

Chronic Tumor Necrosis Factor- α Inhibition Enhances NO Modulation of Vascular Function in Estrogen-Deficient Rats

Ivan A. Arenas, Stephen J. Armstrong, Yi Xu, Sandra T. Davidge

Abstract—Tumor necrosis factor- α (TNF- α) is involved in the pathogenesis of vascular disease. Clinical studies have shown that postmenopausal women have higher serum TNF- α levels; however, whether this increase in TNF- α is associated with vascular dysfunction is unknown. We investigated whether estrogen deficiency is associated with increased serum TNF- α levels and tested the effects of in vivo TNF- α inhibition on vascular reactivity. Aged (12 to 15 months) Sprague-Dawley rats were ovariectomized and treated with placebo, estrogen, or a TNF- α inhibitor (Etanercept; 0.3 mg/kg) for 4 weeks. Serum TNF- α was determined by a bioassay, and vascular function was evaluated in the myograph system. Estrogen-deficient animals had higher serum levels of TNF- α compared with either estrogen-replaced animals or animals treated with Etanercept. Moreover, in estrogen-deficient rats, TNF- α inhibition reduced the constriction of mesenteric arteries to phenylephrine, increased the modulation of this vasoconstriction by the NO synthase inhibitor nitro-L-arginine methyl ester, and decreased the modulation by a superoxide scavenger (Mn(III)tetrakis(4-benzoic acid) porphyrin chloride). Furthermore, endothelium-dependent relaxation was also enhanced by TNF- α antagonism. Additionally, vascular expression of endothelial NO synthase was increased in animals treated with Etanercept, whereas the expression of NAD(P)H oxidase gp91phox and p22phox subunits was decreased. These data show that estrogen-deficient female rats have higher bioactive serum TNF- α levels compared with estrogen-replaced animals. Moreover, a decrease in serum bioactive TNF- α by a soluble TNF- α receptor (Etanercept) results in increased modulation of vascular function by NO. These observations suggest that TNF- α could be a mediator of vascular dysfunction associated with estrogen deficiency. (*Hypertension*. 2005;46:76-81.)

Key Words: aging ■ nitric oxide ■ tumor necrosis factor ■ estrogen ■ vascular function

Cardiovascular disease is the leading cause of death for women in developed countries. Although menopause is considered a major risk factor for vascular disease, the pathophysiological mechanisms linking the decrease in ovarian hormones that occurs in menopause with alterations in vascular function are still unclear.

NO is an important vasodilator and has an important role in the control of vascular homeostasis, including its ability to modulate the actions of vasoconstrictors such as the α -1-adrenergic agonist phenylephrine (PE).¹ In fact, a decrease in NO modulation of vascular function is associated with higher risk of developing vascular disease.² Animal and human studies have shown that the decline of the ovarian function is associated with decreased NO.³⁻⁵ Although the mechanisms remain unknown, the decrease in bioavailable NO seems to be attributable to a decrease in its production or enhanced inactivation by superoxide anion that results in formation of peroxynitrite.

Tumor necrosis factor- α (TNF- α) is a proinflammatory cytokine proposed to be involved in the pathogenesis of vascular dysfunction. In endothelial cells in culture, TNF- α decreases the

expression of endothelial NO synthase (eNOS),⁶ which is the primary enzyme involved in NO production in the vasculature.⁷ Moreover, TNF- α can induce the activity of NAD(P)H oxidase,^{8,9} an enzyme that is a major source of superoxide anion in the vasculature.¹⁰

Studies have shown that postmenopausal women have higher TNF- α levels compared with premenopausal women.^{11,12} However, in those studies, vascular function was not evaluated, and therefore, whether the increase in TNF- α levels with estrogen deficiency is associated with changes in vascular function is largely unknown. Thus, we tested whether in vivo TNF- α inhibition improves vascular function in estrogen-deficient rats. The aim of the present study was to investigate whether estrogen deficiency is associated with an increase in circulating levels of TNF- α and to determine the effects of 4 weeks of in vivo TNF- α inhibition on vascular reactivity. We evaluated the sensitivity of mesenteric arteries to adrenergic constriction and the modulation of this constriction by NO and superoxide anion. Moreover, we investigated changes in endothelial-dependent vasorelaxation and the effects of TNF- α inhi-

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bition on vascular expression of eNOS and NAD(P)H oxidase.

