TNF-α enhances contraction and inhibits endothelial NO-cGMP relaxation in systemic vessels of pregnant rats

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Giardina, Jena B., Gachavis M. Green, Kathy L. Cockrell, Joey P. Granger, and Raouf A. Khalil. TNF-α enhances contraction and inhibits endothelial NO-cGMP relaxation in systemic vessels of pregnant rats. Am J Physiol Regulatory Integrative Comp Physiol 283: R130–R143, 2002. First published February 28, 2002; 10.1152/ajpregu.00704.2001.—Tumor necrosis factor-α (TNF-α) is elevated in the plasma of preeclamptic women and may have a role in pregnancy-induced hypertension. However, whether the hemodynamic effects of TNF-α reflect the direct effects on vascular reactivity is unclear. We tested the hypothesis that TNF-α impairs endothelium-dependent relaxation and enhances vascular contraction in systemic vessels of pregnant rats. We measured isometric contraction in aortic strips isolated from virgin and pregnant Sprague-Dawley rats (nontreated vs. treated for 2 h with 10–1,000 pg/ml TNF-α). In endothelium-intact vascular strips, TNF-α caused greater enhancement of phenylephrine (Phe) contraction in pregnant than virgin rats. TNF-α caused significant inhibition of ACh- and Bradykinin-induced vascular relaxation and nitrite/nitrate production that were more prominent in pregnant than virgin rats. NO-nitro-l-arginine methyl ester [L-NAME, 100 μM, an inhibitor of nitric oxide (NO) synthase] or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 1 μM, an inhibitor of cGMP production in smooth muscle) inhibited ACh relaxation and enhanced Phe contraction in nontreated but to a lesser extent in TNF-α-treated vessels, particularly those of pregnant rats. Endothelium removal enhanced Phe contraction in nontreated but not TNF-α-treated vessels, especially those of pregnant rats. Relaxation of Phe contraction with the NO donor sodium nitroprusside was not different between nontreated and TNF-α-treated vessels. Thus TNF-α enhances vascular contraction and inhibits endothelium-dependent NO-cGMP-mediated vascular relaxation in systemic vessels, particularly those of pregnant rats. The results support a direct role for TNF-α as a possible mediator of increased vascular resistance associated with pregnancy-induced hypertension.

cytokines; endothelium; nitric oxide; pregnancy; arterial pressure; tumor necrosis factor-α

NORMAL PREGNANCY IS OFTEN associated with decreased systemic vascular resistance and arterial pressure and reduced vascular reactivity to circulating vasocostric...

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elevated TNF-α during pregnancy reflect direct effects of the cytokine on the mechanisms of vascular reactivity. Although studies in TNF-α-infused pregnant rats have suggested a possible association between the endothelial cell dysfunction and the hypertension (19), it is not clear whether the reduction in endothelium-dependent vascular relaxation is caused by TNF-α or whether it is merely a consequence of the hypertension that is developed during chronic TNF-α infusion. Also, other cytokines such as interleukin 6 (IL-6), which is activated by TNF-α, have been shown to be elevated in the plasma of preeclamptic women (14, 29, 66), which raises the possibility that the chronic vascular effects of TNF-α may not be caused directly by TNF-α but rather by another cytokine. This made it necessary to investigate the direct effects of TNF-α on the mechanisms of vascular reactivity in systemic vessels during late gestation.

The purpose of the present study was to test the hypothesis that TNF-α directly impairs endothelium-dependent relaxation and enhances the contraction in systemic vessels of pregnant rats. We used vascular strips isolated from virgin and late-pregnancy rats to investigate 1) whether TNF-α enhances the vascular contraction, particularly in vascular strips of pregnant rats; 2) whether TNF-α inhibits endothelium-dependent vascular relaxation, particularly in vascular strips of pregnant rats; and 3) whether the TNF-α-induced changes in vascular relaxation and contraction involve alterations in the endothelium-dependent NO-cGMP pathway, because normal pregnancy is associated with increased NO production (1, 18, 54, 62, 69).

METHODS

Animals. Female virgin (nonpregnant, 12 wk old, ~200–250 g; n = 18) and time-pregnant (day 12 of gestation, ~350 g; n = 18) Sprague-Dawley rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN). The rats were housed individually in the animal facility and maintained on ad libitum standard rat chow and tap water on a 12:12-h light-dark cycle. On day 14 of gestation or the equivalent in virgin rats, all rats were anesthetized with isoflurane and underwent a surgical procedure for catheter implantation. A small polyethylene 50 (PE-50) catheter was placed in the carotid artery for measurement of arterial pressure. The catheter was filled with heparin and exteriorized at the back of the neck. Rats were then housed individually, allowed to recover, and studied five days later (days 19–20 of pregnancy or the equivalent in virgin rats). All procedures were performed in accordance with the guidelines of the Animal Care and Use Committee at the University of Mississippi Medical Center and the American Physiological Society.

