17β-Estradiol increases nitric oxide-dependent dilation in rat pulmonary arteries and thoracic aorta

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Gonzales, Rayna J., Benjimen R. Walker, and Nancy L. Kanagy. 17β-Estradiol increases nitric oxide-dependent dilation in rat pulmonary arteries and thoracic aorta. Am J Physiol Lung Cell Mol Physiol 280: L555–L564, 2001.—Past studies have demonstrated that 17β-estradiol (E₂β) increases endothelial nitric oxide (NO) synthase (eNOS) activity in uterine, heart, and skeletal muscle and in cultured human endothelial cells. However, little is known about E₂β regulation of NO synthesis in the pulmonary vasculature. The present study evaluated E₂β regulation of eNOS function in pulmonary arteries and thoracic aortas. We hypothesized that E₂β upregulates vascular NO release by increasing eNOS expression. To test this, NO-dependent vasodilation was assessed in isolated perfused lungs and aortic rings from ovariectomized Sprague-Dawley rats treated for 1 wk with 20 μg/24 h of E₂β or vehicle. Expression of eNOS was evaluated by Western blot and immunohistochemistry. Also, a RNase protection assay determined eNOS mRNA levels in lung and aortic homogenates from control and treated rats. Vasodilation to ionomycin in lungs from the E₂β-treated group was enhanced compared with that in control animals. Endothelium-intact aortic rings from E₂β-treated animals also demonstrated augmented endothelium-dependent dilation. Both responses were blocked with NOS inhibition. Immunostaining for eNOS was greater in pulmonary arteries and aortas from E₂β-treated compared with control rats. However, mRNA levels did not differ between groups. Thus we conclude that in vivo E₂β treatment augments endothelium-dependent dilation in aorta and lung, increasing expression of eNOS independently of sustained augmented gene transcription.

endothelium-dependent vasodilation; isolated rat lungs; endothelial nitric oxide synthase

PREMENOPAUSAL WOMEN have a significantly lower risk of heart disease compared with age-matched men and postmenopausal women (38). A portion of the cardio-protective action of estrogen appears to be mediated by actions on the vascular wall. This study and others have shown that gender influences the incidence of cardiovascular disease and that this gender effect appears to be mediated primarily by estrogen. A clinical study (5) has shown that intracoronary infusions of 17β-estradiol (E₂β) increase endothelium-dependent relaxation to acetylcholine (ACh) in women compared with that in men. In addition, Gilligan et al. (11) demonstrated that physiological doses of E₂β administered in vivo enhanced ACh vasorelaxation of large conductance and coronary resistance arteries. Therefore, the beneficial effect of estrogen in the vasculature appears to be due in part to upregulation of endothelial nitric oxide (NO) production. This is supported by a study (37) demonstrating that E₂β increases the activity of endothelial NO synthase (eNOS) in uterine artery, heart, and skeletal muscle. Similarly, cultured human vascular endothelial cells incubated for 24 h with E₂β had increased expression of eNOS that was dose dependent (16). In addition, E₂β treatment increased eNOS mRNA levels in skeletal muscle (37), suggesting that increases in NO activity are caused by the induction of gene transcription. Together, these studies provide strong evidence that E₂β enhances NO-mediated vasodilation in systemic vascular beds.

Although many studies have explored the mechanisms of estrogen modulation of vascular tone in the systemic circulation, estrogen regulation of vasomotor responses in the pulmonary circulation is less clear. Early studies by Huigen et al. (17) and Ergueta et al. (9) demonstrated that the incidence of chronic mountain sickness and high-altitude pulmonary edema were significantly less in women compared with men. In addition, female rats exposed to chronic hypoxia exhibited less pulmonary arterial hypertension (24) and attenuated right ventricular hypertrophy (22) compared with age-matched males. Wetzel and Sylvester (39) demonstrated that pulmonary vasomotor responses to hypoxia were attenuated in isolated lungs from adult female sheep compared with lungs from males. However, another study (10) suggested that E₂β augments vasoconstriction in pulmonary arteries, so the effect of E₂β on pulmonary vascular resistance is unclear.