Methods

Animal Model

This study was approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee and was in accordance with the Canadian Council on Animal Care. Female Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Montreal, Quebec, Canada) and were housed in the facilities of the University of Alberta until experimentation at 12 to 15 months of age. This age was chosen because the animals have attained their state of reproductive senescence (ie, similar to the postmenopausal state of women); however, rats experience constant estrus at the end of their reproductive age and continue to produce variable levels of estrogen. Thus, to decrease the variability on estrogen levels in these aged animals, we remove the ovaries at the time of the initial treatment. Then we randomly assigned the animals to different treatments.

Experimental Design

To investigate the effects of estrogen deficiency/replacement on circulating levels of TNF- α , rats were treated with either a placebo pellet (n=8) or an estrogen pellet (1.5 mg/pellet; 60-day release; Innovative Research of America; n=8), which results in maximal serum estrogen levels (\approx 80 pg/mL), similar to that of intact cycling rats. Moreover, to evaluate the role of TNF- α inhibition, rats were treated with either Etanercept (a TNF- α inhibitor; Immunex Corporation), subcutaneously administered at 0.3 mg/kg, 3 \times per week (n=12), or placebo (subcutaneous injection of double distilled H₂O; n=15) for 4 weeks before experimentation. Etanercept is composed of the extracellular ligand-binding portion of the human 75-kDa (p75) TNF receptor 2. Thus, Etanercept binds and inactivates circulating TNF- α . The Etanercept dose for chronic studies was chosen based on effective TNF- α inhibition from previous studies in humans and rats.^{13,14} Rats were euthanized by exsanguination while under anesthesia (sodium pentobarbital; \approx 60 mg/kg body weight). A blood sample was taken and serum was obtained by centrifugation. Samples were snap-frozen (-80°C) for subsequent measurement of TNF- α levels.

Vessel Preparation

A portion of the mesentery was excised and immersed in ice-cold HEPES- buffered physiological saline solution (HEPES-PSS), which contained the following (in mmol/L): 142 NaCl, 4.7 KCl, 1.17 MgSO₄, 1.56 Ca₂Cl, 1.18 KH₂PO₄, 10 HEPES, and 5.5 glucose. Resistance-sized arteries (diameter \approx 200 μm) were dissected and connected to an isometric myograph system (Kent Scientific Corp.) as described previously.¹⁵ Four separate baths were used to study arterial segments simultaneously. Force production was recorded on a data acquisition system (Workbench; Strawberry Tree Inc.).

Vascular Function Studies

Sensitivity of mesenteric arteries to vasoconstriction was evaluated with PE. Cumulative concentrations of PE (0.1 to 50 $\mu\text{mol/L}$) were added to the bath and force was measured. After completion of each dose-response curve, a 30-minute recovery period was allowed, during which the baths were changed every 10 minutes with fresh HEPES-PSS. To investigate the modulation of PE constriction by NO and superoxide anion, vessels were preincubated with inhibitors for 15 minutes before PE concentration-response curves. PE constriction curves were generated in the absence or presence of the NOS inhibitor nitro-L-arginine methyl ester (L-NAME; 100 $\mu\text{mol/L}$; Calbiochem)¹⁶ and a superoxide scavenger (MnTBAP; 100 $\mu\text{mol/L}$; Calbiochem).¹⁷ All constriction curves were normalized to 100% for individual vessels.

To evaluate endothelium-dependent and -independent vasorelaxation, vessels were preconstricted with PE to 50% of maximal

constriction and exposed to cumulative concentrations of bradykinin (0.01 to 1 $\mu\text{mol/L}$) or sodium nitroprusside (0.001 to 1 $\mu\text{mol/L}$).

