Interleukin-6 impairs endothelium-dependent NO-cGMP-mediated relaxation and enhances contraction in systemic vessels of pregnant rats

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Orshal, Julia M., and Raouf A. Khalil. Interleukin-6 impairs endothelium-dependent NO-cGMP-mediated relaxation and enhances contraction in systemic vessels of pregnant rats. Am J Physiol Regul Integr Comp Physiol 286: R1013–R1023, 2004; 10.1152/ajpregu.00729.2003.—IL-6 is elevated in plasma of preeclamptic women, and twofold elevation of plasma IL-6 increases vascular resistance and arterial pressure in pregnant rats, suggesting a role of the cytokine in hypertension of pregnancy. However, whether the hemodynamic effects of IL-6 reflect direct effects of the cytokine on the mechanisms of vascular contraction/relaxation is unclear. The purpose of this study was to test the hypothesis that IL-6 directly impairs endothelium-dependent relaxation and enhances vascular contraction in systemic vessels of pregnant rats. Active stress was measured in aortic strips isolated from virgin and late pregnant Sprague-Dawley rats and then nontreated or treated for 1 h with IL-6 (10 pg/ml to 10 ng/ml). In endothelium-intact vascular strips, phenylephrine (Phe, 10−5 M) caused an increase in active stress that was smaller in pregnant (4.2 ± 0.3) than virgin rats (5.1 ± 0.3 × 10−3 N/m²). IL-6 (1.00 pg/ml) caused enhancement of Phe contraction that was greater in pregnant (10.6 ± 0.7) than virgin rats (7.5 ± 0.4 × 10−4 N/m²). ACh and bradykinin caused relaxation of Phe contraction and increases in vascular nitrite production that were greater in pregnant than virgin rats. IL-6 caused reductions in ACh- and bradykinin-induced vascular relaxation and nitrite production that were more prominent in pregnant than virgin rats. Incubation of endothelium-intact strips in the presence of Nω-nitro-L-arginine methyl ester (10−4 M) to inhibit nitric oxide (NO) synthase, or 1H-[1,2,4]oxadiazolo[4,3-d]quinazolin-1-one (ODQ, 10−5 M) to inhibit cGMP production in smooth muscle, inhibited ACh-induced relaxation and enhanced Phe-induced stress in nontreated but to a lesser extent in IL-6-treated vessels, particularly those of pregnant rats. Removal of the endothelium enhanced Phe-induced stress in nontreated but not IL-6-treated vessels, particularly those of pregnant rats. In endothelium-denuded strips, relaxation of Phe contraction with sodium nitroprusside, an exogenous NO donor, was not different between nontreated and IL-6-treated vessels of virgin or pregnant rats. Thus IL-6 inhibits endothelium-dependent NO-cGMP-mediated relaxation and enhances contraction in systemic vessels of virgin and pregnant rats. The greater IL-6-induced inhibition of vascular relaxation and enhancement of contraction in systemic vessels of pregnant rats supports a direct role for IL-6 as one possible mediator of the increased vascular resistance associated with hypertension of pregnancy.

cytokines; nitric oxide; pregnancy

NORMAL PREGNANCY is associated with decreased systemic vascular resistance and arterial pressure and reduced vascular contraction in response to vasoconstrictors (15, 32, 41, 43). The hemodynamic and vascular changes associated with normal pregnancy have been attributed, in part, to increased nitric oxide (NO) synthesis by various cells, including vascular endothelial cells (1, 9, 48, 55). This is supported by reports that the tissue expression and activity of NO synthase (NOS) are enhanced during gestation (4, 11) and that the metabolic production and plasma level of cGMP, a second messenger of NO and a cellular mediator of vascular smooth muscle relaxation (30), are increased during pregnancy (13).

In 5–7% of pregnancies, women develop a condition called preeclampsia characterized by proteinuria, increased intravascular coagulation, increased systemic vascular resistance, and hypertension (20, 23, 47). Although preeclampsia is a major cause of maternal and fetal morbidity and mortality, the mechanisms of this disorder have not been clearly identified. Studies in animal models of hypertension during pregnancy have proposed that localized reduction in uteroplacental perfusion pressure and the ensuing placental ischemia-hypoxia during late pregnancy would lead to increased vasoactive factors in the systemic circulation, and these factors would in turn cause systemic vascular changes, increased vascular resistance, and hypertension (3, 14, 35). Although several vasoactive factors have been proposed, a key role for plasma cytokines in the pathogenesis of hypertension of pregnancy has been hypothesized (10, 12, 33, 53). In support of the “cytokine hypothesis” it has been shown that the plasma levels of TNF-α are elevated in women with preeclampsia (10, 12, 33, 56). Although some studies suggest that the maternal and cord sera and placental levels of IL-6 may not be different between preeclamptic and normotensive women (6), several other studies have shown that the plasma levels of IL-6 are elevated approximately two- to threefold in women with preeclampsia (12, 25, 36, 53). Studies have also shown that a two- to threefold elevation in plasma IL-6 or TNF-α in late pregnant rats results in significant increases in systemic vascular resistance and arterial pressure (2, 16, 44). Furthermore, endothelium-dependent vascular relaxation is reduced in systemic vessels of late pregnant rats chronically infused with cytokines such as IL-6 (16, 44). However, a fundamental question is whether the hemodynamic effects of elevated plasma IL-6 during pregnancy reflect direct effects of the cytokine on the mechanisms of vascular contraction/relaxation. Although studies in IL-6-infused pregnant rats have suggested possible association between endothelial cell dysfunction and hypertension (44), it is not clear whether the reduction in endothelium-dependent vascular relaxation is caused by IL-6 or is merely a consequence of the hypertension developed during chronic IL-6 infusion. Also, IL-6 may acti-
vate other vasoactive factors in vivo (31), raising the possibility that the chronic vascular effects of IL-6 may not be caused directly by IL-6 but rather by other vasoactive factor(s), and thus this makes it important to investigate the direct effects of IL-6 on the mechanisms of vascular contraction/relaxation in systemic vessels particularly during gestation.