Similar to the systemic circulation, one mechanism for estrogen regulation of pulmonary vasodilation may be augmented by NO production. For example, fetal pulmonary endothelial cells respond to estrogen by increasing eNOS mRNA levels (20) and eNOS activity (33). Therefore, E₂β may protect the pulmonary vascu-
lature during stresses such as hypoxia or lung disease by increasing the synthesis and release of the potent vasodilator NO. However, the magnitude and mechanisms of in vivo estrogen regulation of eNOS in the pulmonary vasculature are not known. In addition, little is known about in vivo E$_2$b regulation of NO synthesis and release in other vascular beds when E$_2$b is maintained at physiological rather than pharmacological levels. Therefore, these experiments examined eNOS expression and activity in the systemic and pulmonary vasculatures after E$_2$b replacement therapy in ovariectomized (OVX) female rats. We hypothesized that physiological levels of E$_2$b would upregulate eNOS expression, leading to enhanced synthesis and release of NO in both the systemic and pulmonary vasculatures.

**METHODS**

All protocols and animal handling were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico School of Medicine.

**Animal Preparation**

Female Sprague-Dawley rats (250–300 g; Harlan Industries) were anesthetized with halothane, and the ovaries were removed bilaterally. After a 4-wk recovery to deplete endogenous E$_2$b stores, mini-osmotic pumps (ALZET model 2002) containing lipid-soluble E$_2$b (20 μg/24 h; Sigma) or vehicle (polypropylene glycol) were implanted subcutaneously at the base of the neck.

**Isolated Aortic Ring Tissue Bath Preparation**

After 7 days of treatment, E$_2$b- and vehicle-treated animals were anesthetized with pentobarbital sodium (50 mg/kg), heparinized, and exsanguinated. Thoracic aortas were removed and placed in ice-cold physiological salt solution (PSS) containing (in mmol/l) 130 NaCl, 4.7 KCl, 1.18 KH$_2$PO$_4$, 1.17 MgSO$_4$, 7H$_2$O, 14.9 NaHCO$_3$, 5.5 dextrose, 0.026 Ca$_{N_2}$-EDTA, and 1.6 CaCl$_2$. Aortas were cleaned of adventitia and cut into 4-mm rings, and when necessary, endothelium was removed by gently rubbing the lumen with fine-tipped forceps. Next, tissues were suspended in water-jacketed tissue baths filled with PSS maintained at 37°C and aerated with 95% O$_2$ and 5% CO$_2$. Rings were stretched with 2.5 g of passive tension to allow maximal detection of active tension generation as determined in preliminary studies and were equilibrated for 1 h. After a 30-min incubation with indomethacin (10 μmol/l; cyclooxygenase inhibitor), rings were challenged with phenylephrine (PE; 10$^{-6}$ mol/l), and the presence of endothelium was verified by Ach (10$^{-6}$ mol/l) relaxation. Tissues exhibiting <80% relaxation were omitted from the study. Force was continuously recorded with an FT03 transducer (Grass) and chart recorder (Gould RS 3800).

**Isolated Aortic Ring Tissue Bath Protocol**

Contraction studies determine if E$_2$b treatment in vivo enhances endothelium-dependent relaxation. To determine whether chronic in vivo E$_2$b treatment enhances endothelium-dependent relaxation in the systemic circulation, we examined the vasodilatory responses to Ach in contracted aortic rings. Tissues were contracted with PE (10$^{-6}$ mol/l), and cumulative concentration-response curves to Ach (10$^{-10}$ to 10$^{-4.5}$ mol/l) were generated. Tissues were rinsed and incubated with N$^\omega$-nitro-l-arginine (l-NNA; 100 μmol/l) for 30 min before relaxation was again measured to evaluate Ach-stimulated NOS activity in vessels from vehicle- and E$_2$b-treated rats.

**Isolated Perfused Lung Preparation**

The isolated lung preparation was used to assess segmental vascular resistances in rat lungs as previously described by Eichinger and Walker (7). Briefly, lungs were isolated from OVX rats treated with E$_2$b or vehicle for 7 days to determine whether E$_2$b upregulates eNOS activity in the pulmonary vasculature. Each rat was anesthetized with pentobarbital sodium, and the trachea was cannulated to ventilate the lungs at a frequency of 55 breaths/min and a tidal volume of 2.5 ml with warmed and humidified gas (6% CO$_2$, 21% O$_2$, and balance N$_2$). Inspiratory pressure was set at 9 cmH$_2$O and positive end-expiratory pressure at 2 cmH$_2$O. Heparinized whole blood was withdrawn from the heart to measure plasma E$_2$b levels with a radioimmunoassay (DiSorin). The pulmonary artery was cannulated with a 13-gauge needle stub and perfused with PSS containing 4% albumin (4% g/24 h; Sigma) or vehicle (polypropylene glycol) to elicit a 10 Torr increase in tone.