Measurement of TNF- α

To distinguish bioactive TNF- α from that bound to Etanercept (inactive), serum TNF- α was measured using the L929-8 bioassay, which allows distinguishing between free (bioactive) or bound forms of TNF- α .¹⁸ Briefly, L929-8 cells (an isolated subclone of the murine fibroblastoid cell line L929) were cultured in the wells of a 96-well flat-bottomed microtiter plate in a medium (Iscove Modified Dulbecco Medium) containing 10% FBS plus 2 $\mu\text{g/mL}$ Actinomycin D (kindly donated by the laboratory of Dr Larry Guilbert, University of Alberta) for 2 hours at 37 $^{\circ}$ in 5% CO₂ in air. A total of 50 μL of serum or recombinant TNF- α standards (1.56 to 200 pg/mL) was then added in triplicate to appropriated wells and incubated at 40 $^{\circ}\text{C}$ for 20 hours. Cell viability was assessed by incubation for 2 hours with neutral red dye (0.05% in PBS), which is taken up by vital cells. Then, supernatant vial is removed, the adherent cells are washed with PBS, and color is developed with 0.05 mol/L NaH₂PO₄ in 50% ethanol. The optical density at 570 nm of each well, which reflects the number of cells still viable, was measured on an automated microplate reader. The concentration of TNF- α in the sample can then be calculated by comparison with a standard curve constructed with the TNF- α standards. The lower limit of assay sensitivity is \approx 200 fg/mL of pure recombinant TNF- α . This bioassay also detects TNF- β , but it is not affected by other known cytokines.

Western Blot Analysis

Mesenteric arteries were dissected and homogenized in Eppendorf tubes (containing a protease inhibitor cocktail to inhibit serine, cysteine, and aspartic proteases to prevent degradation; Sigma) using a small tissue homogenizer. The Bradford assay was used to measure protein concentration. A total of 25 μg of protein was loaded onto an SDS-PAGE 9% gel and transferred to a nitrocellulose membrane. Membranes were then probed with goat polyclonal anti-eNOS antibody (1:1000; Santa Cruz Biotechnology), anti-gp91phox (1:100; Santa Cruz Biotechnology), or p22phox (1:100; Santa Cruz Biotechnology). Specificity of primary antibodies was tested in extracts of mesenteric arteries by using specific blocking peptides (Santa Cruz Biotechnology). Primary antibodies were preabsorbed for 30 minutes with a 5 \times higher concentration of specific blocking peptide before probing the membranes. The primary antibody was then detected with a peroxidase-conjugated host-specific secondary antibody (1:2000; Santa Cruz Biotechnology). Membranes were scanned with a Fluor Multi-Imager and bands were quantified by densitometric analysis. After initial exposure to these antibodies, membranes were washed 3 \times with 0.1% tween phosphate buffered saline and then probed with anti- α -actin (as a loading control; 1:500; Santa Cruz Biotechnology).

Data Analysis

Data from each dose-response curve was fitted to the Hill equation and a straight line generated by linear least-squares regression analysis. The concentration that would give 50% constriction (EC₅₀) for each individual artery was determined from this line, and the mean \pm SE calculated from the curves. Tension (T) was calculated using the formula: T=Force (milliNewtons [mN])/2 \times axial length (mm²). ANOVA was used for statistical analysis among groups. Post hoc analysis was performed using Tukey's test. Student's *t* test was used to compare EC₅₀ between 2 groups. Tests were considered significant at *P*<0.05.

Results

Effects of Estrogen Deficiency and Etanercept on TNF- α Levels

Ovariectomy resulted in low serum estrogen levels in animals treated either with placebo (13.7 \pm 1.7 pg/mL) or Etanercept (16 \pm 0.9 pg/mL). However, estrogen-replaced animals have

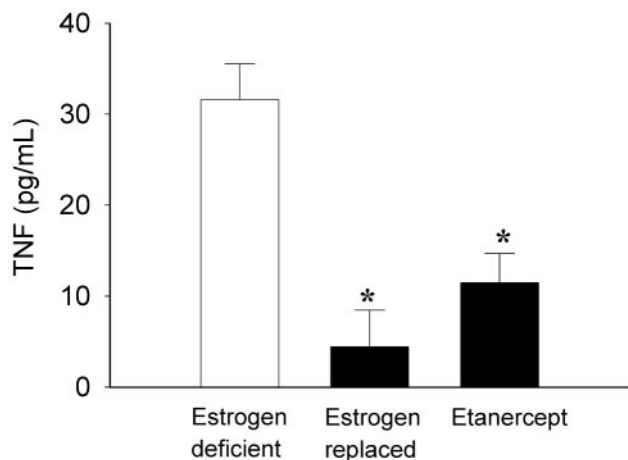


Figure 1. Serum levels of bioactive TNF- α in ovariectomized rats treated for 4 weeks either with 17 β -estradiol or Etanercept (a TNF- α inhibitor). TNF- α was quantified using the L929-8 bioassay by comparison with a pure recombinant TNF- α . Individual samples were tested in triplicate. Results represent means \pm SD. * P <0.05 vs estrogen-deficient animals.

serum estradiol levels (63.2 ± 15.2 pg/mL) within the physiological range.