The purpose of the present study was to test the hypothesis that IL-6 directly impairs endothelium-dependent relaxation and enhances vascular contraction in systemic vessels of pregnant rats. We used vascular strips isolated from virgin and late pregnant rats to investigate 1) whether IL-6 enhances the vascular contraction particularly in vascular strips of pregnant rats, 2) whether IL-6 inhibits endothelium-dependent vascular relaxation particularly in vascular strips of pregnant rats, and 3) because normal pregnancy is associated with increased NO production (1, 9, 48, 55), whether the IL-6-induced changes in vascular contraction/relaxation involve alterations in the endothelium-dependent NO-cGMP pathway.

METHODS

Animals. Female virgin (12 wk, 200–250 g) and time pregnant (day 12 of gestation, ~350 g) Sprague-Dawley rats, 12 rats each, were purchased from Charles River Lab (Wilmington, MA). The rats were maintained on ad libitum standard rat chow and tap water in 12:12-h light-dark cycle. All procedures followed the guidelines of the Institutional Animal Care and Use Committee.

Blood pressure. Systolic blood pressure was measured using a tail-cuff electrophysmomanometer system (ITTC, Woodland Hills, CA). After a 2-day training period, blood pressure was measured on days 14–20 of gestation or the equivalent in virgin rats between 8:00 AM and 11:00 AM. Rats were allowed to rest quietly in a Plexiglas restrainer placed in a warming chamber preset at 30°C. A 1-mm tail cuff with pneumatic pulse transducer was applied to the base of the tail and programmed to inflate to a maximum pressure of 250 mmHg. The pressure cuff was gradually deflated, and the reappearance of pulsations was detected by a photoelectric sensor, amplified, and recorded on a two-channel polygraph. A rest period of 1 min was allowed between inflations. Results from the first five inflation cycles were discarded, and the average from the next five cycles was taken as the systolic blood pressure of each individual rat on any given day. With the use of this protocol, the systolic blood pressure was 132 ± 4 mmHg in virgin rats and was significantly reduced on day 20 of gestation in pregnant rats (117 ± 2 mmHg, P = 0.003).

Measurement of plasma IL-6. On day 20 of pregnancy, blood samples (0.5 ml) were collected for measurement of plasma IL-6 using ELISA system (Cytoscreen, Biosource International, Camarillo, CA). The assay is a solid-phase sandwich-type system that utilizes specific anti-rat IL-6 antibody coated onto the wells of microtiter plates. Serum samples (50 μl) and standards were pipetted in triplicate into appropriate microtiter wells, and the assay was performed according to manufacturer’s instructions. The sensitivity of this IL-6 ELISA system is 0.7 pg/ml, and the upper limit of detection is 150 pg/ml. With the use of this protocol, the plasma levels of IL-6 were not significantly different between virgin (5.6 ± 0.9 pg/ml) and pregnant rats (6.4 ± 1.2 pg/ml, P = 0.599).

Measurement of plasma estradiol-17β and progesterone. Plasma levels of estradiol-17β and progesterone were determined using RIA kits (ICN Biomedicals, Irvine CA). The estrogen assay reactivity with estradiol-17β is 100%, and cross-reactivity with estrone, estriol, and other steroids is 6.4%, 4.5%, and <0.01%, respectively. The progesterone assay reactivity with progesterone is 100%, and cross-reactivity with 17α-hydroxyprogesterone and other steroids is 2.5 and <0.3%, respectively. Plasma estrogen and progesterone were not determined at specific stages of the ovarian cycle in virgin rats because synchronization of the rats would require administering estrogen and progesterone and abortifacient drugs such as PGF2α, which could affect the contraction measurements in the vascular strips (50). Therefore, virgin rats were studied using random selection regardless of the stage of ovarian cycle. With the use of this protocol, plasma estradiol was 121.6 ± 10.2 pg/ml in virgin rats and was significantly elevated in pregnant rats (262.4 ± 8.8 pg/ml, P < 0.001). Also, plasma progesterone was significantly greater in pregnant (283.4 ± 8.5 ng/ml) than virgin rats (98.4 ± 7.6 ng/ml, P < 0.001).

Tissue preparation. On day 20 of pregnancy or the equivalent in virgin rats, the rats were euthanized by inhalation of CO2. The thoracic aorta was excised, placed in Krebs solution, cleaned of connective tissue, and cut into 3-mm-wide strips. For endothelium-intact strips, extreme care was taken to avoid injury of endothelium. For endothelium-denuded strips, the endothelium was removed by rubbing the vessel interior with wet filter paper. Removal of the endothelium was verified by the absence of ACh relaxation in vascular strips precontracted with phenylephrine (Phe).

Isometric tension. One end of the vascular strip was attached to a glass hook and the other end was connected to a Grass force transducer (FT03, Astro-Med, West Warwick, RI). Vascular strips were stretched to Lmax (1.5 the unloaded initial length L) and equilibrated for 1 h in a tissue bath filled with 50 ml Krebs solution continuously bubbled with 95% O2-5% CO2 at 37°C. The changes in isometric contraction were recorded on a Grass polygraph (model 7D, Astro-Med).

Vascular strips were either nontreated or treated with one concentration of IL-6 (10 pg/ml to 10 ng/ml) for 1 h. These IL-6 concentrations were selected to roughly mimic previously reported measurements of plasma IL-6 in human preeclampsia and IL-6-infused pregnant rats (6, 12, 25, 44). Increasing concentrations of Phe were applied, and concentration-contracture curves were constructed. In other experiments, IL-6-treated and nontreated vascular strips were stimulated with half-maximal concentration of Phe (ED50) to elicit a submaximal contraction. Increasing concentrations of ACh, bradykinin, or sodium nitroprusside were added, and the extent of vascular relaxation was measured. In another set of experiments, IL-6-treated and nontreated vascular strips were incubated for 30 min in the presence or absence of Nω-nitro-l-arginine methyl ester (l-NAME, 10−4 M) to inhibit NOS or with 1H-[1,2,4]oxadiazolo[4,3-d]quinazolin-1-one (ODQ, 10−5 M) to inhibit cGMP production in smooth muscle (16, 22, 29), and the effects on Phe-induced contraction and ACh-induced relaxation of Phe contraction were measured.

Nitrite production. Endothelium-intact vascular strips were placed in 1.5 ml Krebs aerated with 95% O2-5% CO2 at 37°C, and the solution was changed every 10 min for 1 h. Samples for basal accumulation of nitrite (NO2−) formed from released NO were taken. Vascular strips were stimulated with ACh for 10 min and then rapidly removed, dabbed dry with filter paper, and weighed. The incubation solutions were assayed for the stable end product of NO, NO2− (16, 22). Briefly, samples of incubation solution (50 μl, in triplicate) were mixed in 96-well microplate with 100 μl Griess reagent. The chromophore generated from the reaction with NO2− was detected spectrophotometrically (535 nm) using THERMOMax microplate reader (Molecular Devices, Sunnyvale, CA). The concentration of NO2− was calculated using a calibration curve with known concentrations of NaNO2.

Western blots. Endothelium-intact aortic strips were transferred to a homogenization buffer containing 20 mM 3-[N-(morpholino)propane sulfonic acid, 4% SDS, 10% glycerol, 2.3 mg dithiothreitol, 1.2 mM EDTA, 0.02% bovine serum albumin (BSA), 5.5 μM leupentin, 5.5 μM pepstatin, 2.15 μM aprotinin, and 20 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride. The tissue was homogenized using 2 ml tight-fitting homogenizer ( Kontes Glass) at 4°C. Protein-matched samples were subjected to electrophoresis on 8% SDS polyacrylamide gels and then transferred to nitrocellulose membranes. The mem-
branes were incubated in 5% BSA in PBS-Tween at 22°C for 1 h and then incubated in the antibody solution at 4°C overnight. PBS-Tween contained (in mM) 80 Na₂HPO₄, 20 NaH₂PO₄, 100 NaCl, and 0.05% Tween. To quantify NOS III, monoclonal anti-endothelial NOS (anti-eNOS) antibody (Transduction Laboratory, Lexington, KY) was used. To maintain the labeling conditions constant, we used the same antibody titer (1:1,000) and protein concentration (10 μg) in all samples. The nitrocellulose membranes were washed 5 × 15 min in PBS-Tween and then incubated in horseradish peroxidase-conjugated anti-mouse IgG for 1.5 h. The blots were visualized with enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). To verify equal loading of sample protein, the immunoblots were stripped and then incubated in horseradish peroxidase-conjugated anti-β-actin antibody (1:5,000, Sigma). Reactive bands were analyzed using a GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA) and expressed as the eNOS/β-actin signal ratio.

**Solutions, drugs, and chemicals.** Normal Krebs contained (in mM) 120 NaCl, 5.9 KCl, 25 NaHCO₃, 1.2 NaH₂PO₄, 11.5 dextrose, 1.2 MgCl₂, and 2.5 CaCl₂ at pH 7.4. Recombinant rat IL-6, IL-6 antibody, and IL-10 were purchased from Biosource International. Stock solutions of Phe, ACh, bradykinin, sodium nitroprusside, and L-NAME (Sigma) were prepared in distilled water. ODQ (Calbiochem, La Jolla, CA) was dissolved in DMSO (>99%, Sigma) and then incubated in horseradish peroxidase-conjugated anti-mouse IgG for 1.5 h. The blots were visualized with enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). To verify equal loading of sample protein, the immunoblots were stripped and then incubated in horseradish peroxidase-conjugated anti-β-actin antibody (1:5,000, Sigma). Reactive bands were analyzed using a GS-700 imaging densitometer (Bio-Rad, Hercules, CA) and expressed as the eNOS/β-actin signal ratio.

**Statistical analysis.** The contractile force was corrected for the cross-sectional area of each vascular strip and expressed as active stress (N/m²) using the following equation, stress = force/cross-sectional area, where cross-sectional area = wet weight/tissue density × length of the strip), and tissue density = 1.05 g/cm³ (14, 16, 32). Data from vascular strips of the same rat were averaged and represented the data for each individual rat. The data from different rats were analyzed and expressed as means ± SE with the n value representing the number of rats. Data were compared using ANOVA with multiple classification criteria [rat type (pregnant vs. virgin), condition of endothelium (intact vs. denuded), and treatment (control vs. IL-6 treatment)] followed by Bonferroni’s post hoc test to compare selected groups. Differences were considered statistically significant if P < 0.05.

**RESULTS**

In endothelium-intact vascular strips of virgin rats, Phe caused concentration-dependent contraction that reached a maximum at 10⁻⁵ M (Fig. 1A). Pretreatment of vascular strips of virgin rats with IL-6 (1,000 pg/ml) for 1 h did not cause further enhancement of Phe contraction in vascular strips of pregnant rats (Fig. 1C) than virgin rats (Fig. 1A). Treatment of the vascular strips with IL-6 (1,000 pg/ml) for 1 h caused a slight upward shift in the baseline tension and enhanced the Phe contraction to a greater extent in pregnant rats (Fig. 1D) compared with virgin rats (Fig. 1B).

To correct for the difference in the size of the vascular strips, Phe contraction was normalized to the cross-sectional area of the vascular strip and presented as active stress (Fig. 2). Phe caused concentration-dependent increases in active stress that were significantly reduced in pregnant compared with virgin rats (P = 0.045) (Table 1). Increasing concentrations of IL-6 (10–1,000 pg/ml) caused concentration-dependent enhancement of Phe-induced active stress in vascular strips of pregnant rats (Fig. 2, A and B). IL-6 (10–1,000 pg/ml) caused greater enhancement of Phe-induced stress in vascular strips of pregnant rats (Fig. 2, A and B, Table 1). IL-6 concentrations >1,000 pg/ml did not cause further enhancement of Phe contraction in virgin or pregnant rats (Fig. 2B).

In vascular strips of virgin or pregnant rats, application of IL-6 (1,000 pg/ml) on top of maximal (10⁻⁵ M) or submaximal (3 to 6 × 10⁻⁷ M) Phe-induced contraction did not cause any rapid increase in contraction over a period of 30 min. Pretreatment of the vascular strips with IL-6 (1,000 pg/ml) for 15 or 30 min did not cause further increase and did not significantly affect
Measurements in IL-6-treated vessels are significantly different (*P < 0.05) from corresponding measurements in control nontreated vessels of virgin and pregnant rats. "Significantly different (P < 0.05) from nontreated or IL-6 plus IL-6 antibody-treated strips of virgin rats. †Significantly different (P < 0.05) from corresponding measurements in control nontreated vessels of virgin and pregnant rats.

**Table 1. Maximal Phe-induced active stress, Phe ED_{50}, and maximal ACh-induced relaxation and nitrite (NOx) production in vascular strips isolated from virgin and pregnant rats and nontreated or treated with IL-6**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Virgin</th>
<th>IL-6</th>
<th>Pregnant</th>
<th>IL-6</th>
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<tr>
<td>Phe (10^{-5}M)-induced active stress, ( \times 10^4 \text{N/m}^2 )</td>
<td></td>
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<tr>
<td>+Endo</td>
<td>5.1±0.3</td>
<td>7.5±0.4*</td>
<td>4.2±0.3</td>
<td>10.6±0.7*</td>
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<tr>
<td>-Endo</td>
<td>6.4±0.4†</td>
<td>8.0±0.6</td>
<td>6.7±0.5†</td>
<td>10.9±0.8*</td>
</tr>
<tr>
<td>L-NAME</td>
<td>6.5±0.6†</td>
<td>8.1±0.7</td>
<td>6.5±0.6†</td>
<td>9.9±0.9*</td>
</tr>
<tr>
<td>ODQ</td>
<td>6.2±0.6</td>
<td>8.4±0.9</td>
<td>7.0±0.6†</td>
<td>11.3±1.0*</td>
</tr>
<tr>
<td>Phe pED_{50} (-\log [M] )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Endo</td>
<td>7.3±0.1</td>
<td>7.6±0.1*</td>
<td>7.1±0.1</td>
<td>7.9±0.2*</td>
</tr>
<tr>
<td>-Endo</td>
<td>7.5±0.1</td>
<td>7.7±0.2</td>
<td>7.5±0.1†</td>
<td>7.9±0.1*</td>
</tr>
<tr>
<td>L-NAME</td>
<td>7.5±0.2</td>
<td>7.7±0.1</td>
<td>7.4±0.1†</td>
<td>7.8±0.1*</td>
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<tr>
<td>ODQ</td>
<td>7.5±0.2</td>
<td>7.9±0.2</td>
<td>7.5±0.1†</td>
<td>8.0±0.1*</td>
</tr>
<tr>
<td>ACh (10^{-5}M)-induced relaxation, %</td>
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<td></td>
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<tr>
<td>+Endo</td>
<td>66.4±1.9</td>
<td>47.2±3.2*</td>
<td>74.9±2.3</td>
<td>25.1±2.2*</td>
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<tr>
<td>L-NAME</td>
<td>23.3±7.5†</td>
<td>17.6±4.5†</td>
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<td>26.6±2.6†</td>
<td>30.4±4.3†</td>
<td>27.7±2.9</td>
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<td>ACh (10^{-5}M)-induced NOx, pmol/mg</td>
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<td></td>
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<td>+Endo</td>
<td>128.6±11.1</td>
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<td>172.6±10.9</td>
<td>76.8±9.6*</td>
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Data represent means ± SE of measurements in vascular strips from 12 virgin and 12 pregnant rats. pED_{50} (−log [M]) is the concentration required to produce half-maximal phenylephrine (Phe) contraction. P values represent the difference between IL-6 (1,000 pg/ml)-treated and control nontreated vascular strips. +Endo, endothelium intact; −Endo, endothelium denuded; L-NAME, N^6-nitro-L-arginine methyl ester; ODQ, 1H-[1,2,4]oxadiazolo[4,3-d]quinazolin-1-one. *Measurements in IL-6-treated vessels are significantly different (P < 0.05) from corresponding measurements in control nontreated vessels of virgin and pregnant rats. †Significantly different (P < 0.05) from corresponding measurement in +Endo vascular strips.