**Isolated Perfused Lung Protocol**

Total, arterial, and venous vasodilatory responses to the endothelium-dependent vasodilator ionomycin. To determine if 7 days of in vivo treatment with E$_2$b increased eNOS activity in pulmonary arteries, we examined total, arterial, and venous vasodilatory responses to eNOS stimulation by ionomycin (calcium ionophore) in lungs from E$_2$b- and vehicle-treated rats. Lungs were isolated and equilibrated as described in Isolated Perfused Lung Preparation. After 30 min of equilibration, lungs were constricted with the thromboxane mimetic U-46619 to elicit a stable pressor response of ~10 Torr above baseline pressure. Lungs that required a dose of U-46619 >8 μg to elicit a 10 Torr increase in tone were eliminated. Once vasoconstriction was stable, NO-dependent vasodilation was measured with ionomycin (1 μmol/l). Pulmonary capillary pressure (Pcap) was estimated with the double occlusion technique (7, 13, 36). Total and segmental pulmonary vascular resistances were calculated during basal, constricted, and dilated conditions with the equations RT = (Pa – Pv)/Q, Ra = (Pa – Pcap)/Q, and Rv = (Pcap – Pv)/Q, where RT is total resistance, Ra is arterial resistance, Rv is venous resistance, and Q is flow. Vasodilatory responses were calculated as percent reversal of U-46619-induced vasoconstriction for total, arterial, and venous segments.

Double occlusion was performed by simultaneously occluding the arterial inflow and venous outflow for ~5 s. Vascular pressures equilibrated to microvascular perfusion pressure...
to yield an assessment of Pcap. According to previous studies (6, 36), this method of vascular occlusion yields accurate estimates of Pcap. In a separate set of experiments, the NO contribution to dilation was assessed by pretreating isolated lungs with L-NNA (300 μmol/l) using the same protocol described in Isolated Perfused Lung Protocol. This dose of L-NNA is effective in inhibiting endothelium-derived NO-dependent pulmonary vasodilation in the isolated perfused rat lung (27).

Western Blot Analysis

eNOS expression in aortas and lung homogenates from E₂β- and vehicle-treated rats. eNOS protein content was evaluated with standard immunoblotting methods. The left lobes of the lungs and the thoracic aortas from E₂β- and vehicle-treated groups were rapidly isolated, rinsed briefly in ice-cold homogenizing buffer, and frozen in liquid nitrogen. Frozen tissue was coarsely ground in liquid nitrogen with a precooled mortar and pestle and then further homogenized in a glass dounce homogenizer in ice-cold Tris-HCl buffer containing EDTA (0.3 mg/ml), leupeptin (5 μg/ml), pepstatin A (0.7 μg/ml), aprotime (2 μg/ml), and phenylmethylsulfonyl fluoride (20 μg/ml). Homogenates were spun at 800 g at 4°C for 1.5 min to remove cellular debris, the supernatant was drawn off, and an aliquot was analyzed for protein concentration with the Bradford method (Bio-Rad protein assay). Samples were dissolved in 5× sample buffer (1 μg/ml), boiled for 5 min, and separated in 15% polyacrylamide gels. In addition to E₂β and vehicle samples (10 μg/lane, aorta; 30 μg/lane, lung), each gel contained molecular weight markers and an eNOS standard (Transduction Laboratories). Separated proteins were transferred to polyvinylidene difluoride membranes, blocked overnight, and probed with a monoclonal antibody specific for eNOS (1:1,000; Transduction Laboratories). Enhanced chemiluminescence development in conjunction with a horseradish peroxidase-labeled secondary antibody (1:5,000) was used to visualize eNOS protein. The relative quantity of protein was determined with SigmaGel software (SPSS). The molecular weight markers and an eNOS protein standard were used to verify antibody detection of proteins of the expected size and to normalize values between gels. In a separate experiment, 5, 10, and 15 μg/lane of protein were analyzed to ensure linearity.

