th>MAP, mmHg</th>
<th>UmVR, mmHg·min(^{-1})·l(^{-1})</th>
<th>HR, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td>42.9 ± 1.0 (8)</td>
<td>198 ± 34 (8)</td>
<td>158 ± 5 (8)</td>
</tr>
<tr>
<td>24 h</td>
<td>43.9 ± 0.6 (8)</td>
<td>210 ± 40 (8)</td>
<td>155 ± 5 (8)</td>
</tr>
<tr>
<td>48 h</td>
<td>42.5 ± 0.9 (4)</td>
<td>154 ± 28 (4)</td>
<td>160 ± 13 (4)</td>
</tr>
<tr>
<td>&gt;72 h</td>
<td>44.3 ± 1.5 (3)</td>
<td>194 ± 46 (3)</td>
<td>168 ± 4 (3)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Numbers in parentheses represent number of measurements.
nist (total dose 1,000 µg) also had no effect on PRA, with values averaging 14.1 ± 4.5 and 10.2 ± 4.2 (n = 6) during and after completion of the study. In contrast, systemic infusion of the AT1 antagonist increased PRA from 9.15 ± 2.6 to 16.9 ± 5.4 ng·ml⁻¹·h⁻¹ after a cumulative dose of 50 µg, and PRA remained elevated throughout the remainder of the dose response (n = 5, P = 0.001), averaging 22.7 ± 5.0 ng·ml⁻¹·h⁻¹ at completion of these studies. This rise in PRA is consistent with inhibition of the RAS negative feedback. Pressor responses were not completely inhibited by systemic infusion of the AT1 antagonist until 125 µg total dose had been infused. AT1 blockade after systemic infusion gradually decreased and was only 27% at 72 h. Although basal MAP fell from 44.6 ± 1.6 to 39.2 ± 1.7 mmHg (P = 0.04) immediately after completing the systemic infusions, basal MAP did not differ from control values at 24 h, although pressor responses were inhibited 64%.

**DISCUSSION**

For the RAS to play an important role in fetal cardiovascular homeostasis, functional AT receptors, which easily bind ANG II and transduce the signal for smooth muscle contraction, must be expressed in fetal VSM. We (35) previously demonstrated the presence of a single class of high-affinity AT in the aorta and placental arteries of fetal sheep throughout the last third of gestation. Furthermore, the binding density and affinity were similar to that in adult aorta and mesenteric artery (9, 27). We, therefore, assumed that AT receptors capable of mediating ANG II-induced increases in stresses were expressed in both the systemic and umbilical-placental vasculature of fetal sheep. Two major AT subtypes are now known to exist, but in adult tissues only AT1 receptors mediate contraction responses (4, 7, 9, 16, 46). Cox et al. (11) recently reported that, in contrast to the adult (9), fetal sheep appear to only express AT2 in systemic arteries. In contrast, the umbilical artery and its primary placental
branches express predominantly AT_1. If only AT_1 receptors mediate smooth muscle contraction, the fetal systemic vasculature may be unable or less capable of responding to ANG II, suggesting that vasoconstrictr responses and thus increases in fetal MAP may primarily reflect increases in umbilical vascular resistance or the release of another vasoconstricting agent, e.g., catecholamines (23), neither of which have been studied. In the present study we determined 1) the AT subtype expressed in umbilical and femoral arteries, the latter representative of a systemic vascular bed, 2) if differences in subtype expression account for the observed differences in umbilical and systemic responsiveness to infused ANG II, and 3) how the umbilical and systemic vasculature contribute to ANG II-induced increases in fetal MAP.

As reported by Cox et al. (11), the predominant AT subtype in umbilical VSM was AT_1, whereas the primary subtype in femoral VSM was AT_2, consistent with observations in several other vascular beds in fetal sheep. The AT_1 receptor is also expressed in the human umbilicoaplacental circulation (24, 25), but systemic artery subtype expression is not yet described. Similar to adult tissues expressing AT_1 receptors, ANG II increased umbilical resistance dose dependently. Contraction responses to ANG II by adult smooth muscle that predominantly express AT_2 receptors, e.g., uterine artery and nonpregnant myometrium, are either absent or markedly attenuated (9, 12). This, however, has not been examined in fetal smooth muscle. In the present study umbilical vascular responses to infused ANG II exceeded those in MAP, confirming prior reports (8, 36, 47) and suggesting that the systemic vasculature is less sensitive to ANG II. This, however, is indirect evidence, and, as seen in the present study, acute increases in MAP can be modified by alterations in cardiac output through baroresponses. We, therefore, implanted a flow probe on a femoral artery and examined for the first time fetal vascular responses to several doses of ANG II by an apparent AT_2 predominant vascular bed. Although ANG II caused dose-dependent increases in femoral vascular resistance like that in the umbilical vascular bed, the femoral vascular response was less sensitive to ANG II, as evidenced by a downward shift in the dose-response curve. Thus at estimated plasma levels >90 pg/ml, as determined from the rates of infusion used in the present study (36), the umbilical vascular bed demonstrated greater sensitivity to systemic ANG II infusions, especially at estimated plasma levels ≥200 pg/ml. This difference in sensitivity was previously observed by Iwamoto and Rudolph (21), who reported that a 30-min infusion of a single ANG II dose did not affect resistance in the “periphery,” whereas umbilicoaplacental resistance rose 30%. This difference in AT subtype expression may also explain why the umbilicoaplacental vasculature is more sensitive to ANG II than the maternal uteroplacental vasculature (36), which like fetal systemic arteries primarily expresses AT_2 receptors in VSM (10).