We first investigated the effect of estrogen or chronic TNF- α inhibition on serum bioactive TNF- α . Estrogen-replaced rats had TNF- α levels similar to that of intact cycling animals (6.3 ± 4 and 4.4 ± 4 pg/mL, respectively). However, estrogen-deficient animals had higher serum bioactive TNF- α levels compared with either estrogen-replaced or Etanercept-treated animals (31.62 ± 3.9 [placebo] versus 4.4 ± 4 [estrogen] and 11.48 ± 3.2 [Etanercept] pg/mL; Figure 1).

Effects of TNF- α Inhibition on Vascular Reactivity in Estrogen-Depleted Rats

To further assess the pathophysiological role of TNF- α in estrogen-depleted animals, we tested the effects of chronic TNF- α inhibition on vascular function. Treatment with Etanercept decreased the sensitivity to PE compared with placebo control animals (Etanercept versus placebo; $EC_{50}=4.72\pm 0.7$ versus 2.22 ± 0.1 μ mol/L; Figure 2A). However, there were no differences in maximum tension between Etanercept and placebo groups (3.8 ± 0.8 and 3.9 ± 0.7 mN/mm², respectively).

Incubation with L-NAME resulted in a similar increase in maximum tension in Etanercept and placebo animals (5.0 ± 0.8 and 5.0 ± 1.1 mN/mm² respectively). However, L-NAME significantly increased the sensitivity to PE only in Etanercept-treated animals (EC_{50} without and with L-NAME 4.72 ± 0.7 and 1.98 ± 0.27 μ mol/L; Figure 2B) but not in placebo animals (EC_{50} without and with L-NAME 2.22 ± 0.1 and 1.83 ± 0.3 μ mol/L; Figure 2B). These data indicate that TNF- α is involved in the decrease of NO modulation of vasoconstriction in estrogen-depleted animals.

The effect of superoxide anion on PE-induced constriction was evaluated by preincubating the vessels with a superoxide scavenger (MnTBAP) before the PE challenge. MnTBAP pretreatment did not affect maximum tension to PE (Etanercept 4.0 ± 0.8 mN/mm² versus placebo 3.7 ± 0.7 mN/mm²). However, MnTBAP decreased the sensitivity to PE in mes-