Immunohistochemistry

Immunostaining for eNOS in aortas from E₂β- and vehicle-treated rats. To further establish if E₂β increases eNOS, we assessed eNOS immunostaining in aortas and pulmonary arteries. Cleaned aortic segments were placed in specimen molds that contained embedding medium (Tissue-Tek O.C.T. compound) and frozen in isobutane cooled with liquid nitrogen. Transverse sections of the vessels were cut (10 μm) and thaw-mounted on glass slides (Superfrost Plus). Sections were stained for elastin and counterstained with Van Gieson solution (Accustain Elastic Stain; Sigma) to identify arteries and veins. Arteries were identified by the presence of an internal elastic lamina and ranged in size from 200 to 300 μm. Densitometry was performed as described in Immunostaining for eNOS in aortas from E₂β- and vehicle-treated rats.

Ribonuclease Protection Assay

Construction of probe templates for ribonuclease protection assay analysis. Probe templates for ribonuclease protection assays (RPAs) were constructed for rat eNOS and malate dehydrogenase (MDH). Rat cDNA was synthesized from isolated perfused lungs with reverse transcription. Segments of the cDNA obtained were amplified for eNOS and MDH with PCR, and primer sequences were made with archived rat and mouse DNA sequences (GenBank). Probe templates were amplified with Pyrococcus furiosus DNA polymerase (Stratagene), and the primer sequences are listed in Table 1. The eNOS PCR product was inserted into the Srf1 site of the pPCR-Script Amp SK(+)- cloning vector (GenBank accession no. U46017) with the PCR-Script Amp cloning kit (Stratagene). Plasmid DNA was prepared from transformants with the Qiagen Spin miniprep kit (Qiagen) and subjected to restriction analysis to determine the orientation of inserts relative to the vectors T7 and T3 RNA polymerase promoters. eNOS and MDH PCR templates were prepared with reverse PCR primers with a T7 RNA polymerase promoter sequence, 5'-TAATACGACTCACTATAGGG-3', appended to the 5'-end of levels (0 = 100% light transmittance and 225 = 0% light transmittance). A densitized image was generated after subtracting out background, and densitometry values were calculated from the average density of all regions that stained above threshold.

Immunohistochemical staining for eNOS in lungs from E₂β- and vehicle-treated rats. Lungs were prepared for immunohistochemical analysis as described previously (26). After isolation, lungs were perfused with PSS containing 4% bovine serum albumin and papaverine (10⁻⁴ mmol/l) followed by perfusion with phosphate buffer containing paraformaldehyde (5%), glutaraldehyde (0.1%), and papaverine (10⁻⁴ mmol/l). During perfusion with PSS and fixatives, Pv was maintained at 12 Torr to elicit maximal recruitment (8). Lungs were inflated, fixed, and embedded in optimum cutting temperature compound. Transverse sections were cut (10 μm) and thaw-mounted onto glass slides (Superfrost Plus). Lung sections were immunostained following the same protocol described for aortic tissues (see Immunostaining for eNOS in aortas from E₂β- and vehicle-treated rats). Serial sections were stained for elastin and counterstained with Van Gieson solution (Accustain Elastic Stain; Sigma) to identify arteries and veins. Arteries were identified by the presence of an internal elastic lamina and ranged in size from 200 to 300 μm. Densitometry was performed as described in Immunostaining for eNOS in aortas from E₂β- and vehicle-treated rats.

Table 1. Accession nos., expected PCR product sizes of each fragment product length, and primer sequences for eNOS and MDH

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Product Length, bp</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>MDH</td>
<td>M29462</td>
<td>110</td>
<td>5'-TGCGAGACCCCATCAGAGAC-3' (forward)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5'-CTGATACGGCTCATCAGAAG-3' (reverse)</td>
</tr>
<tr>
<td>eNOS</td>
<td>U53142</td>
<td>308</td>
<td>5'-GAGAAGATGGGCAAGCTGGCT-3' (forward)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5'-GCTTACAGTGGCCCTCCACTAG-3' (reverse)</td>
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MDH, malate dehydrogenase; eNOS, endothelial nitric oxide synthase.
the original reverse primer. The new primer was used to reamplify the original PCR products, resulting in the T7 promoter being expressed upstream of the antisense RNA strand. Probe templates were purified with the QIAquick PCR purification kit (QIAGEN). A MAXIscript in vitro transcription kit (Ambion) was used according to the manufacturer’s recommendations to prepare the radiolabeled antisense cRNA. Full-length RNA probes were purified by PAGE on 5% Tris-borate-EDTA (TBE)-urea gels, and the amount of radioactive label was determined by scintillation counting. The eluted probes were stored in elution buffer at −20°C until needed.

