Placental growth factor is a potent vasodilator of rat and human resistance arteries

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Submitted 8 August 2007; accepted in final form 10 January 2008

Early studies from our laboratory have shown that rat uterine resistance arteries are highly sensitive to dilation to vascular endothelial growth factor (VEGF), and that this response is further enhanced during pregnancy through a mechanism linked to estrogen (12, 13).

Placental growth factor (PIGF) is a member of the VEGF growth factor family that was discovered in 1991. Unlike some VEGF isoforms, which bind to VEGF receptor (VEGFR)-1 [fms-like tyrosine kinase (Flt)] and/or VEGFR-2 (kinase insert domain-containing receptor or fetal liver kinase), PIGF binds specifically to VEGFR-1. Thus far, four studies have examined the effects of PIGF on vascular reactivity, with reports of vasodilatory action in isolated renal (10), placental (14), pulmonary (7), and mammary vessels (17). The mechanism of dilation has not been determined. Vasodilation to VEGF has been more widely reported and is associated with signaling through VEGFR-2 via a mechanism that involves activation of phosphoinositide 3-kinase and Akt-dependent phosphorylation of endothelial nitric oxide (NO) synthase, resulting in increased NO production (e.g., Refs. 4, 15). In contrast, one report concluded that the vasodilator response may involve both types of receptor (7). VEGF heterodimerization has also been reported and linked to prostanoid synthesis (11).

The physiological role of PIGF is not known; however, studies have shown that its circulating concentrations exceed those of VEGF by >40-fold during normal gestation, although its affinity for VEGFR-1 is only 1/10th of the affinity of VEGF (1). During preeclampsia, a disease associated with hypertension and uteroplacental underperfusion, free PIGF concentrations are significantly reduced due to elevations of a soluble form of VEGFR-1 (sFlt-1) (10, 16). Adenoviral overexpression of sFlt-1 in rats results in a preeclampsia-like syndrome characterized by hypertension, proteinuria, and renal endothelial glomerular endotheliosis (10). Recently, it has been suggested that a ratio of sFlt-1 to PIGF may be a useful predictive marker for the development of preeclampsia (16).

Based on these observations, we hypothesized that PIGF, like VEGF, may exert a vasodilatory effect on the peripheral vasculature and thus modulate regional blood flow and resistance. In view of previous results of enhanced uterine artery dilation to VEGF in pregnancy (2), we also measured uterine artery reactivity to PIGF as a function of concentration, as well as the expression of both the VEGFR-1 and VEGFR-2 message in both humans and rats. Its potency and mechanism vary with physiological state and vessel location and are mediated solely by the VEGFR-1 receptor subtype. Gestational changes in the uterine circulation suggest that this factor may play a role in modulating uterine vascular remodeling and blood flow during the pregnant state.

vascular endothelial growth factor; nitric oxide; uterine circulation; rat; human resistance arteries; vascular endothelial growth factor receptor-1; fms-like tyrosine kinase-1

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the placenta. Due to placental production and secretion of this growth factor, uterine veins are likely to contain the highest concentrations of PlGF before systemic dilution.

METHODS

Isolated rat uterine and mesenteric artery reactivity. Adult (12- to 14-wk-old) pregnant and nonpregnant Sprague-Dawley rats were purchased from Charles River Canada and shipped to the University of Vermont. All procedures were approved by the Institutional Animal Care and Use Committee. Vessels were obtained from late pregnant (LP; day 20/22 of gestation; n = 23) and age-matched nonpregnant animals (NP; n = 13) following euthanasia with an injection of methohexital sodium (Brevital, 50 mg/kg ip) and decapitation in a small-animal guillotine. Vessels from pregnant animals were used for reactivity experiments; in addition, those from NP animals were used to test for the effects of pregnancy on PlGF reactivity (n = 5) and for determination of receptor message (n = 8).

The uterus and a section of the gut 5–10 cm distal to the pylorus were removed and placed in separate petri dishes containing cold (4°C) HEPES-buffered physiological salt solution (PSS). HEPES-PSS reactivity experiments; in addition, those from NP animals were used to test for the effects of pregnancy on PlGF reactivity (n = 5) and for determination of receptor message (n = 8).

The uterus and a section of the gut 5–10 cm distal to the pylorus were removed and placed in separate petri dishes containing cold (4°C) PSS that was continuously bubbled with 5% CO2–95% O2. Following a 30-min equilibration period, a passive circumference-tension curve was created for each segment to set optimum resting tension. Endothelium-dependent vasodilatation was assessed by the addition of a single concentration of bradykinin (1 μmol/l) to each chamber after preconstriction with norepinephrine (NE; 1 μmol/l). Arteries that did not achieve at least 70% vasorelaxation to bradykinin were excluded from the study.