Although both the umbilical and systemic vasculature responded to infused ANG II, the difference in their dose-response curves and, thus, sensitivity to this agent raises questions regarding the mechanism(s) whereby ANG II increases fetal MAP. The present data suggest, but do not conclusively prove, that it may occur primarily by increasing umbilical vascular resistance, a vascular bed that accounts for ~40% of cardiac output. Systemic infusions of saralasin, a nonspecific AT antagonist and losartan, an AT_1 receptor blocker, inhibit ANG II-induced pressor responses in fetal sheep (32, 39). However, systemic infusion of AT antagonists will neither separate umbilical and systemic responses, nor will it differentiate the differences in AT subtype expression. To address this we inhibited only the umbilical responses to ANG II with an AT subtype-specific antagonist, L-158,809. Although we also blocked the hindlimb vasculature, it accounts for only 5–8% of cardiac output (5, 44) and would have had minimal effects on the pressor response. Local infusion of the AT_2 antagonist did not affect the rise in umbilical resistance or the pressor response after a cumulative dose 10-fold greater than the total dose of the AT_1 antagonist. Thus it is unclear if the AT_2 receptor modulates fetal VSM responsiveness. In contrast, infusion of small cumulative doses of the AT_1 antagonist into the umbilical circulation dose dependently inhibited...
ited all of the responses to infused ANG II. Furthermore, the relative inhibition of the pressor response was directly and linearly related to inhibition of the rise in umbilical resistance, suggesting that as much as 80% of the ANG II-induced rise in fetal MAP may be due to increases in umbilical vascular resistance, while responses in the systemic vasculature account for the remaining portion of the pressor response. It is notable that, as early as 1962, Dawes (14) suggested a substantial role for the umbilicalplacental vascular bed in modulating systemic vascular resistance and thus arterial pressure.

For this conclusion to be correct, it is necessary to demonstrate that the AT\textsubscript{2} antagonist did not spill over into the systemic circulation, thereby resulting in systemic AT blockade. In fetal sheep the RAS negative-feedback system is functional in late gestation (34). Furthermore, it can be competitively inhibited by systemic infusion of the nonspecific receptor antagonist saralasin, resulting in increases in PRA (20, 22, 26, 36). Thus we evaluated the spillover of the AT antagonists by comparing the effects of local and systemic AT blockade on the levels of arterial PRA. The AT\textsubscript{2} antagonist had no effect on PRA. Although local AT\textsubscript{1} infusion inhibited both the umbilical and pressor responses after a total cumulative dose of 125 µg, PRA was unchanged. In contrast, after systemic infusion of 50 µg of the AT\textsubscript{1} antagonist arterial PRA nearly doubled and remained elevated with subsequent doses, whereas the vascular responses were minimally affected until the cumulative dose exceeded 50 µg. It is unlikely the infused ANG II or the rise in MAP played a role in masking a rise in renal renin release, because samples for PRA were taken 20 min after the pressor response to each dose of the blocker. At this time MAP was at basal values and plasma ANG II should have returned to preinfusion levels because its half-life is ~20 s and clearance would be complete by 1.5 min, i.e., >15 min before sampling. These data, therefore, not only support the view that local infusion of small cumulative doses of the AT\textsubscript{1} antagonist inhibited only the umbilical and hindlimb vascular beds, but also that the RAS negative-feedback system in fetal sheep is mediated through AT\textsubscript{1} receptors, consistent with its presence in the fetal kidney at this age (33). Furthermore, complete inhibition of the negative-feedback mechanism occurred before inhibition of vascular responses to ANG II. We, therefore, have demonstrated in fetal sheep for the first time that the negative-feedback mechanism is more sensitive to competitive inhibitors than the VSM, confirming in the fetus observations made in adult animals more than 20 years ago (23, 28).