eNOS mRNA expression in thoracic aortas and lungs from E2β- and vehicle-treated rats. Total RNA was isolated from lungs and aorta by homogenization in TRIzol reagent (GIBCO BRL). A RPA Kit II (Ambion) was used to quantitate mRNA in homogenates from treated and control groups according to the manufacturer’s recommendations. In brief, aliquots of the labeled probe containing 2–8 × 10⁶ counts/min (cpm) were added to 5 μg of total RNA. Samples were precipitated in ethanol, resuspended in hybridization buffer, and heated to 95°C for 4 min. The hybridization reactions were incubated overnight at 42°C, then digested with RNase A (2.5 U/ml), and cloned with RNase T1 (100 U/ml). Digested products were precipitated, resuspended in 4–6 μl of formamide loading buffer, loaded onto 5% TBE-urea polyacrylamide minigels (Bio-Rad), and electrophoresed. Dried gels were exposed to a phosphor storage screen (Molecular Dynamics). Bands were quantitated with ImageQuant software, and products are expressed as a percent of the internal control gene MDH. Additional hybridizations containing 5, 10, and 15 μg of total RNA from lung tissue were performed to ensure linearity.

**Drugs and Chemical Solutions**

Indomethacin (Sigma) was dissolved in ethanol. PE, ACh, and L-NNA (all from Sigma) were dissolved in double-distilled water (ddH₂O). Ionomycin (Calbiochem) was diluted in dimethyl sulfoxide (Sigma) and stored at 4°C. Meclofenamate (Sigma) was prepared in normal saline. U-46619 was diluted in dimethyl sulfoxide (Sigma) and stored at 4°C. Meclofenamate was dissolved in ethanol, resuspended in hybridization buffer, and heated to 95°C for 4 min. The hybridization reactions were incubated overnight at 42°C, then digested with RNase A (2.5 U/ml), and cloned with RNase T1 (100 U/ml). Digested products were precipitated, resuspended in 4–6 μl of formamide loading buffer, loaded onto 5% TBE-urea polyacrylamide minigels (Bio-Rad), and electrophoresed. Dried gels were exposed to a phosphor storage screen (Molecular Dynamics). Bands were quantitated with ImageQuant software, and products are expressed as a percent of the internal control gene MDH. Additional hybridizations containing 5, 10, and 15 μg of total RNA from lung tissue were performed to ensure linearity.

**Statistics**

Data are reported as means ± SE, with P ≤ 0.05 considered significant. Groups were compared with Student’s t-test of significance. In the contractility studies, individual points on concentration-response curves for the two groups were compared with Student’s t-test. Data expressed as percentages were normalized with the arcsine transformation before statistical analysis. The n value is the number of animals used for each study.

**RESULTS**

**Plasma E2β Concentrations in OVX Female Rats**

E2β plasma levels were significantly higher in rats treated with E2β (102 ± 11 pg/ml) compared with those treated with vehicle (<5 pg/ml). E2β replacement resulted in plasma levels in the physiological range (1, 3, 15, 18, 30), demonstrating that E2β replacement via osmotic pumps delivered a dose of E2β in OVX rats that mimicked endogenous levels.

**Effects of E2β In Vivo on Endothelium-Dependent Relaxation Induced by ACh in Aortic Rings**

Vasodilatory responses to ACh in endothelium-intact aortic rings from OVX rats treated with E2β or vehicle are summarized in Fig. 1A. Cumulative concentration-response curves to ACh (10⁻¹⁰ to 10⁻⁴.⁵ mol/l) were generated in tissues contracted with PE (10⁻⁶ mol/l). Force generated by PE was not different be-

![Image](http://ajplung.physiology.org/DownloadedFrom/10.220.33.6)
Effects of E2 from vehicle- and E2 was no difference in baseline resistance between lungs vasodilatory response to ionomycin in lungs from E2-treated animals. To elicit an increase in total, arterial, and venous vascular resistances from baseline resistance. Additionally, the change in vascular resistances induced by U-46619 was similar in lungs from both groups. However, a smaller dose of U-46619 was needed to achieve the same 10 Torr constriction in lungs with L-NNA compared with L-NNA vehicle-treated lungs (299 ± 24 vs. 605 ± 35 nmol/l for vehicle and 276 ± 9 vs. 534 ± 21 nmol/l for E2 under L-NNA and L-NNA vehicle conditions, respectively). After the U-46619 constriction stabilized, vasodilatory responses to ionomycin (1 μmol/l) were assessed. L-NNA treatment annulled the differences in ionomy-
cin-induced vasodilation in lungs from E2β-treated compared with vehicle-treated rats (Fig. 2B). However, the vasodilatory response to ionomycin was not completely blocked by l-NNa in either group of lungs, suggesting that a vasodilatory component other than NO is involved in ionomycin-induced pulmonary vasodilation.

