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Am J Physiol Heart Circ Physiol, April 1, 2010; 298 (4): H1235-H1248.

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T. Lahm

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J. B. Snow, V. Kitzis, C. E. Norton, S. N. Torres, K. D. Johnson, N. L. Kanagy, B. R. Walker and T. C. Resta

J Appl Physiol, January 1, 2008; 104 (1): 110-118.

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Maturation changes in the regulation of pulmonary vascular tone by nitric oxide in neonatal rats

L. G. Chicoine, M. L. Paffett, M. R. Girton, M. J. Metropoulos, M. S. Joshi, J. A. Bauer, L. D. Nelin, T. C. Resta and B. R. Walker

Am J Physiol Lung Cell Mol Physiol, November 1, 2007; 293 (5): L1261-L1270.

[Abstract] [Full Text] [PDF]

Endogenous estrogen attenuates pulmonary artery vasoreactivity and acute hypoxic pulmonary vasoconstriction: the effects of sex and menstrual cycle

T. