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Endothelin and platelet-activating factor: Significance in the pathophysiology of ischemia/reperfusion-induced fetal growth restriction in the rat

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Abstract

Objective—The objective of the study was to evaluate the role of endothelin-1 and platelet-activating factor in ischemia/reperfusion-induced fetal growth restriction in the rat.

Study design—On day 17 of gestation, the right uterine and ovarian arteries were occluded for 30 minutes in experimental but not sham-operated rats. All rats received endothelin receptor A antagonist, A-127722 (10 mg/kg per day), platelet-activating factor antagonist, WEB-2086 (1 mg/kg), or vehicle. On gestational day 21, litter size, fetal viability, and fetal and placental weights were recorded. Reverse transcription–polymerase chain reaction for phospholipase A₂-IIA and preproendothelin-1 messenger ribonucleic acid was performed on uterus and placentas from each uterine horn. Groups were compared statistically by analysis of variance.

Results—Ischemia/reperfusion reduced fetal weights, in both the ischemic horn and the nonischemic horn ($P < .001$). Antagonism of either endothelin receptor A or platelet-activating factor normalized fetal growth in both horns. Neither placental weight nor the incidence of fetal demise was affected by ischemia/reperfusion. Phospholipase A₂-IIA and preproendothelin-1 messenger ribonucleic acid expression did not differ between right and left uterine horns in any group. Uterine and placental tissues in the ischemia/reperfusion group exhibited increased phospholipase A₂-IIA ($P < .01$) but not preproendothelin-1.

Conclusion—Endothelin-1 and platelet-activating factor are both important mediators in the pathophysiology of ischemia/reperfusion-induced fetal growth restriction in the rat, contributing to the fetal growth restriction observed in both the ischemic and nonischemic horns. Antagonism of either mediator produces normal fetal growth in this model of fetal growth restriction.

Keywords

Endothelin; Platelet-activating factor; Ischemia/reperfusion; Fetal growth restriction; Rat

Fetal growth restriction (FGR) remains a common and important cause of perinatal morbidity and mortality.^{1–3} This is particularly true when growth restriction complicates a pregnancy remote from term.⁴ Suboptimal uteroplacental perfusion is the most common identifiable cause of FGR. Histologic evaluation of placentas from pregnancies complicated by FGR commonly demonstrates evidence of placental ischemia and/or infarction. The severity of fetal growth restriction correlates with number and severity of ischemic lesions observed.⁵ The distribution of these ischemic findings implicates the limitation of blood flow through the maternal spiral

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arteries.⁶ Because the redundancy of uteroplacental vessels allows for collateral blood flow to placental units, the presence of an infarct suggests not only the obstruction of a uteroplacental vessel but also compromise of collateral blood flow.⁷ Evidence for redistribution of blood flow within the placental lobules is commonly observed, suggesting that the dynamics of ischemia/reperfusion (IR) are at work within the substance of the placenta.⁸ The mechanisms regulating IR in the placenta have not been elucidated.

Animal models have been developed to evaluate the role of uterine ischemia in FGR. Wigglesworth⁹ originally demonstrated that ligation of the uterine artery in the rat results in fetal growth restriction. More recently temporary uterine ischemia by unilateral occlusion of uterine perfusion followed by reperfusion of the ischemic horn¹⁰ has been shown to result in FGR in both uterine horns, not just the one subjected to ischemic insult.¹¹ This model is more analogous to the pathophysiology occurring in human FGR than the previous uterine artery ligation model, particularly because it reveals the impact of IR on regional blood flow in the placenta. The occurrence of FGR even after relatively brief occlusion of uterine blood flow suggests that it is not only the restriction of blood flow per se that results in a compromise of fetal growth but the long-term consequences of IR that is at play. Furthermore, the restriction of fetal growth in the contralateral uterine horn suggests that circulating factors or mediators are involved.

Endothelin-1 (ET-1) is a vasoactive mediator produced primarily by endothelial cells. ET-1 is the most potent endogenous vasoconstrictor known and, along with its receptors, is expressed in the placenta. ET-1 infusion in maternal rats results in FGR independent of maternal hypertension.¹² ET-1 also plays a primary role in the pathophysiology of both nitric oxide synthase inhibition as well as hypoxia-induced FGR.^{13,14} Ischemia is a potent stimulus to ET-1 production. ET-1 has previously been shown to play an important role in the pathophysiology of IR injury in the lung.^{15,16} Consequently, we hypothesize that ET-1 may also play a role in the pathophysiology of IR-induced FGR.

Platelet-activating factor (PAF) is a proinflammatory mediator that also has vasoactive properties by virtue of its effect on vascular smooth muscle. We have previously demonstrated that maternal PAF infusion in the rat results in FGR.¹⁷ PAF has previously been shown to act synergistically with ET-1 to decrease local perfusion in other models, including bacteremia-induced pulmonary hypertension in the lung,¹⁸ acute pancreatitis,¹⁹ and IR in transplanted lung.¹⁶ Ishimoto et al²⁰ demonstrated that PAF plays a role in the pathophysiology of IR-induced FGR, ameliorating FGR by administration of a PAF receptor antagonist. The relative contributions of ET-1 and PAF in this model have not been evaluated. We hypothesize that PAF, acting synergistically with ET-1, contributes to the pathophysiology of IR-induced FGR. The purpose of this study was to evaluate the significance of ET-1 and PAF in IR-induced FGR.

Material and methods

Endothelin receptor and PAF receptor antagonists

A-127722 (provided by Abbott Laboratories, Abbott Park, IL) is a nonpeptide compound with a high affinity for ET_A receptors ($K_i = 0.069$ nM) and a low affinity for ET_B receptors ($K_i = 139$ nM). It has a 35% bio-availability in rats and a plasma half-life of 3.5 hours.²¹ WEB-2086 (provided by Boehringer-Ingelheim, Ingelheim, Germany) is a thieno-triazolodiazepine PAF receptor antagonist with a median inhibitory concentration of 170 nM and a plasma half-life of 1 hour.²²⁻²⁴

Animals

Nonpregnant Sprague-Dawley female and male rats were purchased from Harlan Laboratories (Madison, WI), housed in the Evanston Northwestern Healthcare Research Institute Center for Comparative Medicine, maintained in 12-hour light/12-hour dark cycles, and allowed free access to a standard laboratory rodent diet and water. Animal care and the conduct of all experiments were in accord with guidelines approved by the Evanston Northwestern Healthcare Research Institute Institutional Animal Care and Use Committee. Rats were bred at an age of 11 to 20 weeks and a weight of 225 to 250 g. Thirty-six maternal rats were used for this study, 6 in each experimental group.

IR

On day 17 of gestation, a laparotomy was performed under general anesthesia consisting of a single intraperitoneal injection of xylazine, ketamine, and acepromazine in combination (8, 40, and 1.3 mg/kg, respectively). A midline abdominal incision was utilized to expose both uterine horns. Vascular clamps were then applied to both the uterine and ovarian arteries of the right uterine horn to totally occlude perfusion. The uterine horns were replaced into the abdominal cavity, with the occlusion clamps in place. After 30 minutes, the vascular clamps were removed. The abdominal incision was closed. Rats in the sham-operated group had the uterine horns exposed and returned to the abdomen, and then, after 30 minutes, the abdomen was closed.

ET and PAF receptor antagonism

The ET_A antagonist, A-127722 (10 mg/kg per day), was administered intraperitoneally on days 17 to 21 of gestation via an Alzet osmotic pump (5.1 cm long and 1.4 cm in diameter, Durect Corp, Cupertino, CA) placed into the abdomen during IR surgery (sham-ET_A antagonist and IR-ET_A antagonist groups). The PAF receptor antagonist, WEB-2086 (1 mg/kg), was administered intravenously into the femoral vein just prior to the IR surgery (sham-PAF antagonist and IR-PAF antagonist groups). Control rats received only vehicle (20% ethyl alcohol, 40% propylene glycol, and 0.04 M NaOH in H₂O, intraperitoneally by osmotic pump and saline intravenously).

Pregnancy outcome

On gestational day 21, hysterotomies were performed, litter size was noted, fetal viability was determined for each pup, and fetal and placental weights were obtained for each live pup.

Prepro-ET-1 and phospholipase A₂ (PLA₂) expression analysis reverse transcription–polymerase chain reaction (PCR)

Placental and uterine tissues were frozen in liquid nitrogen and stored at –80°C. Total ribonucleic acid (RNA) was extracted using RNA STAT-60 (TEL-TEST, Friendswood, TX), according to the manufacturer's instructions. The concentration of RNA was determined by absorbance at 260 nm, and the purity was checked by the 260:280 nm ratio (greater than 1.8). RNA integrity was verified by electrophoresis in a 1% agarose gel. For each sample, 3 µg of total RNA were reverse transcribed at 37°C for 1 hour in a total of 20 µL of reaction mixture (50 mM Tris-HCl, 75 mM potassium chloride, 2 mM magnesium chloride, 10 mM dithiothreitol, 1.25 mM of each deoxynucleotide-triphosphate, 7.5 pM random hexamer, 1 U/µL RNasin [RNase inhibitor], and 10 U/µL Moloney-murine leukemia virus reverse transcriptase [Invitrogen, Carlsbad, CA]).

Complementary deoxyribonucleic acid (DNA) was amplified by PCR with 0.1 U/µL AmpliTaq DNA polymerase on a GeneAmp 5700 real-time sequence detector (Applied Biosystems, Foster City, CA) in a total volume of 50 µL consisting of 1.0 µL reverse transcription product,

10 mM Tris-HCl, 50 mM potassium chloride, 2 mM magnesium chloride, 0.1 mM of each deoxynucleotide-triphosphate, and 1.0 μ M of each primer.

The primer sequences were, for rat prepro-ET-1, sense 5'-GACCAGCGTCCTTGTTCCAA-3' and antisense 5'-TTGCTACCAGCGGATGCAA-3' for rat PLA₂-IIA, sense 5'-CCGTCTGGAGAAACGTGGAT-3' and antisense 5'-GTTCCGGGCAAACATTTCAG-3'. TaqMan MGB probes were, for rat prepro-ET-1, 6FAM-TCCAAGAGAGGTTGAGGTGT-MGBNFQ and for rat PLA₂-IIA, 6FAM-TGGCACAAAGTTTC-MGBNFQ. The reaction mixtures were heated at 50°C for 2 minutes to allow UNG to degrade dUTP-containing double-stranded DNA and then heated at 95°C for 10 minutes and immediately carried through 60 cycles of PCR with 15 seconds denaturation at 95°C, 20 seconds annealing at 60°C, and 40 seconds extension at 72°C. Quantification was on the basis of standard curves generated from copy number standards. Control ribosomal RNA reagents (Applied Biosystems) were used to normalize all results, which are expressed as the ratio of a specific messenger RNA (mRNA)/ribosomal RNA.

Statistical analysis

Results are presented as mean \pm SEM. Statistical comparisons among groups were made using an analysis of variance (ANOVA) followed by a post hoc Newman-Keuls test or a Kruskal-Wallis nonparametric ANOVA with post hoc Dunn's test, as appropriate. All statistical tests were 2-tailed and results were considered statistically significant at $P < .05$.

Results

Fetal weights were reduced in response to IR, in both the ischemic uterine horn and the contralateral, nonischemic horn ($P < .001$, compared with all other groups). With IR, treatment with either ET_A antagonist or PAF receptor antagonist resulted in normalization of fetal growth in both uterine horns (Figure 1). In the absence of IR, neither ET_A antagonism alone nor PAF receptor antagonism alone had any impact on fetal weight.

Placental weight was not affected by IR. Placental weights in rats treated only with the ET_A antagonist were not significantly different from the control (sham operated, vehicle treated) rats but were significantly lower than those in the IR group ($P < .05$; Figure 2). This reduced placental size in the ET_A antagonist-treated rats had no impact on fetal growth in this group (Figures 1 and 2).

Litter size and numbers of live pups in each litter did not differ among the groups or between right and left uterine horns in any individual group (Table). Fetal demise occurred with similar frequencies in each of the groups in the study.

The transcription of both PLA₂-IIA and prepro-ET-1 mRNA exhibited considerable variability within each group at 4 days after ischemia. There was no difference in the transcription of either PLA₂-IIA or prepro-ET-1 mRNA between the right and left uterine horn in any of the experimental groups; therefore, the results are presented as single values for each tissue and group. Uterine PLA₂-IIA expression was increased in the IR group ($P < .001$), compared with all other groups (Figure 3). There was a trend toward increased prepro-ET-1 mRNA expression in the IR group, compared with the sham-operated, vehicle-treated control group ($P < .09$; Figure 4). Placental PLA₂-IIA expression increased in the IR group, compared with all other groups ($P < .01$), but prepro-ET-1 expression was not significantly different among any of the groups (Figures 5 and 6).

Comment

IR injury in the maternal rat leads to fetal growth restriction. The temporary interruption of uterine blood flow to one horn of the rat uterus leads to reduced fetal growth in not only the temporarily ischemic horn but also the contralateral horn. This suggests that the reduction of fetal growth is caused by not only the temporary interruption of blood flow to the uteroplacental unit(s) but also circulating mediators that are produced in response to this ischemic insult. In this study we have demonstrated that both ET-1 and PAF contribute to the pathophysiology of IR-induced FGR.

Antagonism of either ET-1 or PAF results in normalization of fetal growth in this animal model of FGR. PAF receptor antagonism has been shown previously to ameliorate IR-induced FGR.²⁰ Using a different PAF receptor antagonist, we have observed the same result. ET_A receptor antagonism ameliorates FGR equally well. There are several possible reasons for the complete efficacy of either ET_A or PAF receptor antagonists. Prevention of the vasoconstrictive actions of either of these mediators could result in improved placental perfusion and thereby function. Furthermore, decreased activity of the inflammatory effects of these mediators may also improve placental function. Whether by improving placental perfusion or decreasing inflammation, either of these antagonists improves placental function in the setting of IR sufficiently to normalize fetal growth. Our observation that IR did not have an impact on placental weight is consistent with that of Lewis et al.¹¹ Thus, IR appears to have its primary impact on placental function rather than on placental size, and administration of either antagonist normalizes this function.

ET-1 and PAF may act synergistically to decrease local perfusion and augment the inflammatory response. Stimulation of PLA₂ expression and PAF synthesis by ET-1 via the ET_A receptor has been shown in a variety of cells and tissues.²⁵⁻³¹ PAF and ET have been shown to contribute synergistically to posttransplant lung IR injury in the rat.¹⁶ Studies in rat models of bacteremia-induced lung injury have demonstrated rapid induction of pulmonary hypertension by PAF but mediated in part by ET-1.¹⁸ Similarly, PAF and ET-1 both affect capillary blood flow and vascular permeability in rat models of acute pancreatitis.¹⁹ With respect to IR-induced FGR in the rat, if ET-1 and PAF are functioning synergistically, the expectation would be additional improvement in fetal growth with addition of a second antagonist rather than complete normalization of growth with either antagonist. However, if these antagonists improve placental perfusion and/or function sufficiently to meet the demands of the developing fetus, this additive effect may not be observed. The lack of observation of an additive effect does not rule out synergism between these mediators.

In this study, we evaluated transcription of PLA₂-IIA and prepro-ET-1 mRNA in the uterus and placenta 4 days after the IR insult. We demonstrated that transcription of both of these mediators was responsive to the IR insult. Uterine and placental PLA₂-IIA was significantly increased by IR, but uterine prepro-ET-1 mRNA, even though it appeared elevated in the IR group, did not achieve statistical significance because of high variability. The high degree of variation may in part be due to the timing of our evaluation. After 4 days, compensatory mechanisms may be actively moderating the molecular response. Furthermore, the response of these mediators to the initial insult may be waning. The expression of these mediators may best be studied at a time closer to the ischemic insult to capture the early molecular responses. This is the subject of ongoing investigation in our laboratory.

The fact that expression of these mediators was similar in both horns of the uterus in spite of the ischemic insult occurring in only 1 horn supports the concept that the observed growth restriction is secondary to circulating mediators rather than to the ischemic insult itself. This

study demonstrates that both ET-1 and PAF are prominent mediators in the pathophysiology of this model of FGR.

Other mediators may also contribute to the pathophysiology of IR-induced FGR. Tanaka et al³² demonstrated that thromboxane plays a salient role in the pathophysiology of this model. Leukocytes are known to accumulate in response to tissue reperfusion and to limit postischemic perfusion and fetal growth.³³ Furthermore, other proinflammatory proteins, free radical oxygen species, or nitric oxide (either directly or indirectly) may all contribute to the pathophysiology of this model, as they have in other models of IR.³⁴ Their role in this model of FGR, however, has not been evaluated. IR is likely to set in motion a cascade of events. The prominence of any individual component in these events likely varies in proportion to its contribution to the overall process. However, factors having the greatest impact on perfusion are likely to have the greatest significance in the pathophysiologic process as a whole.

The uterine IR model of FGR in the rat is uniquely suited to study the contribution of vascular mediators to decreased uteroplacental perfusion in FGR. This model may provide valuable insights into the mechanisms operative in uteroplacental dysfunction in humans as well. Inadequate uteroplacental perfusion ultimately leads to ischemia and/or infarction of the placenta, resulting in a compromised capacity for maternal-fetal gas and nutrient exchange. Histologic evidence of placental ischemia is commonly observed in cases of human FGR. Salafia et al⁵ evaluated placentas from nonanomalous preterm singleton live-born fetuses for evidence of uteroplacental ischemia. They found that growth restriction is primarily related to the cumulative number and severity of placental ischemic lesions.

Vascular pathology associated with obliteration of small muscular arteries in the tertiary stem villi,³⁵ and resulting in reduced vascularization of the intermediate and terminal villi,^{36,37} implies that IR may occur in relatively localized areas of the placenta. The mediators produced in response to IR in these localized areas may affect perfusion and function of not only these but also surrounding areas of the placenta. The uterine IR model in the rat is then an attractive model to investigate the pathophysiology of FGR caused by suboptimal uteroplacental perfusion (the most common identifiable cause of FGR in humans) and specifically to study the role of mediators affecting regional perfusion and function and ultimately fetal growth.

Ischemic insults to the placenta result in compromised nutrient and gas exchange in the affected area but also result in the expression of mediators, which may further compromise the function of surrounding areas of the placenta. ET-1 and PAF, possibly acting synergistically, both play a prominent role in this pathophysiologic process. Antagonists to these mediators have been shown to improve pregnancy outcome in several animal models of FGR and deserve consideration as therapeutic modalities in human FGR as well.

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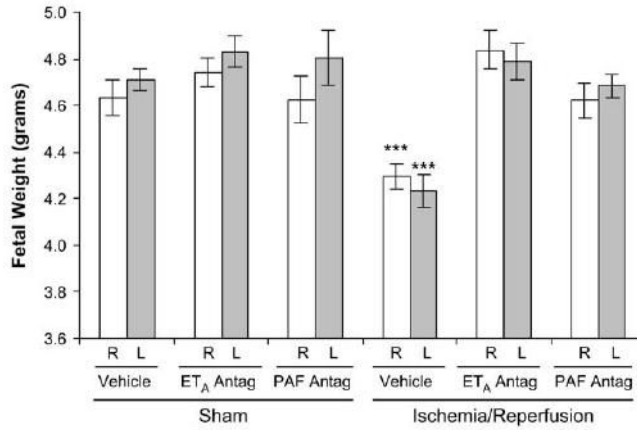


Figure 1.

Fetal weights on gestational day 21 in the right (R, *white bars*) and left (L, *gray bars*) uterine horns 4 days after maternal sham operation or 30-minute IR of the right uterine horn. Fetal weights in both uterine horns of IR rats were significantly lower than those in all other groups. ET_A antagonism and PAF receptor antagonism each prevented fetal growth restriction. Data are mean \pm SEM. ***, $P < .001$ versus all other groups.

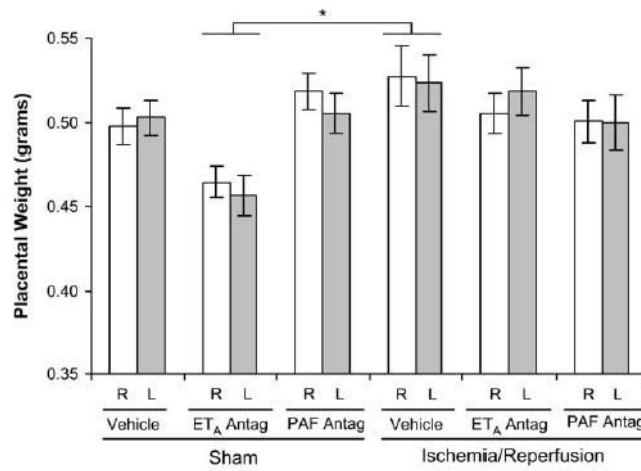


Figure 2.

Placental weights on gestational day 21 in the right (R, *white bars*) and left (L, *gray bars*) uterine horns 4 days after maternal sham operation or 30-minute IR of the right uterine horn. Placental weights in ET_A antagonist-treated rats were significantly reduced, compared with those in vehicle-treated, IR rats. None of the placental weights were significantly different from those in the sham/vehicle-treated rats. Data are mean \pm SEM. *, $P < .05$ for sham/ET_A antagonist versus IR/vehicle groups.

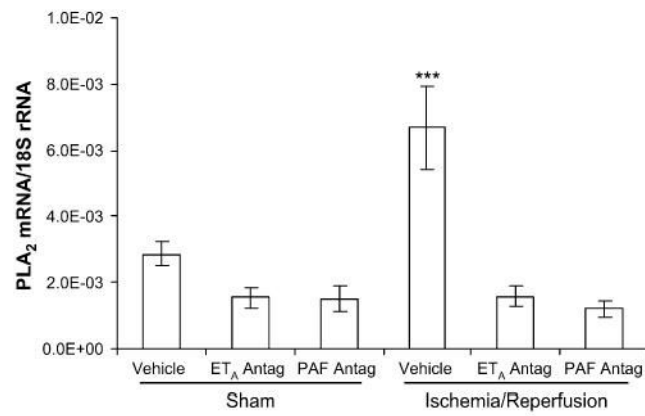


Figure 3.

Uterine PLA₂-IIA mRNA expression on gestational day 21, 4 days after maternal sham operation or 30-minute IR of the right uterine horn. PLA₂-IIA expression was significantly increased in the IR group, compared with all other groups. ET_A antagonism and PAF receptor antagonism each prevented the increase in PLA₂-IIA mRNA. Data are mean ± SEM. ***, $P < .001$ versus all other groups.

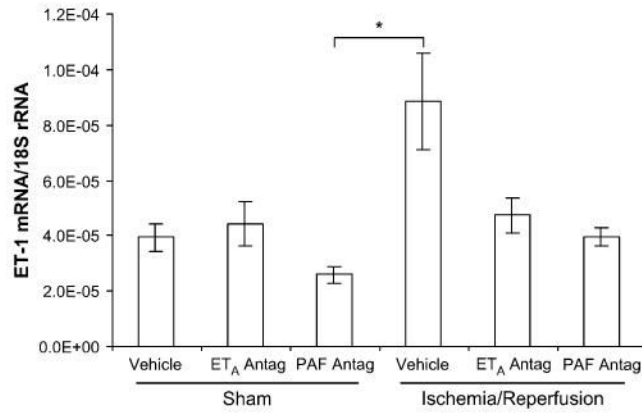


Figure 4. Uterine prepro-ET-1 mRNA expression on gestational day 21, 4 days after maternal sham operation or 30-minute IR of the right uterine horn. Prepro-ET-1 expression was significantly different only between the sham-operated/PAF antagonist-treated rats and the IR/vehicle-treated rats. Data are mean \pm SEM. *, $P < .05$.