Measurement of mean arterial pressure. On the day of the experiment, each rat was placed in a Plexiglas restrainer. The carotid arterial catheter was connected to a Statham pressure transducer and the mean arterial pressure was continuously recorded in conscious rats on a Grass polygraph (model 7D, Astro-Med, West Warwick, RI). The mean arterial pressure values were 107 ± 4 mmHg in virgin rats and 96 ± 3 mmHg in pregnant rats.

Tissue preparation. The rats were anesthetized by inhalation of isoflurane. The thoracic aorta was rapidly excised, placed in oxygenated Krebs solution, and cleaned of connective tissue. The aorta was cut transversely into 3-mm-wide rings. Aortic rings were cut open into strips. For endothelium-intact vascular strips, extreme care was taken throughout the procedure to avoid injury of the endothelium. For endothelium-denuded vascular strips, the endothelium was removed by gently rubbing the vessel interior with wet filter paper. Removal of the endothelium was routinely verified by the absence of ACh relaxation in vascular strips precontracted with submaximal concentrations of phenylephrine (Phe).

Isometric tension. One end of the vascular strip was attached to a glass hook using a thread loop, and the other end was connected to a Grass force transducer (model FT03, Astro-Med). Vascular strips were stretched to Lmax (1.5× the unloaded initial length, L). To determine Lmax, the vascular strips were stretched to different lengths and then stimulated with 96 mM KC1; the tissue length at which no further increase in KC1 response was observed was considered Lmax. Lmax was measured separately in vascular strips of virgin and pregnant rats. Lmax in virgin rats was not significantly different from that in pregnant rats. Vascular strips were allowed to equilibrate for 1 h in a water-jacketed, temperature-controlled tissue bath filled with 50 ml of Krebs solution that was continuously bubbled with 95% O2-5% CO2 at 37°C. The changes in isometric tension were recorded on a Grass polygraph (model 7D).

A control contraction was elicited by applying Phe (10–5 M) to the tissue bath solution. Once the Phe contraction reached a plateau, the tissue was rinsed with Krebs solution three times for 10 min each time. The whole procedure of contraction and washing was repeated twice. The tissues were then either nontreated or were treated with one concentration of TNF-α (10–1,000 pg/ml) for 2 h. Increasing concentrations of Phe were applied, the contractile responses were recorded, and concentration-response curves were constructed.

In other experiments, nontreated and TNF-α-treated vascular strips were stimulated with Phe to elicit a submaximal contraction. Increasing concentrations of ACh, bradykinin, or sodium nitroprusside were added, and the extent of vascular relaxation was measured. In other experiments, nontreated and TNF-α-treated vascular strips were incubated for 30 min in the presence or the absence of N0-nitro-l-arginine methyl ester (l-NAME, 100 μM), to inhibit NOS, or with 1H-[1,2,4]oxadiazolo[4,3-b]quinazolin-1-one (ODQ, 1 μM), to inhibit cGMP production in smooth muscle (32, 52), and the effects of the Phe-induced contraction and the ACh-induced relaxation of Phe contraction were measured. The concentrations of l-NAME and ODQ were selected based on previous studies, which have shown that these inhibitors are effective and specific at the concentrations used in this preparation (26, 52, 58, 64).

Nitrite/nitrate production. Endothelium-intact vascular strips were placed in test tubes containing 1.5 ml Krebs solution with or without TNF-α, incubated with 95% O2-5% CO2 at 37°C, and the solution was changed every 10 min for 2 h. Samples for basal accumulation of nitrite formed from released NO were first taken. The Krebs solution was replaced, and the strips were stimulated with ACh for 10 min. The vascular strips were rapidly removed, dabbed dry with filter paper, and weighed. The incubation solutions were assayed for the stable end product of NO, NO2−. Briefly, samples of the incubation solution (50 μl, in triplicate) were mixed in a 96-well microtiter plate with 100 μl of the Griess reagent (26). The chromophore generated by the reaction with nitrite was detected spectrophotometrically (550 nm) using a microtiter plate reader (BioTek, Winooski, VT). The concentration
of nitrite was calculated using a reference calibration curve with known concentrations of NaNO₂.

Solutions, drugs, and chemicals. Normal Krebs solution 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 TNF-α was purchased from Biosource International (Camarillo, CA). Stock solutions of L-phenylephrine HCl, ACh, bradykinin, sodium nitroprusside, and L-NAME (Sigma) were prepared in distilled water. ODQ (Calbiochem, La Jolla, CA) was dissolved in DMSO (final concentration <0.1). All other chemicals were of reagent grade or better.