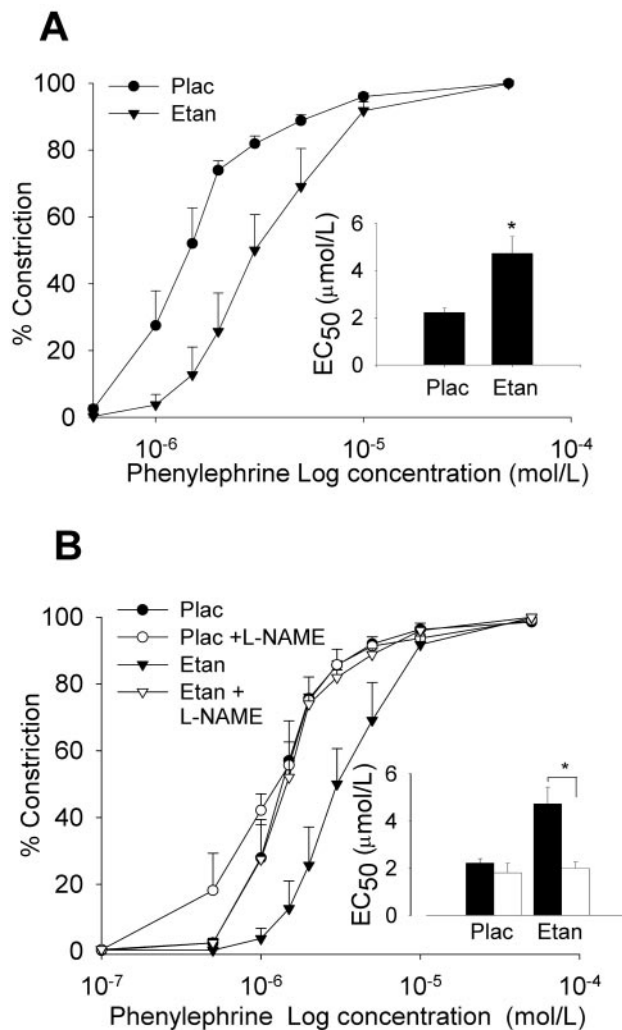


Figure 2. A, PE concentration-response curves and EC_{50} bar graphs of mesenteric arteries from ovariectomized rats treated with Etanercept (Etan; $n=15$) or control animals (placebo [Plac]; $n=12$). Bars represent mean \pm SE. * P <0.05. B, PE concentration-response curves and EC_{50} in the absence (solid symbols and bars) or presence (open symbols and bars) of in vitro L-NAME (100 μ mol/L; $n=8$). Circles represent vessels from placebo-treated animals. Triangles represent vessels from animals treated with Etanercept. Bars represent mean \pm SE; * P <0.05.

enteric arteries from placebo animals (EC_{50} without and with MnTBAP 2.22 ± 0.1 and 4.85 ± 0.5 μ mol/L, respectively; Figure 3) but not in Etanercept-treated animals (EC_{50} without and with MnTBAP 4.72 ± 0.7 versus 3.86 ± 0.3 μ mol/L; Figure 3). Similar findings were obtained in a subset of vessels exposed to the soluble polyethylene glycol-superoxide dismutase (PEG-SOD; EC_{50} Etanercept and placebo 3.6 ± 0.8 and 4.77 ± 0.4 μ mol/L).

Because superoxide anion scavenging only affected the sensitivity of vessels from placebo animals, we tested whether the decreased sensitivity was attributable to NO. A subset of vessels from animals treated with placebo was coincubated with MnTBAP and L-NAME. In the presence of MnTBAP, L-NAME enhanced PE constriction (EC_{50} 4.85 ± 0.5 [MnTBAP] and 2.70 ± 0.7 [MnTBAP+L-

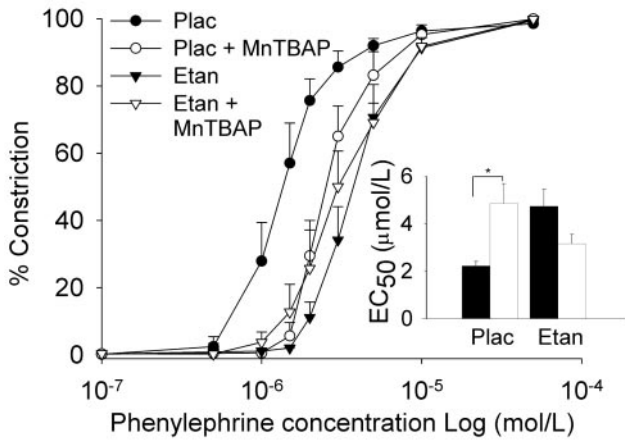


Figure 3. PE concentration-response curves and EC₅₀ bar graphs of mesenteric arteries from ovariectomized rats treated with Etanercept (Etan; n=15) or control animals (placebo [Plac]; n=12) in the absence (solid symbols and bars) or in the presence of in vitro MnTBAP (10 μmol/L; open symbols and bars; n=6). Circles represent vessels from placebo-treated animals. Triangles represent vessels from animals treated with Etanercept. Bars represent mean±SE. *P<0.01 vs PE alone.

NAME] μmol/L; Figure 4), indicating an increase in NO modulation in the vessels of placebo animals in the presence of a superoxide scavenger.

To further determine differences in NO modulation, we evaluated endothelial-dependent vasorelaxation with bradykinin. Relaxation was increased in vessels from Etanercept-treated animals compared with controls (percent maximal relaxation; Etanercept versus placebo, 89±6 versus 65±7; P<0.05; Figure 5A). However, there were no differences in endothelial-independent vasodilation using sodium nitroprusside (percent maximal relaxation; Etanercept versus placebo, 93±3 versus 92±2; Figure 5B).

