Mechanism of uterine vascular refractoriness to endothelin-1 in pregnant sheep

SHERRIE McELVY,1 SUZANNE G. GREENBERG,1 JOHN L. MERSHON,1 DA SENG YANG,1 CATHERINE MAGILL,2 AND KENNETH E. CLARK1

1Department of Obstetrics and Gynecology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0526; and 2Axys Pharmaceuticals, San Francisco, California 94080

Received 21 September 2000; accepted in final form 25 April 2001

Since the discovery of endothelin-1 (ET-1) (10, 30), a potent vasoconstrictor and produces marked pressor responses when given systemically. Studies in sheep have demonstrated that during pregnancy the uterine vasculature is refractory to exogenously administered ET-1. We hypothesize that this pregnancy-dependent refractoriness is due to an upregulation of local uterine metabolism of ET-1 and/or ETB receptors and/or downregulation of local uterine ETA receptors. To investigate these possibilities, 21 nonpregnant and 17 pregnant sheep were used. Dose-response curves to intravenous infusion of ET-1 and phenylephrine were generated for pregnant and nonpregnant sheep. ET-1 infused systemically demonstrated vasoconstriction in the systemic and renal vasculature of pregnant and nonpregnant animals and vasoconstriction in the uterine vasculature of nonpregnant animals. The pregnant animals showed no uterine vascular response to ET-1. In contrast, phenylephrine showed vasoconstriction in the systemic, renal, and uterine circulations in both pregnant and nonpregnant sheep. After experimentation, the animals were euthanized, and tissues were harvested for Western blot and activity analysis of neutral endopeptidase (NEP) or RT-PCR analysis of endothelin-converting enzyme (ECE) and ETA and ETB receptors. The content and activity of NEP in the uterine and renal vasculature of pregnant and nonpregnant animals were similar. RT-PCR demonstrated the presence of ECE in the uterine vasculature of pregnant and nonpregnant sheep. ETA receptor mRNA was significantly reduced in pregnant compared with nonpregnant sheep, whereas ETB receptor mRNA remained unchanged. We conclude that the uterine vascular refractoriness seen in the pregnant sheep is due to a downregulation of ETA receptors.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
ing a role for NEP in regulating uterine smooth muscle cell contraction in late pregnancy (19). This led us to speculate that an increase in NEP during pregnancy may increase metabolism of ET-1 in the uterine vasculature of pregnant animals, thus offering a potential explanation for our previously reported uterine refractoriness to exogenously administered ET-1 (31).

ET receptors belong to the superfamily of G protein-coupled receptors and exist in two subtypes, ETA and ETB. In vascular tissue, ETA receptors are expressed on vascular smooth muscle and are responsible for vasoconstriction (23). ETB receptors are expressed on the vascular endothelium and mediate the transient vasodilator response to ET-1 through the release of nitric oxide (NO) and/or prostacyclin (23).

Because it is unknown whether the uterine vascular refractoriness seen in the pregnant sheep is due to an increased metabolism of ET-1 by NEP or an alteration of ETA and/or ETB receptors, we undertook this study to compare the content and activity of NEP and to determine the presence and quantity of ETA and ETB receptors in the uterine arteries of pregnant and non-pregnant sheep. These cellular results were compared with the hemodynamic responses to ET-1 in the renal and uterine vasculature.

MATERIALS AND METHODS

Animals and Surgical Preparation

Twenty-one nonpregnant (50–70 kg) and seventeen pregnant ewes (110–115 days gestation) were purchased from two commercial vendors (Joy Russell; Williamsburg, OH; and Tom Morris; Reistertown, MD) and used in various portions of this investigation. All studies were conducted under an approved Institutional Animal Care and Use Committee protocol, and the sheep were housed in a facility approved by the American Association for Accreditation of Laboratory Animal Care. Our surgical procedures and instrumentation of pregnant and nonpregnant sheep with maternal femoral artery and vein catheters, fetal femoral artery and vein catheters, and maternal uterine and renal artery flow probes have been previously published (9).