Both myometrial and subcutaneous arteries were preconstricted with NE (3 μM), and concentration-response curves to PlGF (0.01–10 nM) were generated before and after a 30-min incubation with a solution containing a combination of Nω-nitro-L-arginine methyl ester (L-NAME; 0.3 mM) and indomethacin (Indo, 10 μM) to inhibit production of endothelial prostanoids and NO.

Measurement of message for VEGFR-1 and VEGFR-2 by RT-PCR. Main uterine arteries were isolated and flash-frozen in liquid nitrogen for subsequent extraction and purification of mRNA, followed by
real-time RT-PCR Taqman assay to evaluate the mRNA expression of VEGFR-1 and VEGFR-2. Proprietary sequences were purchased from ABI. Total RNA was extracted using TRIzol (Invitrogen), as described by the manufacturer. RNA concentrations were determined by measuring the absorbance at a wavelength of 260 and 280 nm. RNA was then converted to cDNA using random hexamer primers and the Superscript First-strand Synthesis System and stored at −70°C until ready for use. Real-time quantification of target mRNA transcripts was performed using an ABI Prism 7000 Sequence Detection System. All samples were quantified in a multiplex reaction with 18s ribosomal RNA to control for differences in sample preparation. Control (ribosomal VIC RNA control; ABI) and targeted cDNA were amplified in a single reaction tube. The efficiency of amplification of each target transcript was evaluated by plotting cycle threshold to log (sample concentration) for each standard curve. Reactions were carried out using Universal Master Mix (ABI). Amplification was performed over 50 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s. Cycle threshold values were determined for each reaction using real-time plots of fluorescence vs. cycle. Relative quantification was determined using the standard curve method. Target cDNA expression was normalized to 18s ribosomal RNA, and each sample was run in triplicate to obtain an average value.

Drugs and solutions. All chemicals were purchased from Sigma Chemical (St. Louis, MO), including salts for buffer preparation, L-NNA, Indo, phenylephrine, diltiazem, and papaverine. PlGF-2 (mouse) and antibodies to VEGFRs were purchased from R&D Systems; VEGF-E was obtained from Research Diagnostics and used for both animal and human tissue studies.

Statistical analysis. Data are expressed as means ± SE, where n is the number of arterial segments studied. The n values refer to both number of vessels and number of animals; wherever possible, two vessels were used from one animal to evaluate the effects of NO inhibition (i.e., one vessel with and one without preincubation in L-NNA). Hence, paired or unpaired Student’s t-tests were used as appropriate to determine the significance of differences between sets of data, and differences were considered significant at P ≤ 0.05. When more than two treatment groups were evaluated, as in the VEGF-E and antibody studies, differences in responses between groups were determined with ANOVA followed by a multiple-comparisons test (Tukey’s) to evaluate the significance of differences between treatment means.

In wire-mounted human vessels, relaxation to PIGF was expressed as percent inhibition of the contraction induced by NE. Differences in responses between groups were compared by determining concentration-response curves using a two-way repeated-measures ANOVA, using PIGF concentration as a within-subject factor and artery type or incubation conditions as a between-subject factors. All data are presented as means ± SE; n represents the number of vessels (one vessel per woman).

Calculations. Pharmacological sensitivity to vasodilation by PIGF (IC_{50}) was calculated for each vessel by normalizing the fractional response to a particular concentration of PIGF to the maximal response obtained at the highest concentration of drug tested. Efficacy was defined as the percentage of maximal dilation, which was determined at the end of each experiment by the addition of diltiazem (10 μM) and papaverine (100 μM), a relaxing solution that induces complete vascular smooth muscle relaxation. In wire-mounted vessels, efficacy was defined as the percentage of maximal relaxation relative to preconstrictor level of tension.

RESULTS

PIGF induced dilation in all arterial types studied, although there were significant differences in sensitivity, efficacy, and relative contribution of NO to the observed effects.

Vasodilatory effects of PIGF on rat uterine vessels; effects of gestation; expression of message for VEGFR-1 vs. VEGFR-2. Arcuate arteries and veins obtained from the uterine circulation dilated readily to PIGF. As previously observed with VEGF
arteries obtained from LP animals were significantly (1,000×) more sensitive than those from age-matched NP controls (IC50 values: NP = 2.7 ± 1.4 nM; LP = 0.003 ± 0.0015 nM; P < 0.01), although the extent of maximal dilation (efficacy) was comparable (NP = 86 ± 5.8%; LP = 78 ± 11%; P > 0.05).

Moreover, the sensitivity of LP uterine arteries to the vasodilator effects of PlGF was significantly (200×) greater than that of veins (IC50 value: 0.6 ± 0.17 nM in veins; Fig. 1B) and dilated to a greater extent than veins (efficacy averaged 78 and 39% of maximal dilation, respectively, at maximal concentrations; Fig. 1A).