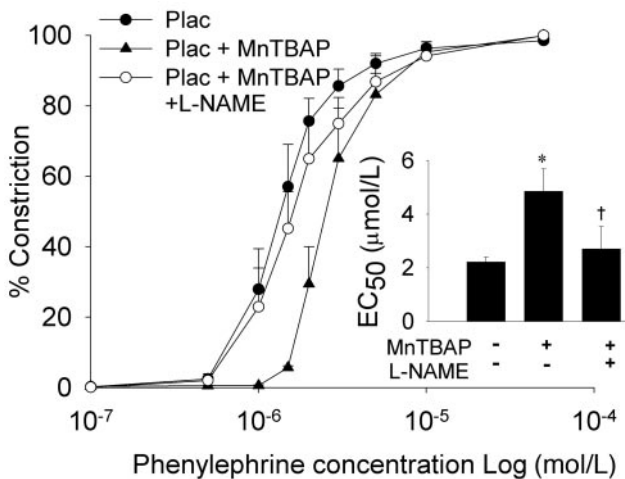


Figure 4. PE concentration-response curves and EC₅₀ bar graphs from mesenteric arteries from ovariectomized rats treated with placebo (Plac). PE in the absence (n=12) or presence of in vitro MnTBAP (10 μmol/L; n=6) or L-NAME (100 μmol/L; n=8) or MnTBAP plus L-NAME (n=3). Bars represent mean±SE. *P<0.05 vs placebo. †P<0.05 vs MnTBAP plus L-NAME.

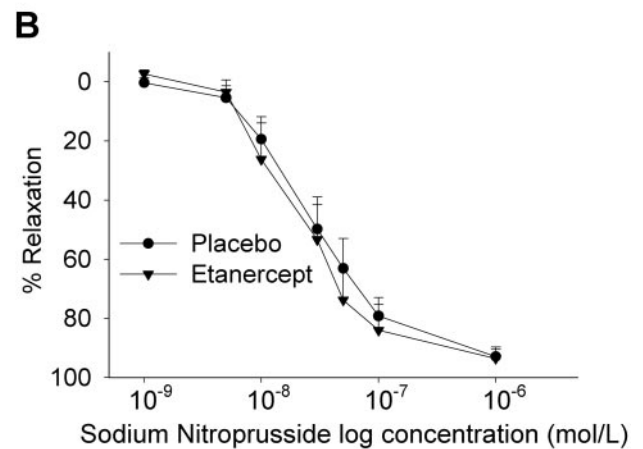
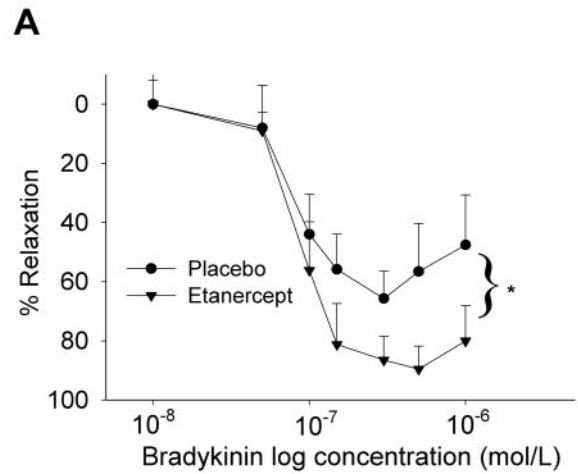


Figure 5. A, Endothelium-dependent relaxation to bradykinin of mesenteric arteries from ovariectomized rats treated for 4 weeks with Etanercept (n=6) or placebo (n=6). B, Endothelium-independent relaxation to sodium nitroprusside of mesenteric arteries from ovariectomized rats treated for 4 weeks with Etanercept (n=6) or placebo (n=6). Circles represent vessels from placebo-treated animals. Triangles represent vessels from animals treated with Etanercept. *P<0.05.