Fig. 2. Phe-induced contraction in vascular strips isolated from virgin and pregnant (Preg) rats and nontreated or treated with IL-6. Endothelium-intact strips were either nontreated or treated for 1 h with IL-6 and then stimulated with increasing concentrations of Phe (A). Effect of different concentrations of IL-6 on Phe contraction was measured (B). Data points represent means ± SE of measurements in vascular strips from 12 virgin and 12 pregnant rats. +IL-6-treated strips are significantly different (P < 0.05) from nontreated or IL-6 plus IL-6 antibody-treated strips of virgin rats. †IL-6-treated strips of pregnant rats.

Removal of the endothelium enhanced Phe-induced stress in control nontreated vascular strips of virgin rats (Fig. 3A), and maximum Phe-induced stress was significantly greater in endothelium-denuded than endothelium-intact vessels of virgin rats (P = 0.036) (Table 1). Endothelium removal caused greater enhancement of Phe-induced stress in nontreated vascular strips of pregnant rats (Fig. 3B), and maximum Phe-induced stress was significantly greater in endothelium-denuded than endothelium-intact vessels of pregnant rats (P < 0.001) (Table 1). Endothelium removal did not cause any significant increase in Phe-induced stress in IL-6-treated vessels of virgin or pregnant rats (Fig. 3, A and B, Table 1). When the Phe response was presented as percentage of maximum, analysis of Phe ED_{50} indicated that Phe was more potent in IL-6-treated than nontreated vessels of virgin rats (P = 0.045) and far more potent in IL-6-treated than nontreated vessels of pregnant rats (P = 0.002) (Fig. 3, C and D, Table 1). Analysis of Phe ED_{50} indicated that Phe potency was not significantly different between endothelium-denuded and endothelium-intact vascular strips of virgin rats (P = 0.171), but Phe was significantly more potent in endothelium-denuded than endothelium-intact vascular strips of pregnant rats (P = 0.01) (Fig. 3, C and D, Table 1). In contrast, the potency of Phe in causing contraction was not significantly different between endothelium-denuded and endothelium-intact IL-6-treated vascular strips of virgin (P = 0.659) and pregnant rats (P = 0.999) (Fig. 3, C and D, Table 1).
contraction was significantly greater in the presence than in the absence of L-NAME \((P = 0.045)\) (Table 1). In vascular strips of pregnant rats, L-NAME caused greater enhancement of Phe-induced stress (Fig. 4B), and maximal Phe contraction was significantly greater in the presence than in the absence of L-NAME \((P = 0.005)\) (Table 1). Also, in vascular strips of virgin rats, analysis of Phe ED\(_{50}\) indicated that Phe potency was not significantly different in the presence and absence of L-NAME \((P = 0.381)\) (Table 1). On the other hand, in vascular strips of pregnant rats, Phe was significantly more potent in the presence than in the absence of L-NAME \((P = 0.045)\) (Table 1). Maximal Phe-induced stress and Phe ED\(_{50}\) in the presence of L-NAME were not significantly different from that in the absence of L-NAME in IL-6-treated vascular strips of virgin or pregnant rats (Fig. 4, Table 1).

In endothelium-intact vascular strips of virgin rats, incubation with ODQ \((10^{-5} \text{M})\) to inhibit cGMP production in smooth muscle (16, 22, 29), enhanced Phe-induced stress only slightly (Fig. 4A), and maximal Phe contraction was not significantly different in the presence and absence of ODQ \((P = 0.115)\) (Table 1). In vascular strips of pregnant rats, ODQ caused greater enhancement of Phe-induced stress (Fig. 4B),
and maximal Phe contraction was significantly greater in the presence than in the absence of ODQ (P < 0.001) (Table 1). Analysis of Phe ED50 in vascular strips of virgin rats indicated that Phe potency was not significantly different in the presence and absence of ODQ (P = 0.381) (Table 1). In vascular strips of pregnant rats, Phe was significantly more potent in the presence than in absence of ODQ (P = 0.01) (Table 1). In contrast, maximal Phe-induced stress and Phe ED50 in the presence of ODQ were not significantly different from that in absence of ODQ in IL-6-treated vascular strips of virgin and pregnant rats (Fig. 4, Table 1).

In endothelium-intact vascular strips of virgin rats, ACh caused concentration-dependent relaxation of submaximal Phe (3 × 10^-7 M)-induced contraction (Fig. 5A). Because the magnitude of Phe contraction was different between vascular strips of virgin and pregnant rats and between IL-6-treated and nontreated vascular strips, the Phe concentration was adjusted to produce a submaximal contraction that is roughly equal in magnitude to that in virgin rats (Fig. 5). Pretreatment of vascular strips with IL-6 (1,000 pg/ml) for 1 h reduced the ACh relaxation of Phe contraction in vascular strips of virgin rats (Fig. 5B). ACh-induced relaxation appeared to be greater in vascular strips of pregnant rats (Fig. 5C) than virgin rats (Fig. 5A). Pretreatment of vascular strips with IL-6 (1,000 pg/ml) reduced ACh-induced relaxation of Phe contraction to a greater extent in pregnant rats compared with nontreated vascular strips of pregnant rats (Fig. 5B).