In this study, all nonpregnant animals were ovariocto-

mized to control for cyclic fluctuations in endogenous estrogen. All but eight of the nonpregnant sheep were instrumented with chronic indwelling catheters and flow probes as described above; these instrumented ewes received estradiol-17β (1 μg/kg iv in ethanol) each evening postsurgery to prevent uterine atrophy. To determine whether NEP, ECE, or ET receptors were affected by estradiol-17β, the eight nonpregnant sheep not instrumented were divided into two groups: 1) an estrogen-depleted group receiving no estrogen and euthanized 7 days postovariectomy; and 2) an estrogen-treated group receiving estradiol-17β (1 μg/kg iv) each evening for 7 days and euthanized after the administration of an estrogen bolus (1 μg/kg iv), which assured that tissue harvesting was at peak estrogen response (~2 h after giving estrogen).

All pregnant animals were instrumented as stated above. The pregnant and nonpregnant animals that were surgically instrumented were given at least 7 days to recover before participating in experimental protocols. The pregnant animals were euthanized after experimentation and before 138 days gestation to prevent labor and fetal delivery. In all animals used for tissue collection, the uterine artery and renal cortex were rapidly removed after euthanasia, and tissues were immediately frozen in liquid nitrogen and stored at −80°C.

Experimental Protocols

ET-1 and phenylephrine dose-response curves. To confirm the uterine-specific refractoriness to systemically administered ET-1, dose-response curves were generated in conscious chronically instrumented animals. Six pregnant and six nonpregnant sheep, all instrumented as described above, were allowed to recover, and baseline recordings of mean arterial pressure (MAP), heart rate, and uterine and renal blood flow were obtained. After baseline measurements, animals received intravenous infusion of either ET-1 in a series of six doses (0.1, 0.3, 1.0, 3.0, 10, and 30 ng·kg⁻¹·min⁻¹) or phenylephrine in a series of four doses (0.1, 0.3, 1.0, and 3.0 μg·kg⁻¹·min⁻¹). Each drug was administered for a period of 10 min per dose, with data recorded as above. Sheep were allowed a 3-h reequilibration period, which was more than adequate for all parameters to return to baseline.

ET-1 dose-response experiments were repeated before and after administration of Nω-nitro-l-arginine methyl ester (l-NNAME) in a cohort of six pregnant sheep. After baseline measurements, animals received an intravenous infusion of ET-1 in an abbreviated series of four doses (1.0, 3.0, 10.0, and 30.0 ng·kg⁻¹·min⁻¹) for a total of 10 min per dose, with data recorded as above. Sheep were allowed a 3-h reequilibration period, which was more than adequate for all parameters to return to baseline. l-NNAME (10 mg/kg) was then administered as an intravenous bolus, and new baseline measurements were recorded. The ET-1 cumulative dose-response curve was then repeated.

Western blot analysis of NEP. Twelve pregnant, eight nonpregnant estrogen-treated, and five nonpregnant estrogen-depleted animals were used for this analysis. Frozen renal cortex and secondary uterine arteries were pulverized on dry ice, homogenized, and suspended in 50 mM Tris-HCl with 5 mg/ml each of aprotonin and leupeptin and 0.1 M phenylmethylsulfonyl fluoride. The protein concentration of each homogenate was determined using a modification of the Lowry technique (detergent compatible protein assay, Bio-Rad; Hercules, CA).