Statistical analysis. The developed force was corrected for the cross-sectional area of each individual strip and was expressed as active stress (N/m²) using the equation stress = force/cross-sectional area, where cross-sectional area = wet weight/(tissue density × length of strip), and tissue density = 1.055 g/cm³ as previously described (35, 59). Data from vascular strips of the same animal were averaged and presented as data from one animal, and n represented the number of animals. Data were analyzed and expressed as means ± SE. Data were compared using ANOVA with multiple classification criteria [rat type (pregnant vs. virgin), condition of endothelium (intact vs. denuded), and treatment (control nontreated vs. TNF-α treated, or in presence vs. absence of L-NAME or ODQ)] 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 increases in contraction (Fig. 1A). Application of TNF-α on top of Phe contraction did not immediately augment the contraction in the precontracted vascular strips. Treatment of the vascular strips with TNF-α (1,000 pg/ml) for 30 min, 1 h, or 2 h did not cause significant changes in tension. Pretreatment of the vascular strips with TNF-α (1,000 pg/ml) for 30 min or 1 h did not significantly enhance the Phe contraction. On the other hand, pretreatment of the vascular strips of virgin rats with TNF-α (1,000 pg/ml) for 2 h enhanced the Phe contraction (Fig. 1A). The Phe-induced contraction appeared to be smaller in vascular strips of pregnant rats (Fig. 1B) than virgin rats (Fig. 1A). Treatment of the vascular strips with TNF-α (1,000 pg/ml) for 2 h enhanced the Phe contraction to a greater extent in pregnant rats (Fig. 1B) compared with virgin rats (Fig. 1A).

To correct for the difference in the size of the vascular strips, the Phe contraction was normalized for the cross-sectional area of the vascular strip and presented as active stress (Fig. 2). In virgin rats, Phe caused concentration-dependent increases in active stress to a maximum of (5.0 ± 0.3) × 10⁴ N/m² (Fig. 2A). The
Phe-induced active stress was reduced to a maximum of \((4.2 \pm 0.4) \times 10^4\) N/m\(^2\) in pregnant rats (Fig. 2B). Increasing concentrations of TNF-\(\alpha\) (10–1,000 pg/ml) caused concentration-dependent enhancement of the Phe-induced active stress in vascular strips of virgin rats (Fig. 2A). The maximum Phe-induced stress in TNF-\(\alpha\)-treated vascular strips of virgin rats \([(7.4 \pm 0.5) \times 10^4\) N/m\(^2\)] was significantly greater \((P = 0.002)\) than that in nontreated strips of virgin rats (Fig. 2A). Increasing concentrations of TNF-\(\alpha\) (10–1,000 pg/ml) caused greater enhancement of Phe-induced stress in vascular strips of pregnant rats (Fig. 2B). The maximum Phe-induced stress was significantly greater \((P < 0.001)\) than that in nontreated strips of pregnant rats (Fig. 2B). TNF-\(\alpha\) concentrations >1,000 pg/ml did not cause any further enhancement of Phe contraction in virgin or pregnant rats. When the Phe response was presented as a percentage of the maximum Phe contraction, Phe appeared to be more potent in causing contraction in TNF-\(\alpha\)-treated than nontreated vessels of pregnant rats (Table 1).

Removal of the endothelium enhanced the Phe-induced stress slightly in control (nontreated) vascular strips of virgin rats (Fig. 3A). The maximum Phe-induced stress in endothelium-denuded vessels \([(6.1 \pm 0.5) \times 10^4\) N/m\(^2\)] was not significantly different \((P = 0.122)\) from endothelium-intact vessels of virgin rats \([(5.0 \pm 0.3) \times 10^4\) N/m\(^2\)]. In contrast, removal of the endothelium significantly enhanced the Phe-induced stress in control (nontreated) vascular strips of pregnant rats (Fig. 3B). The maximum Phe-induced stress was significantly greater \((P = 0.003)\) in endothelium-denuded \([(6.7 \pm 0.5) \times 10^4\) N/m\(^2\)] than endothelium-intact vessels of pregnant rats \([(4.2 \pm 0.4) \times 10^4\) N/m\(^2\)]. Removal of the endothelium did not cause any significant increase in Phe-induced stress in TNF-\(\alpha\)-treated vessels of virgin or pregnant rats (Fig. 3A and B). When the Phe response was presented as a percentage of the maximum Phe contraction, Phe appeared to be more potent in causing contraction in endothelium-denuded than endothelium-intact vascular strips, particularly those of pregnant rats (Fig. 3C and D). Analysis of the half-maximally effective dose (ED\(_{50}\)) value for Phe indicated that the Phe potency was not significantly different \((P = 0.392)\) between endothelium-denuded and endothelium-intact vessels.

**Fig. 2.** Phe-induced contraction in aortic strips of virgin (A, C) and pregnant (B, D) rats nontreated or treated with TNF-\(\alpha\). Endothelium-intact strips were either nontreated or treated for 2 h with 10–1,000 pg/ml TNF-\(\alpha\) and then stimulated with increasing concentrations of Phe. Phe contraction was measured and presented as active stress (A, B) or as % of maximum Phe contraction (C, D). Data points represent means \pm SE of measurements in 18–24 vascular strips from 6 virgin and 6 pregnant rats.