Cumulative data in vascular strips of virgin rats showed that ACh caused concentration-dependent relaxation of Phe contraction (Fig. 6A). ACh-induced relaxation was significantly greater in pregnant than virgin rats (P = 0.009) (Table 1). IL-6 (10^-10 to 1,000 pg/ml) caused concentration-dependent inhibition of ACh-induced relaxation in virgin rats (Fig. 6), and maximal ACh (10^-5 M) relaxation was significantly reduced in IL-6-treated compared with nontreated vascular strips of virgin rats (P < 0.001) (Table 1). IL-6 caused greater inhibition of ACh-induced relaxation in pregnant rats (Fig. 6B), and maximal ACh (10^-5 M) relaxation was significantly reduced in IL-6-treated compared with nontreated vascular strips of pregnant rats (P < 0.001) (Table 1). Higher concentrations of IL-6 (3,000 pg/ml to 10 ng/ml) did not cause further inhibition of ACh-induced relaxation in vascular strips of virgin or pregnant rats (Fig. 6B). Treatment of vascular strips of virgin or pregnant rats for 1 h with IL-6 (1,000 pg/ml) in the presence of neutralizing anti-IL-6 antibody (10 μg/ml) or with IL-10 (10 to 1,000 pg/ml) did not significantly affect ACh relaxation (Fig. 6B).

Similarly, bradykinin caused concentration-dependent relaxation of Phe contraction (maximum 63.4 ± 2.7%) in vascular strips of virgin rats and significantly greater relaxation in pregnant rats (maximum 74.8 ± 3.6%) (P = 0.019). Maximal bradykinin-induced relaxation was significantly reduced in IL-6 (1,000 pg/ml)-treated (46.3 ± 5.8%) compared with nontreated vascular strips of virgin rats (P = 0.014) and far more reduced in IL-6 (1,000 pg/ml)-treated (21.7 ± 2.4%) compared with nontreated vascular strips of pregnant rats (P < 0.001).

Incubation of endothelium-intact strips in the presence of l-NAME (10^-4 M) or ODQ (10^-3 M) for 30 min significantly inhibited ACh-induced relaxation of Phe contraction in nontreated and to a lesser extent in IL-6-treated vessels of virgin rats (Fig. 7A, Table 1). In contrast, incubation of endothelium-intact strips in the presence of l-NAME or ODQ significantly inhibited ACh-induced relaxation of Phe contraction in nontreated but not IL-6-treated vessels of pregnant rats (Fig. 7B, Table 1). Removal of the endothelium abolished ACh-induced

**Fig. 5.** Representative traces of ACh-induced relaxation of Phe contraction in vascular strips isolated from virgin (A and B) and pregnant rats (C and D) and nontreated or treated with IL-6. Endothelium-intact strips nontreated or treated with IL-6 1,000 pg/ml for 1 h were stimulated with a submaximal concentration of Phe to produce a contraction roughly equal in magnitude in virgin and pregnant rats. Increasing concentrations of ACh were added, and the relaxation of Phe contraction was measured. At the end of the experiment, sodium nitroprusside (SNP, 10^-6 M) was added to ensure the ability of the smooth muscle to relax.
relaxation in control nontreated and IL-6-treated vessels of virgin and pregnant rats.

Western blot analysis using tissue homogenates of endothelium-intact vascular strips and anti-eNOS antibody showed a prominent band at ~140 kDa with optical density significantly greater in pregnant than virgin rats ($P = 0.003$) (Fig. 8A). The optical density of eNOS was not significantly different between IL-6-treated and nontreated vascular strips of virgin ($P = 0.873$) or pregnant rats ($P = 0.877$) (Fig. 8A).

In endothelium-intact vascular strips of virgin rats, basal nitrite production was $31.6 \pm 5.6$ pmol/mg tissue weight, and ACh caused concentration-dependent increases in nitrite production (Fig. 8B). The basal ($52.1 \pm 6.5$ pmol/mg) and ACh ($10^{-5}$ M)-induced nitrite production was significantly enhanced in vascular strips of pregnant compared with virgin rats ($P < 0.05$) (Fig. 8B). IL-6 reduced ACh-induced nitrite production in vascular strips of virgin rats ($P = 0.029$) and more significantly in pregnant rats ($P < 0.001$) (Fig. 8B, Table 1).

In endothelium-denuded vascular strips, sodium nitroprusside, an exogenous NO donor and a standard guanylate cyclase activator (30), caused concentration-dependent relaxation of submaximal Phe contraction that was not significantly different between nontreated and IL-6-treated vascular strips of virgin or pregnant rats (Fig. 9).

**DISCUSSION**

The main findings of the present study are 1) IL-6 enhances vascular contraction particularly in vascular strips of pregnant rats, 2) IL-6 inhibits endothelium-dependent vascular relaxation particularly in vascular strips of pregnant rats, and 3) the IL-6-induced reduction in vascular relaxation and enhancement of vascular contraction involve alterations in the endothelium-dependent NO-cGMP pathway.