Effects of TNF- α Inhibition on Vascular Expression of eNOS and NAD(P)H Oxidase

To evaluate whether changes in function were accompanied by alterations in tissue protein levels, we evaluated the expression of eNOS and NAD(P)H by Western blot. eNOS expression in animals treated with Etanercept was increased compared with placebo (Figure 6A). In contrast, the expression of NAD(P)H oxidase p22phox and gp91phox was decreased by TNF- α inhibition (Figure 6B and 6C).

Discussion

TNF- α levels have been shown to be higher in postmenopausal women compared with premenopausal women.^{11,12} However, in those studies, vascular function was not evaluated; thus, whether this increase in TNF- α levels is associated with vascular dysfunction is unknown. In the present study, estrogen-depleted animals had a 2-fold increase in serum TNF- α levels compared with estrogen-replaced animals. Moreover, elevated TNF- α levels impair vascular function in

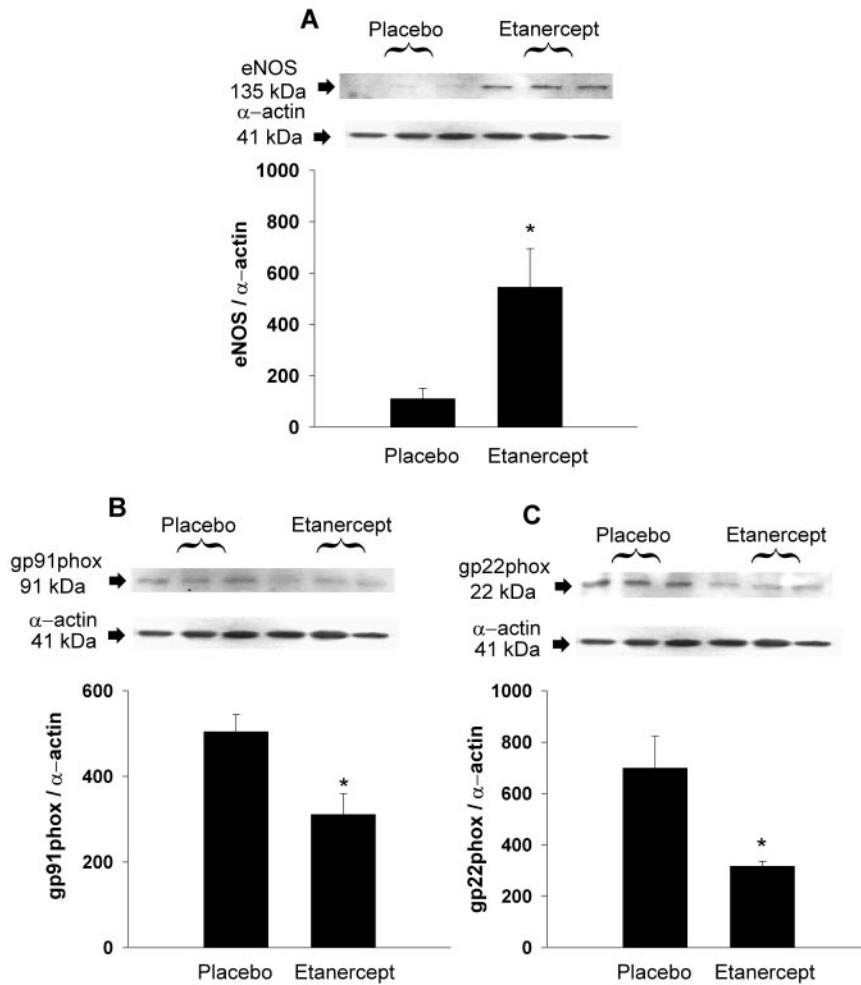


Figure 6. Tissue expression of eNOS and NAD(P)H oxidase on mesenteric arteries of ovariectomized rats treated for 4 weeks with Etanercept or control animals. Representative Western blot and summary of densitometric analysis for eNOS (A), NAD(P)H gp91phox (B), or NAD(P)H p22phox subunits (C). Bars represent mean \pm SD. * $P < 0.05$ vs placebo.

estrogen-depleted animals, as evidenced by the chronic TNF- α inhibition studies.