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vascular strips of virgin rats, but Phe was significantly more potent ($P = 0.005$) in endothelium-denuded than endothelium-intact vascular strips of pregnant rats (Table 1). In contrast, the potency of Phe in causing contraction was not significantly different between endothelium-denuded and endothelium-intact TNF-α-treated vascular strips of virgin and pregnant rats (Fig. 3, C and D, and Table 1).

In endothelium-intact vascular strips, incubation with L-NAME (100 μM) for 30 min to inhibit NOS enhanced the Phe-induced stress only slightly in vascular strips of virgin rats (Fig. 4 A). The maximal Phe-induced active stress and Phe ED$_{50}$ in vascular strips of virgin and pregnant rats nontreated or treated with TNF-α

<table>
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<th>Variable</th>
<th>Virgin</th>
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<th>Pregnant</th>
<th>Pregnant + TNF-α</th>
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<td>Phe, 10$^{-5}$ M</td>
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<td>5.0 ± 0.3</td>
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<td>8.0 ± 0.7</td>
<td>6.2 ± 0.5†</td>
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<td></td>
<td>- Endo</td>
<td>6.5 ± 0.7</td>
<td>8.5 ± 0.8</td>
<td>6.9 ± 0.6†</td>
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<tr>
<td>Phe pED$_{50}$ (–log [M])</td>
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<td>7.3 ± 0.2</td>
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<td>7.5 ± 0.1</td>
<td>7.8 ± 0.2</td>
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<td>7.9 ± 0.2</td>
<td>7.5 ± 0.1†</td>
<td>8.0 ± 0.2*</td>
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Values are means ± SE of measurements in 12-24 vascular strips from 6 virgin and 6 pregnant rats. pED$_{50}$ (–log [M]) is the concentration required to produce half-maximal phenylephrine (Phe) contraction. *$P < 0.05$, measurements in tumor necrosis-α (TNF-α; 1,000 pg/ml)-treated vessels are significantly different from corresponding measurements in control TNF-α nontreated vessels of virgin and pregnant rats; †$P < 0.05$, significantly different from corresponding measurements in endothelium-intact (+ Endo) vascular strips. l-NAME, N$\tilde{G}$-nitro-l-arginine methyl ester; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one.

**Fig. 3.** Effects of endothelium removal on Phe-induced contraction in aortic strips of virgin (A, C) and pregnant (B, D) rats nontreated or treated with TNF-α. Endothelium-intact (+ Endo) and endothelium-denuded (– Endo) strips were nontreated or treated with 1,000 pg/ml TNF-α for 2 h and then stimulated with increasing concentrations of Phe. Phe contraction was measured and presented as active stress (A, B) or as % of maximum Phe contraction (C, D). Data points represent means ± SE of measurements in 12–24 vascular strips from 6 virgin and 6 pregnant rats.

**Table 1.** Maximal Phe-induced active stress and Phe ED$_{50}$ in vascular strips of virgin and pregnant rats nontreated or treated with TNF-α.
induced contraction in vascular strips of virgin rats was not significantly different ($P = 0.106$) in the presence or the absence of L-NAME (see Table 1). Incubation with L-NAME (100 μM) significantly enhanced the Phe-induced stress in vascular strips of pregnant rats (Fig. 4B). The maximal Phe-induced contraction in vascular strips of pregnant rats was significantly greater ($P = 0.011$) in the presence than absence of L-NAME (see Table 1). Also, plotting of the Phe response as a percentage of maximum response showed that Phe was more potent in causing contraction in the presence than absence of L-NAME, particularly in vascular strips of pregnant rats (Fig. 4, C and D). Analysis of the ED$_{50}$ value of Phe in virgin rats indicated that the Phe potency was not significantly different ($P = 0.496$) in the presence or absence of L-NAME (see Table 1). In vascular strips of pregnant rats, Phe was significantly more potent ($P = 0.018$) in the presence than absence of L-NAME (see Table 1). In contrast, the maximal Phe-induced stress and the ED$_{50}$ value of Phe in the presence of L-NAME were not significantly different from those in the absence of L-NAME in TNF-α-treated vascular strips of pregnant rats (Fig. 4, Table 1).