In LP rat uterine arcuate arteries, the vasodilatory actions of PlGF were completely eliminated by preincubation with L-NNA (0.2 mM), an inhibitor of NO synthase. Conversely, in LP veins, ~30% of the dilation remained (Fig. 2).

This vasodilatory effect was due to activation of VEGFR-1, as preincubation of LP vessels with an antibody to the R1 receptor eliminated >90% of the observed dilation, while preexposure to an anti-R2 antibody was completely without effect (Fig. 3). The addition of a selective agonist for VEGFR-2 (VEGF-E) was also without effect, and there was no amplification of the response to PlGF + VEGF-E vs. PlGF alone (Fig. 4).

Real-time RT-PCR measurement of mRNA showed that message for both receptor subtypes was present in the uterine artery wall. VEGFR-1 but not VEGFR-2 message was upregulated during gestation (Fig. 5, A and B, respectively).

Effects of PlGF on reactivity of LP rat mesenteric arteries. Third-order mesenteric arteries dilated to PlGF (~50% of maximal dilation at the highest concentration, 10 nM) to a lesser extent than comparably sized uterine arteries (compare Figs. 6A vs. 1A); dilation was also considerably slower than that observed in uterine vessels, taking 15–45 min instead of 5–15 min to stabilize. Preincubation in L-NNA (0.2 mM) did not affect efficacy, but decreased sensitivity (IC50 values: control: 0.22 ± 0.04 nM; L-NNA: 1.20 ± 0.47 nM; P < 0.05; Fig. 6, A and B, respectively).
Effects of PlGF on human myometrial and subcutaneous arteries. Both types of human vessels relaxed to PlGF in a concentration-dependent manner (Fig. 7). The extent of dilatation to the maximal concentration of PlGF (10 nM) was almost twice as large in myometrial vs. subcutaneous arteries (79 ± 7 vs. 45 ± 8%; P < 0.05; Fig. 7). Myometrial arteries were also more sensitive to PlGF-induced vasodilation (IC50: 1.1 ± 0.38 vs. 3.0 ± 0.45 nM; P < 0.05, Figs. 7 and 8).

Preincubation with L-NAME (300 μM) and Indo (10 μM) eliminated ~50% of the relaxation response to PlGF in myometrial arteries (e.g., at 10 nM: 79 ± 7% PSS vs. 44 ± 14% L-NAME + Indo; n = 5, P < 0.05, ANOVA); however, it had no effect on efficacy in subcutaneous arteries (51 ± 7% PSS vs. 48 ± 9% in L-NAME + Indo; n = 6).

The data shown in Fig. 7 indicate the extent of dilation under each condition relative to preconstriction level (efficacy). Calculation of pharmacological sensitivity requires the normalization of this parameter to the maximal effect observed in response to the compound in question (PlGF) for each vessel. Hence, the data in Fig. 7 were normalized in this manner to assess sensitivity and are replotted in Fig. 8. As indicated above, there was a difference in sensitivity to PlGF between subcutaneous and myometrial arteries (P < 0.05, ANOVA); however, after preexposure to these inhibitors, the sensitivity of myometrial arteries was comparable to that obtained in subcutaneous arteries before and after L-NAME + Indo (P > 0.05, ANOVA). After preincubation with L-NAME + Indo, IC50 values were 2.2 ± 0.65 nM (myometrial) to 2.4 ± 0.5 nM (subcutaneous) and were no longer statistically different (P > 0.05).

DISCUSSION

The main findings of this study are as follows. 1) PlGF is a potent vasodilator of resistance arteries and veins from the rat uterine circulation, with arteries displaying significantly greater sensitivity and efficacy than veins. 2) In rat uterine arteries, pregnancy significantly enhanced sensitivity to PlGF without altering its efficacy. 3) PlGF dilation is principally mediated by the release of NO in rat uterine arteries, while other, still unidentified factors contribute to the vasodilation of uterine veins. 4) In rat tissues, message for both VEGFR subtypes is present in the uterine artery wall, and VEGFR-1 (but not VEGFR-2) message is significantly increased in pregnancy. 5) Human uterine (myometrial) arteries also dilate in response to PlGF, although the contribution of NO to the vasorelaxation, while significant, is proportionately less in human vs. rat uterine arteries. 6) Finally, rat mesenteric and human subcutaneous arteries dilate to PlGF in a NO-independent manner. Together, these observations demonstrate the broad and mechanistically diverse actions of PlGF on the vascular wall of resistance arteries and veins. They also support the importance of PlGF/VEGFR-1 signaling in uterine blood flow regulation during pregnancy and raise the potential of an influence on blood pressure and peripheral resistance, as well as venous capacitance.
In this study, we were interested in probing the role of tissue (nonsoluble) VEGF-R1 in uterine circulatory function during pregnancy. An in vitro approach using isolated vessels allowed us to quantify the vasodilatory effects of PI GF, a selective agonist for VEGF-R1. As the results indicate, PI GF is an extremely potent vasodilator, with significant effects documented at subnanomolar concentrations, particularly in uterine arteries. The observation of a venodilatory effect is novel, and the fact that PI GF levels would be highest in the veins that drain the placenta suggests that it may play a role in regulating venous tone. Furthermore, uterine venorelaxation is associated with an increased permeability of the vascular wall (2), and, in view of the well-documented proximity of arteries to veins in the uterine circulation of several animal species, as well as in the human, its permeability-enhancing action may facilitate signal transfer from vein to artery (3) and thereby potentially alter uterine resistance artery tone, placental blood flow, and vascular structure.