**Western Blot Analysis of Endothelial Expression of eNOS in Aortas and Lungs From E2β- and Vehicle-Treated OVX Rats**

To determine if enhanced endothelium-dependent relaxation observed in aortas isolated from E2β-treated rats was due to increased eNOS expression, eNOS levels were assessed in tissue homogenates from E2β- and vehicle-treated rats. Figure 3A illustrates eNOS protein expression in aortic homogenates. Consistent with the contractility studies, there was a significant increase in eNOS protein expression in aortic tissue from E2β-treated rats. This suggests that E2β treatment in vivo for 7 days augments aortic endothelium-dependent relaxation by increasing NO production via elevated eNOS enzyme concentrations.

Because lungs from E2β-treated animals exhibited enhanced vasodilation to ionomycin and this response was abolished by l-NNa, eNOS protein levels were also evaluated in lung tissue homogenates (Fig. 3B). However, there was no difference in eNOS protein expression between groups as evaluated by Western analysis.

**Immunohistochemistry for eNOS in Aortas and Lungs From E2β- and Vehicle-Treated OVX Rats**

Figure 4A illustrates eNOS immunoreactivity in aortas from vehicle- and E2β-treated animals. Consistent with the data from Western analysis, quantitative immunohistochemistry demonstrated a greater intensity of eNOS staining in aortas from E2β-treated rats compared with aortas isolated from vehicle-treated rats. Moreover, the staining was localized within the endothelium. Photomicrographs of pulmonary arteries (~300 μm in diameter) stained with the eNOS antibody from vehicle- and E2β-treated rats are illustrated in Fig. 4B. eNOS staining was significantly greater in pulmonary arteries from E2β-treated compared with vehicle-treated rats. This suggests that the upregulation of eNOS occurs within the vasculature but is not detectable by Western analysis of whole lung homogenates.

**Analysis of eNOS mRNA Levels in Aortas and Lungs From E2β- and Vehicle-Treated OVX Rats**

Because data from the aorta suggest that eNOS protein expression is increased in animals treated with E2β, gene expression for eNOS was evaluated in the same tissue harvested for eNOS protein analysis. Figure 5A illustrates RPA data showing eNOS mRNA normalized to MDH mRNA in aortas from vehicle- and E2β-treated OVX rats. There was no significant difference between groups. It appears that the E2β-induced enhanced aortic vasodilation did not require sustained

<table>
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<tr>
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<th>Total Resistance, mmHg·ml⁻¹·min⁻¹·kg</th>
<th>Arterial Resistance, mmHg·ml⁻¹·min⁻¹·kg</th>
<th>Venous Resistance, mmHg·ml⁻¹·min⁻¹·kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>O VX vehicle l-NNa (300 μmol/l)</td>
<td>8 0.11 ± 0.01 0.07 ± 0.01 0.04 ± 0.01</td>
<td>0.45 ± 0.02 0.22 ± 0.01 0.24 ± 0.02</td>
<td>0.34 ± 0.00 0.18 ± 0.00 0.17 ± 0.01</td>
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<tr>
<td>O VX E2b l-NNa (300 μmol/l)</td>
<td>8 0.11 ± 0.01 0.07 ± 0.00 0.04 ± 0.01</td>
<td>0.47 ± 0.03 0.25 ± 0.02 0.22 ± 0.01</td>
<td>0.36 ± 0.00 0.18 ± 0.01 0.18 ± 0.01</td>
</tr>
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Values are means ± SE; n, no. of rats. L-NNa, Nω-nitro-l-arginine.
elevation of eNOS transcription. mRNA for eNOS was also assessed in total RNA isolated from lung tissue. Figure 5B illustrates eNOS mRNA normalized to MDH mRNA in lungs from vehicle- and E₂β-treated female rats. Similar to those observed in aortic tissue, eNOS mRNA levels were not different between groups.