Samples (10 μg protein/well) were separated by electrophoresis on 8–16% gradient gels. After separation, samples were transferred to nylon membranes (MagnaGraph, MSI) by electrophoresis at 0.750 A for 4 h in a cold room. After transferring, the membranes were incubated in a blocking solution [5% nonfat dry milk, 5% gelatin, and 1% normal goat serum in Tris-buffered saline (TBS)] for 1 h at room temperature on an orbital shaker. Membranes then received 3- to 15-min washes with TBS containing 0.5% Tween 20. Washed membranes were incubated in primary antibody (polyclonal NEP antibody B58, 1:50,000; gift from Axys Pharmaceutical; San Francisco, CA) overnight at room temperature. Membranes were washed in Tween 20-TBS for 3- to 15-min washes and incubated in secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase, 1:10,000; Pro-
phosphorylated on 1% agarose gels and stained with ethidium bromide.

Quantitation of the PCR products was performed by computerized densitometry (AlphaImager 2200, Alpha Innotech; San Leandro, CA), with all reactions run on a single gel for comparison. The densities were normalized to the corresponding actin bands.

Statistical analysis. Data were analyzed by one-way ANOVA, two-way ANOVA with repeated measures, or Student’s t-test as appropriate. Data are presented as means ± SE. Results were considered significant at the P < 0.05 level.

RESULTS

Dose-Response Curves

When ET-1 was infused systemically in a cumulative series of six doses, nonpregnant sheep responded with dose-dependent uterine vasoconstriction, whereas pregnant sheep were almost unresponsive to the vasoconstrictive effect of ET-1. Figure 1 demonstrates this ET-dependent increase in uterine vascular resistance, which resulted in a marked and progressive decrease in uterine blood flow in the nonpregnant animals and no effect on uterine blood flow or vascular resistance in

Fig. 1. Changes in uterine blood flow (A) and uterine vascular resistance (B) in response to increasing doses of endothelin-1 (ET-1) infusion in nonpregnant and pregnant sheep. *P < 0.05 and ** P < 0.01 vs. control period. P < 0.0001, pregnant vs. nonpregnant dose-response curves.
the pregnant animals. This pregnancy-associated refractoriness to ET-1 appeared to be specific to the uterine vasculature, because measurement of renal blood flow and renal vascular resistance in response to ET-1 showed very similar responses between nonpregnant and pregnant sheep (Fig. 2). In both groups, renal blood flow fell from baseline in a dose-dependent fashion by up to 40–50% as a result of the increase in renal vascular resistance. As in the uterine vasculature, the majority of the response occurred at the highest three doses of ET-1. Both pregnant and nonpregnant sheep exhibited a dose-dependent increase in MAP in response to ET-1 (Fig. 3). This response was slightly, although not significantly, blunted in pregnant animals.

It is possible that the uterine vascular refractoriness to ET-1 observed in the pregnant sheep is due to compensatory release of a local vasodilator such as NO. Therefore, ET-1 dose-response curves were repeated in pregnant animals in the presence of L-NAME, an inhibitor of NO synthase. In this cohort of six sheep, uterine blood flow again did not change in response to increasing doses of intravenous ET-1, and this refractoriness was not affected by administration of L-NAME (Fig. 4). As expected, baseline uterine blood flow was slightly decreased after NO inhibition (from 540 ± 58 to 403 ± 44 ml/min), and in fact the entire dose-response curve was shifted to lower values in the presence of L-NAME. This suggests that while endogenous NO does contribute to the regulation of basal uterine tone in the pregnant sheep, it does not appear to be involved in the mechanism of pregnancy-associated uterine vascular refractoriness to ET-1.

To determine whether the uterine vascular refractoriness to ET-1 observed in pregnant animals was specific to ET-1, dose-response experiments were repeated using a second vasoconstrictor, phenylephrine. While the uterine vasculature was slightly less sensitive to phenylephrine during pregnancy, this was not significant and was quite different from the complete refractoriness observed in response to ET-1. By the highest dose of phenylephrine, uterine vascular resistance more than doubled in pregnant sheep, and this was associated with an ~50% reduction in uterine blood flow (Fig. 5). In the renal vascular bed, the response to phenylephrine was also slightly but not significantly

Fig. 2. Changes in renal blood flow (A) and renal vascular resistance (B) in response to increasing doses of ET-1 infusion in nonpregnant and pregnant sheep. *P < 0.05 and **P < 0.01 vs. control period.