Similarly, in endothelium-intact strips, incubation with ODQ (1 μM) for 30 min to inhibit cGMP produc-

tion in smooth muscle (26, 32, 52) enhanced the Phe-induced stress slightly in vascular strips of virgin rats (Fig. 4A). The maximal Phe-induced contraction in vascular strips of virgin rats was not significantly different ($P = 0.08$) in the presence or absence of ODQ (see Table 1). Incubation with ODQ (1 μM) significantly enhanced the Phe-induced stress in vascular strips of pregnant rats (Fig. 4B). The maximal Phe-induced contraction in vascular strips of pregnant rats was significantly greater ($P = 0.002$) in the presence than absence of ODQ (see Table 1). Also, Phe was more potent in causing contraction in the presence than absence of ODQ, particularly in vascular strips of pregnant rats (Fig. 4, C and D). Analysis of the ED$_{50}$ value of Phe in virgin rats indicated that the Phe potency was not significantly different ($P = 0.392$) in the presence and absence of ODQ (see Table 1). In vascular strips of pregnant rats, Phe was significantly more potent ($P = 0.005$) in the presence than absence of ODQ (see Table 1). In contrast, the maximal Phe-induced stress and the ED$_{50}$ value of Phe in the presence of ODQ were not significantly different from those in the absence of ODQ in TNF-α-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 TNF-α-treated and -nontreated vascular strips, the Phe concentration was adjusted to produce a submaximal contraction that was roughly equal in magnitude to that in virgin rats (Fig. 5). Treatment of vascular strips with TNF-α (1,000 pg/ml) for 2 h reduced the ACh relaxation of Phe contraction in vascular strips of virgin rats (Fig. 5A). Treatment of the vascular strips with TNF-α (1,000 pg/ml) reduced the ACh relaxation of Phe contraction in vascular strips of pregnant rats (Fig. 5B) compared with virgin rats (Fig. 5A).

Cumulative data in vascular strips of virgin rats showed that ACh caused concentration-dependent relaxation of Phe contraction with 10⁻⁵ M ACh causing 66.3 ± 2.9% relaxation (Fig. 6A). The ACh-induced relaxation appeared to be enhanced in pregnant rats (Fig. 6B). The ACh (10⁻⁵ M)-induced relaxation in pregnant rats (75.2 ± 1.9%) was significantly greater (0.027) than that in virgin rats. Increasing concentrations of TNF-α (10⁻⁵ M) caused concentration-dependent inhibition of ACh-induced relaxation in virgin rats (Fig. 6A). The ACh (10⁻⁵ M)-induced relaxation in vascular strips of virgin rats treated with TNF-α (1,000 pg/ml) was significantly reduced to 48.4 ± 5.3% (P = 0.015) compared with nontreated vascular strips of virgin rats. Increasing concentrations of TNF-α caused greater inhibition of ACh-induced relaxation in pregnant rats (Fig. 6B). The ACh (10⁻⁵ M)-induced relaxation in vascular strips of pregnant rats treated with TNF-α (1,000 pg/ml) was significantly reduced to 25.1 ± 2.3% (P < 0.001) compared with nontreated vascular strips of pregnant rats. Higher concentrations of TNF-α (3,000 pg/ml) did not cause any further significant inhibition of ACh (10⁻⁵ M)-induced relaxation in vascular strips of virgin rats (46.7 ± 4.9%) or pregnant rats (24.6 ± 3.4%).

Similarly, bradykinin caused concentration-dependent relaxation of Phe contraction to a maximum of 63.4 ± 2.7% in vascular strips of virgin rats and significantly greater (P = 0.03) relaxation in pregnant rats (74.8 ± 3.6%). The maximal bradykinin-induced relaxation in vascular strips of virgin rats treated with TNF-α (1,000 pg/ml) was significantly reduced to

Fig. 5. Representative traces of ACh-induced relaxation of Phe contraction in aortic strips of virgin (A) and pregnant (B) rats nontreated or treated with TNF-α. Endothelium-intact strips nontreated or treated with TNF-α 1,000 pg/ml for 2 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.
46.3 ± 3.5% (P = 0.003) compared with nontreated vascular strips of virgin rats. The maximal bradykinin-induced relaxation in vascular strips of pregnant rats treated with TNF-α (1,000 pg/ml) was significantly reduced to 21.7 ± 2.4% (P < 0.001) compared with nontreated vascular strips of pregnant rats.

In endothelium-intact strips, incubation with L-NAME (100 µmol/l) or ODQ (1 µM) for 30 min significantly inhibited the ACh-induced relaxation of Phe contraction in nontreated but to a less extent in TNF-α-treated vessels of virgin rats (Fig. 7A). The ACh (10⁻⁵ M)-induced relaxation in TNF-α-nontreated vessels of pregnant rats was significantly reduced in the presence of L-NAME (17.7 ± 3.5%; P < 0.001) or ODQ (33.4 ± 5.2%; P < 0.001) compared to that in the absence of L-NAME or ODQ (75.2 ± 1.9%). The ACh (10⁻⁵ M)-induced relaxation in TNF-α-treated vessels of pregnant rats was not significantly different in the presence of L-NAME (30.5 ± 4.4%; P = 0.307) or ODQ (21.7 ± 2.8; P = 0.374) compared to that in the absence of L-NAME or ODQ (25.1 ± 2.3%). Removal of the endothelium abolished the ACh-induced relaxation in control (nontreated) vir-

Fig. 6. ACh-induced relaxation of Phe contraction in aortic strips of virgin (A) and pregnant (B) rats nontreated or treated with TNF-α. Endothelium-intact strips nontreated or treated for 2 h with 10–1,000 pg/ml TNF-α were stimulated with Phe to elicit a submaximal contraction. Increasing concentrations of ACh were added and the percent relaxation of Phe contraction was measured. Data points represent means ± SE of measurements in 12–24 vascular strips from 6 virgin and 6 pregnant rats.