Consistent with previous studies on female rats, we have found that the blood pressure and vascular contraction are reduced in pregnant rats compared with virgin rats (15, 16, 22, 32). Although previous studies have shown that the vascular reactivity to vasoconstrictor agonists is enhanced in rat aortic and pulmonary artery strips treated with the cytokine TNF-α (22, 54), little information is available on the acute vascular effects of the cytokine IL-6. We investigated whether acute application of IL-6 could directly enhance the vascular contraction in vascular strips, particularly those of pregnant rats. The present results have shown that IL-6 enhances vascular contraction to the β-adrenergic agonist Phe in vascular strips of virgin rats. Also, the enhancement of Phe contraction by acute treatment with IL-6 is greater in vascular strips of pregnant compared with virgin rats, suggesting greater enhancement of the mechanisms of vascular contraction in IL-6-treated vessels of pregnant rats. The IL-6-induced enhancement of vascular contraction appears to be cytokine specific because it could not be reproduced in vascular strips treated with IL-6 plus neutralizing IL-6 antibody or with similar concentrations of IL-10.
In the search for the possible mechanisms involved in the IL-6-induced enhancement of vascular contraction, we found that removal of the endothelium enhanced Phe-induced contraction slightly in vascular strips of virgin rats but to a larger extent in vascular strips of pregnant rats. In contrast, removal of the endothelium did not cause any significant increase in Phe-induced contraction in IL-6-treated vessels of virgin or pregnant rats. Also, IL-6 caused inhibition of ACh-induced relaxation that was greater in vascular strips of pregnant rats than those of virgin rats. The IL-6-induced inhibition of ACh relaxation did not appear to be due to blockade of endothelial cholinergic receptors because IL-6 caused inhibition of bradykinin-induced relaxation that was also more prominent in pregnant than virgin rats. These results suggest that IL-6 inhibits an endothelium-dependent relaxation pathway particularly in blood vessels of pregnant rats.

One important vasodilator released from endothelial cells is NO (21, 30, 42), and significant increases in endothelial NO production have been shown during pregnancy (1, 15, 48, 55). Thus it seems reasonable to assume that the IL-6-induced inhibition of ACh-induced relaxation could be due to a decrease in the synthesis/release of NO from endothelial cells or a change in the sensitivity of vascular smooth muscle to relaxation by NO. The observation that relaxation of endothelium-denuded vascular strips by sodium nitroprusside was not significantly different between nontreated and IL-6-treated vessels of virgin and pregnant rats provided evidence that IL-6 does not affect the sensitivity of vascular smooth muscle to relaxation and thereby suggests that the reduced ACh-induced relaxation in IL-6-treated vessels of pregnant rats is more likely due to decreased synthesis/release of NO from endothelial cells.

To further investigate the possible role of NO synthesis/release in the impaired endothelium-dependent relaxation in IL-6-treated vessels of pregnant rats, we found that l-NAME, which blocks NO synthesis, significantly inhibited ACh-induced relaxation and enhanced Phe-induced contraction, particularly in nontreated vascular strips of pregnant rats, but had minimal effects in IL-6-treated vessels. These results suggest that NO synthesis by endothelial cells is intact in control nontreated vascular strips of pregnant rats but is impaired during treatment of the vascular strips with IL-6. This is further supported by the observation that IL-6 caused significant reduction in both basal and ACh-induced nitrite production in vascular strips, particularly those of pregnant rats. The precise mechanism by which IL-6 could inhibit endothelial NO synthesis/release is not clear but could be related to changes in the amount and/or activity of eNOS. Although high levels of IL-6, as observed during septic shock or after administration of a high dose of LPS (19), may activate gene expression of inducible NO synthase and promote vasodilation (8, 59), modest levels of LPS, which activate the cytokines IL-6 and TNF-α, may downregulate eNOS (27). This is supported by reports that modest levels of TNF-α downregulate eNOS (5, 58). Although previous studies have shown that small dose infusion of TNF-α in pregnant rats is associated with decreased expression of renal neuronal NOS, no significant differences in the amount of eNOS protein could be detected between pregnant rats nontreated or treated with TNF-α (2). Also, given the relatively short time frame of the present experiments, it seems improbable that the acute vascular effects of IL-6 are due to inhibition of eNOS expression. Additionally, the present Western blot analysis has confirmed that the amount of eNOS protein is not different between IL-6-treated and nontreated vascular strips of virgin or pregnant rats. Thus the acute effects of IL-6 on NO production appear to be more likely related to potential IL-6 mediated posttranscriptional and/or posttranslational modifications of eNOS activity. For example, IL-6 has been localized in the plasma membrane caveolae (46), which
may promote tighter association of eNOS with the inhibitory protein caveolin-1 and thereby prevent the initial activation of eNOS and its dissociation from the plasma membrane (39). IL-6 may also affect the activity of mitogen-activated protein kinase or the phosphatidylinositol-3-kinase (PI3-kinase)-dependent Akt/protein kinase B pathway and thereby prevent phosphorylation of eNOS and its relocation to the plasma membrane, a process required for its full activation (39). Furthermore, IL-6 could alter eNOS activity via a protein kinase C (PKC)-dependent mechanism. This is supported by reports that IL-6 activates endothelial PKC and that PKC causes eNOS phosphorylation and inhibition of eNOS activity (17, 40).