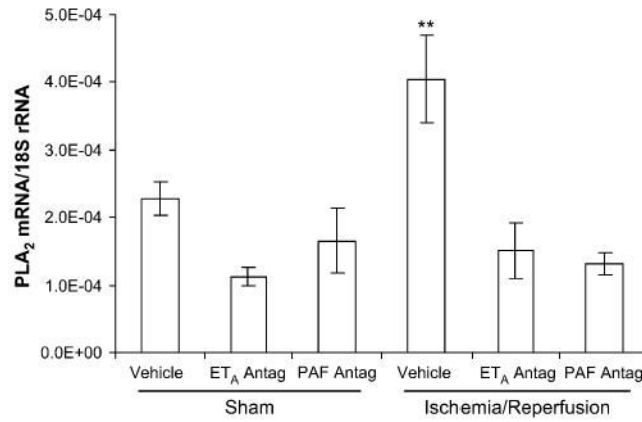


Figure 5. Placental PLA₂-IIA mRNA expression on gestational day 21, 4 days after maternal sham operation or 30-minute IR of the right uterine horn. PLA₂-IIA expression was significantly increased in the IR group, compared with all other groups. ET_A antagonism and PAF receptor antagonism each prevented the increase in PLA₂-IIA mRNA. Data are mean ± SEM. **, $P < .01$ versus all other groups.

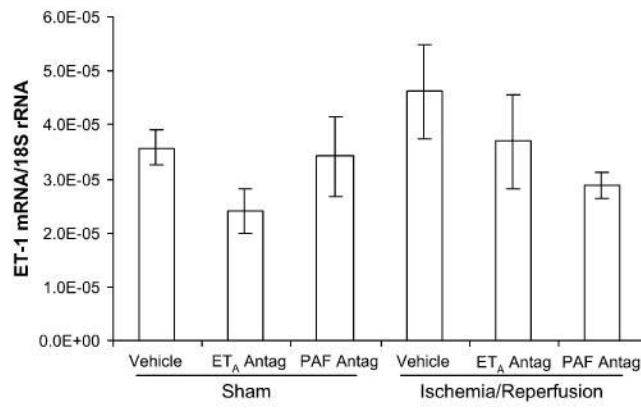


Figure 6. Placental prepro-ET-1 mRNA expression on gestational day 21, 4 days after maternal sham operation or 30-minute IR of the right uterine horn. Prepro-ET-1 expression was not significantly different among any of the groups. Data are mean \pm SEM.

Table
Litter size and fetal viability on gestational day 21, 4 days after maternal sham operation or 30-minute IR of the right uterine horn

| | Sham | | | IR | | |
|--------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| | Vehicle | A-127722 | WEB-2086 | Vehicle | A-127722 | WEB-2086 |
| Right uterine horn | | | | | | |
| Litter size | 7.0 ± 1.2 | 8.0 ± 0.7 | 7.2 ± 0.6 | 6.4 ± 0.8 | 5.5 ± 0.9 | 5.4 ± 0.6 |
| Live births | 6.5 ± 1.1 | 7.5 ± 0.7 | 6.3 ± 0.8 | 5.4 ± 0.6 | 5.5 ± 0.9 | 5.1 ± 0.5 |
| Fetal deaths | 0.5 ± 0.2 | 0.5 ± 0.3 | 0.8 ± 0.5 | 1.1 ± 0.7 | 0.5 ± 0.5 | 0.3 ± 0.2 |
| Left uterine horn | | | | | | |
| Litter size | 6.7 ± 1.0 | 6.8 ± 0.7 | 5.7 ± 1.1 | 6.6 ± 0.8 | 7.2 ± 1.2 | 6.1 ± 0.8 |
| Live births | 6.2 ± 1.1 | 6.1 ± 0.9 | 5.0 ± 1.0 | 5.9 ± 1.2 | 7.2 ± 1.2 | 6.0 ± 0.8 |
| Fetal deaths | 0.5 ± 0.2 | 0.6 ± 0.3 | 0.7 ± 0.7 | 0.9 ± 0.4 | 0.3 ± 0.3 | 0.1 ± 0.1 |

Data are mean ± SEM, n = 6 litters/group. There were no statistically significant differences among these groups by ANOVA.