Fig. 7. Effects of N⁵-nitro-L-arginine methyl ester (L-NAME) and ODQ on ACh-induced relaxation in aortic strips of virgin (A) and pregnant (B) rats nontreated or treated with TNF-α. Endothelium-intact strips nontreated or treated for 2 h with 10–1,000 pg/ml TNF-α were stimulated with Phe to elicit a submaximal contraction. Increasing concentrations of ACh were added and the percent relaxation of Phe contraction was measured. Data points represent means ± SE of measurements in 12–24 vascular strips from 6 virgin and 6 pregnant rats.
gin rats (1.1 ± 0.2%) and pregnant rats (1.5 ± 0.4%) and in TNF-α-treated vessels of virgin rats (1.2 ± 0.1%) and pregnant rats (1.3 ± 0.3%).

In endothelium-intact vascular strips of virgin rats, the basal nitrite/nitrate production was 31.6 ± 5.6 pmol/mg tissue weight, and ACh caused concentration-dependent increases in nitrite/nitrate production (Fig. 8A). At 10−5 M concentration, ACh significantly increased (P < 0.001) nitrite/nitrate production to 122 ± 9.1 pmol/mg (Fig. 8A). In vascular strips of pregnant rats (Fig. 8B), the basal (52.1 ± 6.5 pmol/mg) and ACh (10−5 M)-induced nitrite/nitrate production (181 ± 10.9 pmol/mg) were significantly enhanced (P < 0.05) compared with virgin rats (Fig. 8A). TNF-α reduced the basal and ACh-induced nitrite/nitrate production slightly in vascular strips of virgin rats (Fig. 8A) but more significantly in pregnant rats (Fig. 8B).

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 TNF-α-treated vessels of virgin (Fig. 9A) or pregnant (Fig. 9B) rats.

**DISCUSSION**

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

Consistent with previous studies, we have found that the vascular reactivity is reduced in vascular strips of pregnant rats compared with virgin rats (17, 18, 35). An aim of the present study was to investigate whether acute application of TNF-α enhances the vascular reactivity in vascular strips of virgin or pregnant rats. Although a previous study (67) has shown that the
vascular reactivity to vasoconstrictors is enhanced in TNF-α-treated aorta and pulmonary artery of male rats, little information is available on the acute vascular effects of TNF-α in females. The present study has shown that TNF-α enhances the vascular contraction to the α-adrenergic agonist Phe in vascular strips of virgin rats. Also, the enhancement of Phe contraction by acute treatment with TNF-α is greater in vascular strips of pregnant compared with virgin rats, which suggests greater enhancement of the mechanisms of vascular contraction in TNF-α-treated vessels of pregnant rats.

In the search for possible mechanisms involved in the TNF-α-induced enhancement of vascular contraction, we found that removal of the endothelium enhanced the Phe-induced contraction slightly in vascular strips of virgin rats but to a larger extent in vascular strips of pregnant rats. However, removal of the endothelium did not cause a significant increase in Phe-induced contraction in TNF-α-treated vessels of virgin or pregnant rats. Also, TNF-α caused an inhibition of ACh-induced relaxation that was more prominent in vascular strips of pregnant rats than those of virgin rats. The TNF-α-induced inhibition of ACh relaxation did not appear to be due to blockade of endothelial cholinergic receptors, because TNF-α caused inhibition of bradykinin-induced relaxation, which was also more prominent in pregnant than virgin rats. These results suggest that TNF-α inhibits an endothelium-dependent relaxation pathway, particularly in blood vessels of pregnant rats.

One important vasodilator released from endothelial cells is NO (25, 33, 48, 53). Also, significant increases in endothelial NO production have been shown during pregnancy (1, 18, 54, 62, 69). Thus it seems reasonable to assume that the TNF-α-induced inhibition of ACh-induced relaxation could be due to a decrease in the synthesis or release of NO from endothelial cells or a change in the sensitivity of vascular smooth muscle to relaxation by NO. The sensitivity of vascular smooth muscle to relaxation by NO could be evaluated by exogenous NO donors such as sodium nitroprusside. The observation that the relaxation of endothelium-denuded vascular strips by sodium nitroprusside was not significantly different between nontreated and TNF-α-treated vessels of virgin and pregnant rats provided evidence that TNF-α does not affect the sensitivity of vascular smooth muscle to relaxation, and thereby suggests that the reduced ACh-induced relaxation in TNF-α-treated vessels of pregnant rats is most likely due to a decrease in the synthesis or release of NO from endothelial cells.