DISCUSSION

It has been suggested that estrogen directly modulates vascular tone by enhancing endothelium-dependent vasodilation. We observed that physiological levels of E₂β replacement in OVX rats augmented endothelium-dependent vasodilation and enhanced eNOS activity in aortas and pulmonary blood vessels. The observation that E₂β treatment in vivo increased endothelium-dependent relaxation in the aorta is consistent with results from previous studies with isolated uterine arteries from guinea pigs (4) and femoral arteries from rabbits (12). Our results in the pulmonary circulation demonstrate for the first time that endothelium-dependent vasodilation in the lung is enhanced by chronic E₂β treatment. These results expand the role for E₂β in protecting cardiovascular function.

The mechanisms by which E₂β increases endothelium-dependent relaxation have not been clearly elucidated. Some studies suggest that E₂β in vivo selectively enhances NO-dependent relaxation. For example, Cheng et al. (3) observed that aortic rings from rats chronically treated with E₂β demonstrated enhanced relaxation to ACh but not to histamine, an endothelium-dependent vasodilator that activates phospholipase A₂ production of prostacyclin. This indirectly suggested that long-term administration of E₂β enhances endothelium-dependent relaxation of blood vessels by upregulating eNOS. Our direct measures of eNOS protein provide additional evidence for eNOS upregulation by E₂β.

Reports of estrogen actions in other vascular beds have demonstrated that E₂β relaxes uterine, skin, and...
coronary vessels (28, 31, 40). During pregnancy, when circulating levels of estrogen are high, pulmonary vasodilation to hypoxia and angiotensin II is decreased (23, 34). Moreover, hypoxic pulmonary vasoconstriction in lungs isolated from female sheep is less compared with constriction in lungs from males (39). Together, these studies provide indirect evidence that estrogen enhances vasodilation in multiple vascular beds including the pulmonary circulation. Thus we evaluated chronic E$_2$ treatment on vasoactivity and eNOS expression in both the aorta and the pulmonary vasculature. We hypothesized that if E$_2$ upregulates eNOS expression, then endothelium-dependent vasodilators would elicit greater NO-dependent relaxation in arteries from E$_2$-treated animals.

In support of this hypothesis, we found that lungs isolated from E$_2$-treated rats exhibited augmented vasodilatory responses to ionomycin and that aortas from E$_2$-treated rats had augmented endothelium-dependent relaxation. Because E$_2$ elicits an endothelium-dependent cardioprotective effect in the systemic and coronary vasculatures, these results suggest it does the same in the pulmonary vasculature. In this way, E$_2$ may maintain low pulmonary vascular resistance in times of stress such as lung disease and hypoxia.

Previous studies have demonstrated gender differences in hypoxic pulmonary hypertension (24) and in hypoxia-induced pulmonary vascular remodeling (39). Although our study did not investigate the role of E$_2$ during hypoxia, it did study the mechanisms associated with E$_2$ regulation of pulmonary endothelial function, which is modified by hypoxia. We found that NOS inhibition with L-NNA attenuated ionomycin-induced vasodilation in lungs from both vehicle- and E$_2$-treated animals and that this treatment abolished the enhanced vasodilation in lungs from E$_2$-treated rats. Although the ionomycin vasodilatory response was not completely abolished by NOS inhibition, there was no difference in vasodilation between groups when NOS activity was inhibited, suggesting that E$_2$ enhances pulmonary endothelial cell NOS activity.

In aortic studies, the augmented component of the ACh-induced aortic relaxation by E$_2$ was also completely abolished by NOS inhibition. This is in agreement with the observation (19) that E$_2$ enhances flow-induced dilation in the brachial arteries of postmenopausal women only in the absence of a NOS antagonist. Together, these observations show that the enhanced endothelium-dependent relaxation that follows E$_2$ treatment requires NOS.

Interestingly, Chang et al. (2) demonstrated that E$_2$ may stimulate prostacyclin synthesis in VSM. Like NO, prostacyclin is a potent pulmonary vasodilator capable of contributing to the enhanced vasodilatory effects that follow E$_2$ treatment. However, in our preparation, prostacyclin is unlikely to have contributed to either the pulmonary dilation or the augmented aortic response because all preparations were treated with a cyclooxygenase inhibitor. In the isolated lungs, the dilation remaining after meclofenamate and L-NNA treatment thus appeared to be dependent on other endothelium-derived factors such as endothelium-dependent hyperpolarizing factor. Therefore, because the difference in the vasodilatory response to ionomycin between lungs from E$_2$- and vehicle-treated rats was abolished by L-NNA treatment but was present during cyclooxygenase inhibition and all aortic relaxation was abolished by L-NNA, we concluded that the enhanced vasodilation induced by estrogen replacement was due to enhanced NO synthesis.