Although a decrease in eNOS activity could explain the decreased NO production and thereby the decreased relaxation in IL-6-treated vascular strips of pregnant rats, we cannot rule out the possibility that IL-6 may also act at a point downstream to decrease the bioactivity/bioavailability of NO. This is supported by reports that cytokines may increase superoxide production (34, 51) and that superoxide decreases the bioactivity of NO and thereby inhibits endothelium-dependent relaxation and promotes vascular contraction (26, 37).

The NO produced by endothelial cells is known to promote vascular relaxation by activating guanylate cyclase and increasing cGMP production in vascular smooth muscle (30). ODQ, which inhibits guanylate cyclase and decreases cGMP production in smooth muscle (16, 29), inhibited ACh-induced relaxation and enhanced Phe-induced contraction in endothelium-intact vascular strips, particularly those of pregnant rats, but not in IL-6-treated vessels of pregnant rats. These results further support the contention that IL-6 decreases NO production/release by endothelial cells and, thereby, reduces the activity of the NO-cGMP pathway in vascular smooth muscle of pregnant rats. The vascular endothelium releases other vasodilator substances such as endothelium-derived hyperpolarizing factor and prostacyclin (52). This may explain why in IL-6-treated vessels some relaxation to ACh was still observed and was not completely inhibited by L-NAME or ODQ. The potential role of NO-independent pathways of vascular relaxation is of particular importance in resistance vessels. Also, removal of the endothelium or incubation of endothelium-intact vascular strips of pregnant rats in the presence of L-NAME or ODQ enhanced Phe-induced contraction to levels that were still less than that in IL-6-treated vessels of pregnant rats. These data suggest that IL-6 may enhance vascular contraction by an additional endothelium-independent mechanism involving direct effects on the cellular mechanisms of vascular smooth muscle contraction. This is supported by reports that cytokines may increase the activity of Ca$^{2+}$-dependent and Ca$^{2+}$-independent protein kinases and enhance contraction in smooth muscle (7, 28, 45).

The plasma levels of IL-6 were not different between virgin and pregnant rats, suggesting comparable prior exposure of the blood vessels to the cytokine. However, the same concentrations of IL-6 produced more dramatic effects in blood vessels of pregnant compared with virgin rats. The cause of the difference in the vascular response to IL-6 in virgin and pregnant rats could be related, in part, to the activity of the NO-cGMP pathway. The tissue expression and activity of NOS (4, 11) and the amount of endothelial NO production are increased during pregnancy (1, 9, 48, 55). Also, cytokines have been shown to downregulate eNOS activity and to inhibit NO release from the vascular endothelium particularly when they are stimulated (5, 24, 57, 58). Another possible cause of the marked effects of IL-6 in vascular strips of pregnant rats may be related to tissue exposure to estrogen and progesterone. Previous studies as well as the present results have shown that plasma estrogen and progesterone levels are higher in pregnant than virgin rats (3). Also, estrogen and progesterone have been shown to modulate eNOS expression/activity and NO production via both genomic and nongenomic mechanisms (38, 50), which may in turn promote feedback regulation of the effects of IL-6 on eNOS activity. Studying the effects of acute and long-term exposure to estrogen and progesterone on the vascular effects of IL-6 should help further identify the mechanisms underlying the possible interactions between sex hormones and IL-6 on the NO-cGMP pathway. Future studies should also explore the effects of IL-6 on vascular strips of ovariecrotomized female rats with or without hormone replacement and should determine whether steroid hormones affect IL-6 activity and/or binding.

The relationship between the observed acute effects of IL-6 in vascular strips of pregnant rats in vitro and the plasma levels of IL-6 and its hemodynamic effects during human pregnancy is still poorly understood. Although several studies have shown increased plasma levels of IL-6 during preeclampsia (12, 25, 36, 53), some studies have shown no change in plasma IL-6 in women with mild preeclampsia (18). Also, while several studies have shown that IL-6 is elevated in the maternal venous circulation (12, 53), some studies have reported differential levels of IL-6 in maternal and cord sera and placenta in women with preeclampsia (6). Additionally, while most studies have suggested that the plasma levels of IL-6 are increased approximately threefold during preeclampsia (12, 25), variations in these levels between 2.56 and 186 pg/ml have been reported (6, 25). In the present study, IL-6 concentrations of 30–1,000 pg/ml were required to produce significant effects, particularly in the vasculature of pregnant rat. Whether IL-6 concentrations similar to those observed in human plasma induce significant effects on human vasculature remains to be investigated. Also, the present results have shown that IL-6 enhances Phe-induced contraction in vascular strips of pregnant rats. On the other hand, preeclampsia is associated with increased plasma levels of endothelin-1 and increased pressor responses to angiotensin II (23, 49). Although the present acute studies did not measure the effects of IL-6 on endothelin-1 release, previous studies have shown that IL-6 stimulates endothelin-1 mRNA expression and endothelin-1 release from endothelial cells (31). Whether IL-6 enhances the vascular contraction to endothelin-1 and other endogenous vasoconstrictors such as angiotensin II in blood vessels of females particularly during pregnancy should be further examined in future investigations.

In conclusion, IL-6 inhibits an endothelium-dependent NO-cGMP-mediated vascular relaxation pathway in systemic vessels of virgin and pregnant rats. The greater IL-6-induced inhibition of vascular relaxation and enhancement of vascular contraction in systemic vessels of pregnant rats supports a direct role for IL-6 as one possible mediator of the increased vascular resistance associated with hypertension of pregnancy.
ACUTE VASCULAR EFFECTS OF IL-6

REFERENCES