Earlier, our laboratory (12) reported a significant effect of pregnancy on uterine artery sensitivity to VEGF and, in a subsequent study, determined this to be secondary to elevated circulating levels of estrogen by studying oophorectomized animals in combination with selective hormone replacement (13). These findings did not distinguish the receptor subtype involved in VEGF vasodilation, since VEGF activates both VEGF-R1 and VEGF-R2. In this study, we demonstrate a similar change in reactivity to PI GF, a selective agonist for the VEGF-R1 receptor subtype, and implicate a transcriptional mechanism as the underlying cause since: 1) VEGF-R1 but not VEGF-R2 message was significantly upregulated in pregnancy, and, as shown by the experiments that utilized receptor blocking antibodies, 2) only VEGF-R1 appears to be involved in the vasodilatory process, at least in the vessels studied herein. This finding does not preclude altered postreceptor signaling as an additional, or complementary, mechanism for enhanced vasodilator sensitivity.

The important question from a physiological standpoint is whether PI GF signaling contributes to the profound increase in uterine blood flow that characteristically occurs in the pregnant state. In this regard, it was recently noted (8) that fetal and placental weights were significantly reduced in a rat model in which PI GF/VEGF signaling was attenuated by adenoviral overexpression of sFlt-1. Although uterine blood flow was not measured in that study, there is a longstanding association between reduced uteroplacental perfusion and reduced fetal and placental weights in studies that utilize a surgical approach to impose a state of reduced uteroplacental perfusion in rats (the reduction of uterine perfusion pressure model; e.g., Refs. 6, 18). More directly pertinent to this study, it was recently shown that, in addition to the hypertension that is associated with reduction of uterine perfusion pressure, there is also a significant (~800%) increase in plasma sFlt-1a concentration. As might be predicted, this excess of soluble receptor was associated with significant reductions in the circulating free concentrations of both PI GF and VEGF (5).

The actions of PI GF extend to the human, as it was a potent vasodilator of human myometrial resistance arteries, with IC50 values in the subnanomolar range. As in rat uterine arteries, in which NO accounted for >90% of the dilation to PI GF, NO contributed significantly to human uterine artery vasodilation, since its inhibition eliminated >40% of the vasodilation. In contrast, endothelial NO synthase inhibition was of little consequence in PI GF-induced dilation of rat mesenteric and human subcutaneous arteries, demonstrating regional differences in its mechanism of action.

The experiments with anti-VEGF-R1 and anti-VEGF-R2 antibodies and the complete absence of a vasodilatory effect of VEGF-E, at least in rat uterine vessels, confirm that dilation is mediated entirely through VEGF-R1. Although this is not surprising in view of the specificity of PI GF for VEGF-R1 signaling, it is noteworthy in that other reports have associated vasodilation and the production of NO most closely with VEGF-R2 signaling through mechanisms involving phospholipase C and Akt (8, 15).

It is also interesting to note that only VEGF-R1, and not VEGF-R2, message was upregulated in gestation, pointing to an unrecognized role for this receptor and of signal transduction pathways subsequent to its activation in the adaptation of the maternal uterine circulation to pregnancy. Combined with two earlier reports that showed an increased sensitivity to VEGF dilation in rat uterine arteries from pregnant rats (12), and of its linkage to estrogen (13), the present findings support the hypothesis that enhanced vasodilation may result from an estrogen-induced increase in VEGF-R1 in the vascular wall. Changes in other signaling pathways downstream to the receptor (e.g., production of NO or other vasodilators) cannot be ruled out and needs to be evaluated in future studies.

ACKNOWLEDGMENTS
The authors acknowledge the excellent technical assistance of Kristen VanWoorst with carrying out small-artery reactivity experiments and of Dr. Huiling Li (Brown University) with real-time RT-PCR measurement of message for VEGF-R1 and VEGF-R2.

GRANTS
This study was supported by National Heart, Lung, and Blood Institute Grant RO1 HL-079253 (G. Osol).

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