The vascular endothelium is affected particularly by the actions of TNF- α . After cytokine stimulation, endothelial cells undergo morphological alterations that result in a prothrombotic and proinflammatory phenotype (activation/dysfunction). However, a common initial change for these endothelial alterations is a decrease in NO availability.^{2,19} TNF- α decreases eNOS expression in endothelial cells⁶ and can also increase the release of superoxide anion that inactivates NO.²⁰ These effects can lead to decreased NO bioavailability. Hence, in vitro administration of TNF- α has been shown to reduce endothelium-dependent vasorelaxation.²¹ However, whether increases in in vivo TNF- α levels in a model of menopause are associated with vascular dysfunction was the focus of the present study.

In this study, TNF- α inhibition with Etanercept was associated with a decrease in serum TNF- α in estrogen-deficient animals. Interestingly, the reduction in TNF- α by this inhibitor was accompanied by an ≈ 3 -fold increase in the tissue expression of eNOS and increased in maximum endothelium-dependent vasorelaxation to bradykinin without changes in smooth muscle sensitivity to NO, as evidenced by no changes in sensitivity to the NO donor sodium nitroprusside. These observations strongly suggest that circulating

TNF- α can target vascular eNOS, leading to a decrease in NO formation.

NO modulates the vascular contraction to α -adrenergic agonists.^{22,23} Moreover, increased NO scavenging by superoxide anion has been shown to increase the constrictor responses to PE.²⁴ On the other hand, superoxide anion could promote vasoconstriction by facilitating the mobilization of cytosolic Ca²⁺ in vascular smooth muscle cells or by causing Ca²⁺ sensitization of contractile elements.²⁵ In the present study, treatment with Etanercept resulted in decreased sensitivity (increased EC₅₀) to PE without affecting maximum contraction. Thus, this suggests that the treatment has modified the endothelial modulation of vasoconstriction rather than having a direct effect on smooth muscle cell contractility.

There was also an absence of NO modulation of PE constriction in estrogen-depleted rats, which was restored in Etanercept-treated animals. Moreover, MnTBAP at concentrations found to reduce the biological effects of superoxide anion in several models of oxidative stress¹⁷ decreased PE constriction in placebo animals and restored in part the modulation by L-NAME of PE constriction. Similar results were obtained using an enzymatic superoxide scavenger (PEG-SOD). These observations suggest that superoxide was involved in the scavenging of NO in these animals.

An important source of superoxide anion in vascular cells is NAD(P)H oxidase.¹⁰ This enzyme can be upregulated by TNF- α . TNF- α activation of NAD(P)H oxidase in vascular cells is associated with an increase in the expression of the membrane-bound component p22phox.^{8,9} In this study, placebo rats have increased tissue expression of p22phox and gp91phox subunits compared with animals treated with Etanercept, suggesting that TNF- α could mediate the upregulation of NAD(P)H oxidase, which could have contributed to increase the levels of superoxide anion in estrogen-deficient animals.

In conclusion, the decrease in NO modulation seen in estrogen-depleted rats could be attributable to a decrease in eNOS expression or to an increase in NO scavenging by free radicals such as superoxide anion. Some studies have shown that estrogen deficiency is associated with decreased NO availability and increased superoxide anion in the vasculature of postmenopausal women and ovariectomized animals.^{3,26,27} However, most of these studies have focused on the direct effects of estrogen on free radical production, and little is known about the role of other potential factors that could mediate these alterations in NO availability. In this study, we show that estrogen-deficient rats have higher serum bioactive TNF- α levels compared with estrogen-replaced animals. Moreover, decrease of bioactive levels of TNF- α by a soluble TNF- α receptor resulted in increased modulation of vascular function by NO, higher expression of eNOS, and decreased expression of NAD(P)H oxidase in mesenteric arteries. These observations suggest that TNF- α could be a mediator of vascular dysfunction associated with estrogen deficiency.

Perspectives

Our results support the interplay of estrogen with immune factors in the control of cardiovascular function. Although estrogen may affect TNF- α transcription directly, future studies should address additional mechanisms that may mediate the increase in TNF- α levels during estrogen deficiency. Some clinical studies have also shown that estrogen deficiency is associated with increased TNF- α levels. The results of the present study suggest that increases in TNF- α levels in postmenopausal women could result in vascular dysfunction.

Acknowledgments

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