In addition to affecting endothelial function, E$_2$ may further enhance vasodilation by altering VSM sensitivity to NO. In postmenopausal women, short-term administration of E$_2$ augments vasodilatory responses to ACh in the coronary (11, 25) and systemic (35) circulations. These studies showed that E$_2$ improved both endothelium-dependent (ACh) and -independent (sodium nitroprusside) vasodilatory responses in coronary and forearm blood vessels, suggesting that E$_2$ can influence both endothelial and VSM function. In contrast to what was shown in postmenopausal women but in agreement with animal studies (3), we observed no differences in sensitivity to the NO donor SNAP in denuded aortic rings from E$_2$- and vehicle-treated rats. In addition, pulmonary vasodilation to NO donors was not augmented by E$_2$ treatment (Resta TC and Walker BR, unpublished data). Therefore, our data cannot be explained by enhanced VSM sensitivity to NO.

Many substances, including E$_2$, can regulate eNOS activity acutely by increasing endothelial cell intracellular calcium concentration ([Ca$^{2+}$]$\text{_{i}}$) to activate eNOS and by stimulating NO release. However, the mechanism of the chronic effect of E$_2$ upregulation of NOS activity is less clear. Classically, the regulation of transcription by E$_2$ is mediated by receptor binding and transport to the nucleus. The subsequent regulation of gene transcription and protein expression is dependent on the presence of an estrogen response element, which is the binding site for the estrogen receptor complex. It has been suggested that the eNOS promoter contains an estrogen response element as well as an activator protein-1 binding site, both of which are thought to regulate enzyme expression and NO release (21). Although a functional study (16) showed E$_2$ upregulation of eNOS (16), an electromobility shift study (21) found that the estrogen receptor does not bind to the NOS promoter. Therefore, E$_2$ regulation of eNOS expression remains controversial. With Western analysis, we found that E$_2$ treatment in vivo increased eNOS protein levels in rat aorta compared with vehicle treatment. We further demonstrated that eNOS-immunoreactive staining was more intense in aortas from E$_2$-treated rats. These findings are in agreement with our observation that ACh relaxation was augmented in the aorta and with previous reports (16, 29, 37) that demonstrated increased eNOS protein expression after chronic administration of higher doses of E$_2$.

To date, no studies have investigated the effect of E$_2$ treatment in vivo on NOS expression in the pulmonary vasculature. However, an in vitro study (20)
has demonstrated that chronic E$_2$B administration upregulates eNOS gene expression in fetal pulmonary arterial endothelial cells. Thus this study suggests that E$_2$B may augment pulmonary vasodilation by upregulating the NO-generating enzyme eNOS.

We found that E$_2$B treatment increased eNOS immunostaining in pulmonary arteries. Arterial and venous morphology, determined in serial sections, made it apparent that eNOS staining was enhanced only in arteries. This was in agreement with our functional studies that showed that only pulmonary arterial vasodilation was enhanced in E$_2$B-treated rats. In addition to immunohistochemical analysis, Western blots were used to quantitate eNOS protein expression. In contrast to immunohistochemical analysis, we found no detectable differences in eNOS expression in lung homogenates from the E$_2$B- and vehicle-treated groups with this method. One possibility for the disparate findings with these two techniques is that the level of E$_2$B induction within the lung is localized within the arterial vascular endothelium and is too small to be detected by Western analysis of whole lung homogenates.

Because eNOS protein levels in E$_2$B-treated rats appeared to be enhanced in both vascular beds, we also examined eNOS mRNA levels with a RPA. In contrast to E$_2$B immunostaining, we found no increases in mRNA in either aorta or lung from rats treated with E$_2$B. It is interesting that other studies (14, 32) reporting increased eNOS protein levels after E$_2$B administration have not detected increased message either. The increase in eNOS expression apparent in immunostained sections suggests that the augmented endothelium-dependent responses associated with E$_2$B treatment are due to elevated NO expression but not to persistent increases in eNOS message.

In conclusion, replacing E$_2$B in OVX rats to produce physiological plasma levels enhances endothelium-dependent relaxation and increases eNOS expression in both thoracic aortas and pulmonary arteries. Thus it is likely that the observed augmentation of the endothelium-dependent vasodilation in the aortas and lungs from E$_2$B-treated female rats is due to increased levels of the NO-generating enzyme eNOS. Enhanced eNOS levels may thus contribute to E$_2$B cardioprotective effects in both systemic and pulmonary arteries.

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