To further investigate the possible role of NO synthesis and release in the observed impaired endothelium-dependent relaxation in the TNF-α-treated vessels of pregnant rats, we found that incubation of the vascular strips in the presence of L-NAME, which is known to block NO synthesis, significantly inhibited vascular relaxation by ACh and enhanced the vascular contraction to Phe, particularly in control (nontreated) vascular strips of pregnant rats, but had minimal effects in TNF-α-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 TNF-α. This is further supported by the observation that TNF-α caused significant reduction in both the basal and the ACh-induced nitrite/nitrate production in vascular strips, particularly those of pregnant rats.

The precise mechanism by which TNF-α could inhibit endothelial NO production and/or release is not clear at the present time, but could be related to changes in the endothelial cells, endothelial NOS (eNOS) protein expression, or changes in NOS activity. TNF-α has been shown to induce endothelial cell apoptosis (56). Although high levels of TNF-α, as observed during septic shock or after administration of a high dose of lipopolysaccharide, activate gene expression of inducible NOS and promote vasodilation, modest levels of TNF-α have been shown to downregulate eNOS mRNA by shortening its half-life (5, 72). We have recently found that small-dose infusion of TNF-α in pregnant rats is associated with a significant decrease in the expression of renal neuronal NOS isoform; however, no significant change in the amount of eNOS protein could be observed between pregnant rats treated and nontreated with TNF-α (2). Also, given the relatively short time frame of the present experiments, it seems improbable that the acute vascular effects of TNF-α are due to inhibition of eNOS expression. Instead, the acute effects of TNF-α are more likely related to one of several potential posttranscriptional and/or posttranslational effects. For example, TNF-α-treated endothelial cells could exhibit a tighter association of eNOS with inhibitory molecules such as caveolin-1 (44). Also, TNF-α may interfere with the myristoylation and palmitoylation of eNOS and its translocation to and association with plasmalemmal caveolae, a process required for its full activation (44, 57). TNF-α may also change the activity of mitogen-activated protein kinase, a signaling pathway that induces eNOS phosphorylation and enzyme inhibition (8). Additionally, pretreatment of bovine aortic endothelial cells with TNF-α has been shown to inhibit the phosphatidylinositol-3-kinase-Akt/protein kinase B (PKB) pathway and to prevent PKB-induced phosphorylation and activation of eNOS (36). Furthermore, TNF-α could inhibit endothelial NO production via a protein kinase C (PKC)-dependent mechanism. This is supported by reports that TNF-α activates endothelial PKC (23) and that PKC can cause eNOS phosphorylation and inhibition of NOS activity (45, 50).

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, 33). We found that ODQ, which is known to inhibit guanylate cyclase and to decrease cGMP production in smooth muscle (26, 32, 52), inhibited the endothelium-dependent vascular relaxation by ACh and enhanced the vascular contraction to Phe in endothelium-intact strips, particularly those of pregnant rats, but not in TNF-α-treated vessels of pregnant rats.
rats. These results further support the contention that TNF-α decreases NO production and/or release by endothelial cells and thereby the activity of the NO-cGMP pathway in vascular smooth muscle of pregnant rats.

Although the present results suggest that TNF-α inhibits an endothelium-dependent NO-cGMP vascular relaxation pathway, several cautionary remarks need to be emphasized regarding these interpretations. First, the vascular endothelium has been shown to release other vasodilator substances in addition to NO, such as endothelium-derived hyperpolarizing factor and prostacyclin (10, 65). This may explain why some relaxation to ACh was still observed in the vascular strips of TNF-α-treated vessels that was not completely inhibited by L-NAME or ODQ. The potential role of these additional NO-independent pathways of vascular relaxation is of particular importance in resistance vessels. Second, although the present data suggest that the enhanced vascular contraction in the TNF-α-treated vessels of pregnant rats is likely due to inhibition of an endothelium-dependent vascular relaxation pathway, we cannot rule out the possibility that TNF-α may increase the release or the sensitivity of vascular smooth muscle to endothelium-derived contracting factors. This is supported by reports that TNF-α stimulates the production of endothelium-derived contracting factors such as endothelin-1 (37, 42). Third, TNF-α may also enhance the vascular contraction by an additional endothelium-independent mechanism. This is supported by the observation that removal of the endothelium or incubation of endothelium-intact vascular strips of pregnant rats in the presence of L-NAME or ODQ caused an enhancement of Phe-induced contraction to levels that were still less than that in TNF-α-treated vessels of pregnant rats. These observations suggest that TNF-α may have direct effects on the cellular mechanisms of vascular smooth muscle contraction. This is supported by reports that TNF-α may increase the activity of Ca²⁺-dependent and -independent protein kinases and enhance contraction in smooth muscle (6, 31, 55). The enhanced endothelium-independent TNF-α-induced effects in pregnant rat smooth muscle cells suggest that the TNF-α-sensitive mechanisms of smooth muscle contraction may be augmented during pregnancy and should represent important areas for future investigations.