Surprisingly, although AT\textsubscript{2} receptors accounted for the majority, if not all, of the AT expressed by the fetal femoral artery, this vascular bed responded to ANG II and this was blocked by local infusion of the AT\textsubscript{1} antagonist. It is possible that AT\textsubscript{1} receptors are expressed in the resistance vessels, that AT\textsubscript{2} receptors function differently in the fetus compared with the adult, or that ANG II locally releases another vasoconstrictor. The first of these has not been addressed and will require the study of much smaller arteries. Alternatively, the responses may reflect an AT\textsubscript{2} receptor that contracts in the presence of ANG II. If this were the case, the hindlimb responses to ANG II should have been inhibited by AT\textsubscript{2} blockade. This, however, was not seen at a dose 10-fold greater than the AT\textsubscript{1} antagonist. Because AT\textsubscript{1} blockade completely inhibited hindlimb responses, it appears that these responses were mediated via AT\textsubscript{1} stimulation. In adults, ANG II can alter the release and reuptake of catecholamines by peripheral sympathetic neurons (23) and AT are present on the endothelium (10). Thus the hindlimb responses could reflect stimulation of local α-receptors or release of an endothelial-derived vasoconstrictor. Neither have been examined in the fetus. Finally, there is the possibility that the peripheral vascular bed is capable of autoregulation, as seen in adult vascular beds that predominantly express the AT\textsubscript{2} receptor (40, 41). This is supported by the finding that increases in femoral resistance paralleled those in perfusion pressure such that femoral blood flow was unchanged over the range of doses studied, and at the highest dose there was a fall in resistance and rise in blood flow. Peeters et al. (31) suggested this possibility some time ago. To address this it will be necessary to perform studies comparing hindlimb responses to local intra-arterial ANG II infusions with those obtained during systemic ANG II infusion.

In the present study we have demonstrated that the umbilical and systemic artery smooth muscle express different AT receptor subtypes. We also have presented data demonstrating that significant differences exist in the sensitivity to infused ANG II by the umbilical and systemic vasculature. This not only confirms earlier reports (21, 36, 47), but also suggests for the first time that this difference in vascular sensitivity to ANG II may parallel differences in AT subtype expression. However, it is unclear if smooth muscle protein expression and function also differ in the two vascular beds, which could further add to the attenuated contraction responses by the systemic vascular bed, and if AT subtype expression in proximal and distal arteries of the hindlimb differ, which could account for the femoral responses to infused ANG II. In addition, the present study provides evidence that the RAS negative-feedback mechanism in fetal sheep is mediated through the AT\textsubscript{1} receptor and that this is more sensitive to receptor blockade than the VSM, a finding consistent with studies in the adult (23, 28). It remains unclear from these studies, however, what role the AT\textsubscript{2} receptor plays in fetal vascular regulation and how ANG II mediates its effects on the systemic vasculature.

Perspectives

The mechanisms regulating fetal blood pressure are not clearly defined. ANG II is considered an important regulatory hormone in fetal sheep, modulating basal blood pressure and its maintenance during hemorrhage (2, 22). The umbilical vascular bed of fetal sheep is more sensitive to ANG II than other vascular beds (1, 20, 21, 47). Thirty-five years ago, Dawes (14) suggested...
this vascular bed played a prominent role in modulating systemic vascular resistance, because it accounts for ~40% of fetal cardiac output (14). Two AT receptor subtypes are now known to exist. AT1 receptors mediate smooth muscle contraction, are expressed in most adult VSM (4, 18, 42), and predominate in umbilical VSM. In contrast, AT2 receptors do not mediate contraction (9, 19), VSM (14, 42), and predominate in umbilical VSM. In smooth muscle contraction, are expressed in most adult delivery or arterial PO2 (44, 47). Thus hypotensive ANG II without altering fetal oxygen uptake and can decrease 40–50% during physiological increases in further investigation. The difference in receptor subtype expression and ANG II sensitivity, therefore, may explain in part how ANG II acutely modulates in fetal tissues without altering tissue oxygen delivery, while diverting 300 ml of well-oxygenated blood to other tissues without altering tissue oxygen delivery, while simultaneously maintaining perfusion pressure. Future studies should be directed toward further understanding the differences in umbilical and systemic VSM maturation and function, especially in small arteries, to test these hypotheses.

We thank Susan Battle for help in the preparation of this manuscript.

This investigation was supported by National Institutes of Health Grant HD-08783. J. R. Kaiser was a postdoctoral trainee in neonatal-perinatal medicine.

These data were presented in part at the 43rd Annual Meeting of the Society for Gynecologic Investigation, Philadelphia, PA, 1995.

References