Fig. 3. Changes in mean arterial pressure in response to increasing doses of ET-1 infusion in nonpregnant and pregnant sheep. **P < 0.01 vs. control period.

Fig. 4. Changes in uterine blood flow in pregnant animals in response to increasing doses of ET-1, either alone or in the presence of 10 mg/kg Nω-nitro-1-arginine methyl ester (L-NAME). n = 6.
blunted in pregnant animals, similar to what was observed in the uterus (data not shown). Overall, the renal vasculature was much less sensitive to phenylephrine than to ET-1, with renal blood flow falling by only 25% from baseline even at the highest dose of phenylephrine. MAP responses to phenylephrine were very similar between pregnant and nonpregnant animals, with a significant increase in pressure observed only at the highest dose in both groups (data not shown).

Western Blot Analysis and Enzymatic Activity for NEP

A representative Western blot is shown in Fig. 6, with bands corresponding to NEP detected in homogenized uterine artery samples shown on the right. In Fig. 6, left, an example of a standard curve generated from a dilutional series of recombinant human NEP (1.250–39 pg) is shown; similar dilutional “standard curves” were included on each gel to allow quantitation of NEP content in tissue samples. Figure 7 demonstrates that there was no significant difference in the content of NEP in uterine arteries from all three groups of sheep. Estrogen-depleted nonpregnant animals were found to have a slightly lower NEP content than the pregnant sheep (35 ± 8.9 vs. 56 ± 7.6 pg/μg protein), whereas the estrogen-treated nonpregnant animals were found to have an insignificant increase in total NEP (69 ± 19.3 pg/μg protein). Similarly, the NEP content measured in the renal cortex was not significantly different in all three groups of sheep (pregnant: 100 ± 24.5; estrogen-depleted nonpregnant:
When the above tissues were further analyzed to determine total NEP activity, it was found that uterine arteries taken from all three groups had similar levels of total NEP activity (pregnant: 54 ± 6; estrogen-depleted nonpregnant: 57 ± 4; estrogen-treated nonpregnant: 51 ± 3 nmol product h⁻¹ mg protein⁻¹; Fig. 7). Thus it does not appear that the uterine vascular refractoriness to ET-1 observed in pregnant sheep is mediated by pregnancy-related differences in the local metabolism of ET-1. In support of this finding, there was no correlation between NEP activity and uterine blood flow measured immediately before death in any of the groups studied (data not shown). In renal cortical tissue, total NEP activity was substantially higher, as expected due to greater content; but again, levels were similar between all three groups of sheep (pregnant: 208 ± 21; estrogen-depleted nonpregnant: 184 ± 87; estrogen-treated nonpregnant: 234 ± 9 nmol product h⁻¹ mg protein⁻¹; Fig. 7).

**RT-PCR Analysis and Quantification**

With the use of RT-PCR, partial cDNA clones for ovine ECE-1 and ETA and ETB receptors were obtained in this study. The sequence for ovine ECE-1 (Genbank Accession No. AF294269) is nearly identical to the bovine cDNA (12) (97% at the nucleotide level and >99% at the protein level). Compared with the human ECE-1 sequence (32), this ovine partial cDNA is 91 and 96% identical at the nucleotide and protein level, respectively. The ovine ETA and ETB receptor partial cDNA clones (Genbank Accession Nos. AF293847 and AF349439, respectively) are also highly homologous to the bovine cDNA (3), 98 and 100% identical at the nucleotide and protein levels, respectively. The ovine ETA receptor shows 90% identity at the nucleotide level and 98% identity at the protein level to the human ETA receptor (11). The ovine ETB receptor shows 90.5% identity at the nucleotide level and 96% identity at the protein level to the human ETB receptor (Genbank Accession No. XM007108).