An important question is why TNF-α caused greater enhancement of vascular contraction and more pronounced inhibition of endothelial NO-cGMP relaxation in blood vessels of pregnant rats compared with those of virgin rats. Although the exact cause of the dramatic effects of TNF-α in blood vessels of pregnant rats is not clear at the present time, it could be related in part to the activity of the NO-cGMP pathway. The tissue expression and activity of NOS (4, 13, 61, 68) and the amount of endothelial NO production (1, 18, 54, 62, 69) as well as the metabolic production and plasma level of cGMP (15) have been shown to be increased during pregnancy. Also, TNF-α has been shown to downregulate eNOS mRNA (5, 72) and to significantly inhibit NO release from the vascular endothelium, particularly when it is stimulated (28, 71).

The question remains as to how the acute vascular effects of TNF-α in pregnant rats relate to human preeclampsia. It has been hypothesized that placental ischemia and hypoxia during late pregnancy may contribute to maternal endothelial cell dysfunction by enhancing the synthesis of cytokines such as TNF-α (12, 14). In support of the cytokine hypothesis, several studies have shown increased plasma levels of TNF-α during preeclampsia (14, 39, 66, 70). However, some studies have shown no change in plasma TNF-α levels in preeclamptic women (22, 29). Also, although several studies have shown that TNF-α levels are elevated in the maternal venous circulation (14, 39, 66, 70), some studies have reported a decrease in TNF-α in the umbilical cord plasma of patients with severe preeclampsia (38). It has also been reported that plasma TNF-α is elevated in 36% of cases of established preeclampsia, but the rise in plasma TNF-α levels occurs only after the syndrome is detected clinically and is not related to the severity of the disease (43), which suggests that circulating TNF-α may not contribute to the development of preeclampsia but may rise as a consequence of the pathological processes of the disease.

Nevertheless, reports that the plasma levels of TNF-α are elevated approximately twofold in women with preeclampsia (14, 39, 66, 70) and that chronic infusion of TNF-α in pregnant rats causes endothelial cell dysfunction and increases the vascular resistance and arterial pressure (2, 19, 27) still support a possible role of TNF-α in pregnancy-induced hypertension. Although the present results suggest that the direct TNF-α-induced inhibition of endothelial vascular relaxation and enhancement of vascular contraction may contribute to the increased vascular resistance and arterial pressure in pregnant rats chronically infused with TNF-α, the relationship between the acute effects of TNF-α in vascular strips of pregnant rats in vitro and the hemodynamic effects during pregnancy in vivo should be interpreted with caution. Clinical studies in humans have suggested that the plasma levels of TNF-α vary between 0.5 and 100 pg/ml (9, 22, 60). In the present study, TNF-α concentrations of 30–1,000 pg/ml were required to produce significant effects, particularly in the vasculature of pregnant rats. Whether TNF-α concentrations similar to those observed in human plasma induce significant effects in the human vasculature remains to be investigated. The present results also suggest that the Phe-induced contraction of aortic strips of pregnant rats is enhanced by TNF-α. On the other hand, preeclampsia is associated with increased plasma levels of endothelin 1 and increased pressor responses to ANG II (40, 63). Whether acute treatment with TNF-α enhances the vascular contraction to other vasoconstrictors such as endothelin 1 and ANG II and in small resistance vessels with more relevance to preeclampsia is unclear and should represent important areas for future investigation. Also, TNF-α may have additional vascular or nonvascular
effects during pregnancy in vivo. For example, TNF-α may activate other vasoactive factors during pregnancy in vivo. This is supported by reports that IL-6, which is activated by TNF-α, is also elevated approximately twofold in the plasma of women with pre-eclampsia (14, 29, 66). Whether acute exposure to IL-6 enhances the vascular contraction is unclear and should be investigated in future studies. TNF-α may also increase the arterial pressure in late pregnancy via additional renal mechanisms that involve decreased renal plasma flow and glomerular filtration rate (27).

In conclusion, TNF-α inhibits an endothelium-dependent NO-cGMP-mediated vascular relaxation pathway in systemic vessels, particularly those of pregnant rats. The greater TNF-α-induced inhibition of vascular relaxation and enhancement of vascular contraction in systemic vessels of pregnant rats supports a direct role for TNF-α as one possible mediator of the increased vascular resistance that is associated with pregnancy-induced hypertension.

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REFERENCES