RT-PCR amplification of total RNA from uterine arteries of pregnant and nonpregnant sheep demonstrated the presence of ECE-1 mRNA in all three groups. The bands in each of these groups demonstrated some variation in density. On the other hand, ET0 receptor mRNA differed significantly between the pregnant and nonpregnant sheep. Figure 8 is representative of these RT-PCR results. This diagram depicts the results from two representative sheep from each group for ECE-1, ETA receptor, ETB receptor, and β-actin (control). Densitometry analysis of ETA receptor products (Fig. 9) revealed that this message was significantly reduced in pregnant compared with nonpregnant control ewes (from 1.45 ± 0.29 to 0.93 ± 0.07 units). Interestingly, ETB receptor mRNA was also somewhat suppressed by estrogen treatment in the nonpregnant animals (from 1.45 ± 0.29 to 0.98 ± 0.09 units), although this did not reach statistical significance. Densitometry analysis of ETB receptor products showed that while the message for this receptor subtype is present in the ovine uterine vasculature, it does not appear to be affected by either estrogen treatment or pregnancy (Fig. 9).

**DISCUSSION**

From earlier studies in our laboratory, we know that there is a local uterine refractoriness to ET-1 in preg-
nant sheep (31). Because we hypothesized that the uterine vascular refractoriness seen in the pregnant sheep is due to increased ET-1 metabolism and/or downregulation of ET_A receptors and/or upregulation of ET_B receptors, we undertook this study to investigate the content and activity of NEP as well as to determine the presence and quantity of ET receptor subtypes in the uterine vasculature. Figure 10 depicts the maturation, degradation, and receptor interaction of ET-1 and should be referenced as we discuss various points in this pathway as potential mechanisms for the uterine refractoriness seen in pregnant sheep. ET-1 is the active metabolite produced by the enzymatic cleavage of Big ET-1. There are two isoforms of this enzyme, ECE-1 and ECE-2. ECE-1 was discovered first and thought only to be extracellular (plasma membrane), but is now known to be intracellular along with ECE-2 (24, 25). A decrease in ECE could lead to decreased conversion of Big ET-1 to ET-1, which would potentiate uterine refractoriness. We looked at the expression of ECE-1 mRNA in uterine arteries and found its expression and quantity to be similar in all groups (pregnant and nonpregnant sheep). These findings allow us to exclude decreased ECE-1 in the pregnant sheep as the mechanism of uterine refractoriness. However, because we did not look at ECE-2, it is uncertain whether a difference in the presence or quantity of ECE-2 exists. Studies by others have shown that the quantity of ECE-2 mRNA is only 1–2% of that of ECE-1 mRNA in cultured endothelial cells (29).

Another mechanism that may result in the uterine refractoriness seen in the pregnant sheep is an upregulation of NEP during pregnancy. Recently, NEP has been found in the ovine uterus including the endometrial stroma, myometrial smooth muscle cells, and vasculature in early pregnancy (22). Our investigation demonstrates a slight estrogenic upregulation of NEP in the uterine arteries, but this did not reach statistical significance. Furthermore, the measured activity in the uterine arteries and renal cortex showed no difference in pregnant and nonpregnant animals. Therefore, NEP upregulation does not appear to be the mechanism responsible for pregnant uterine vasculature refractoriness. It is possible that other ET-1 degradation pathways not addressed by the present study may be upregulated during pregnancy. Clearly, there are a variety of proteases that can inactivate ET peptides (23), and while NEP appears to be the most predominant, it is certainly not specific for ET. Indeed, a novel and seemingly highly specific ET-1-inactivating metalloendopeptidase has been recently described in kidneys from both rats (15) and humans (14). In addition, receptor-mediated mechanisms (i.e., ET_B receptor) may significantly contribute to ET-1 clearance (5). Whether or not any of these pathways is affected by pregnancy remains to be determined.

Another potential mechanism for the observed uterine refractoriness to ET may involve pregnancy-dependent alterations in vascular receptors for ET. ET-1 typically interacts with two receptor types, ET_A or ET_B (Fig. 10). ET_B receptors typically mediate vasodilation via the release of NO; therefore, it is possible that a pregnancy-dependent upregulation of ET_B receptors in the ovine uterine vasculature would serve to offset any local vasoconstrictive response to ET-1 during pregnancy. However, the results of the present study suggest that the ET_B receptor message in the ovine uterine artery is not altered during pregnancy, nor is it influenced by estrogen in nonpregnant animals. With regard to ET_A receptors, interaction of ET-1 with this receptor subtype produces vasconstrictive effects, and these systemic responses are blocked by ET_A receptor-
specific inhibitors in pregnant and nonpregnant animals (17). Whereas ET<sub>A</sub> receptor blockade in nonpregnant animals is associated with reductions in uterine vascular tone, in the pregnant animals there is only minimal effect of blockade of ET<sub>A</sub> receptors on uterine hemodynamics. Therefore, our current study examined the presence and quantity of the ET<sub>A</sub> receptor message. We found that in pregnancy there was a significant reduction in the ET<sub>A</sub> receptor mRNA in the uterine vasculature. It is possible that the mRNA may not correspond with protein expression in the ET system; this could not be ruled out in the present study due to the current unavailability of ovine-specific ET receptor antibodies. However, the RT-PCR findings are consistent with the hemodynamic responses found in this study that demonstrate little or no response to ET-1 in the uterine vasculature of pregnant sheep despite normal dose-dependent systemic and uterine vascular responses in nonpregnant sheep as well as dose-dependent systemic responses in the pregnant sheep. The results of the present study therefore suggest that the uterine refractoriness seen in the pregnant sheep is the result of a downregulation of ET<sub>A</sub> receptors.

Activation by ET-1 of a local uterine vasodilator system is another mechanism that could contribute to the observed uterine refractoriness to ET-1. In other words, if administration of ET-1 were to stimulate the local uterine release of a vasodilator, this would offset the vasoconstrictive effect of ET-1. NO, which is produced by the vascular endothelium, would be a likely candidate for this, particularly because stimulation of ET<sub>B</sub> receptors could mediate this response (23, 27). We explored this possibility in six pregnant animals by repeating the ET-1 dose-response curve in the presence of L-NAME, an inhibitor of NO synthase. In this cohort, we found that the uterine refractoriness to ET-1 was not altered by NO blockade; thus it does not appear that NO is involved in the mechanism. This, however, does not rule out the possible involvement of other potential vaso dilators such as prostacyclin.

ET-1, derived from the vascular endothelium, likely plays an important role in the normal regulation of local as well as global vascular tone. Specific uterine vascular refractoriness to ET-1 during normal pregnancy may serve an important function to protect against sudden and/or substantial decreases in uteroplacental perfusion at times when locally generated or circulating ET-1 becomes elevated. If this refractoriness is the result of a pregnancy-dependent downregulation of uterine ET<sub>A</sub> receptors, as suggested by the present study, then it is possible that disease states associated with decreased uterine blood flow (such as preeclampsia) may involve an aberration in this protective mechanism. This could lead to the uterine vasculature being more sensitive to fluctuations in endogenous ET-1, resulting in increased uteroplacental vascular tone and ultimately in decreased uterine blood flow.

The authors gratefully acknowledge Jeanne Hirth for performing the Western blot and activity analysis for NEP. We also thank R. Scott Baker and Angella Friedman for diligent assistance with the daily maintenance of animals used in this study.

This research work was supported in part by National Heart, Lung, and Blood Institute Grants HL-49901, HL-51051, and HL-56714 as well as by American Heart Association Fellowship Grant #9920620V (to S. McElvy).

REFERENCES


17. McElvy S, Baker RS, Greenberg SG, Khoury J, and Clark KE. Effect of selective endothelin A receptor antagonist on


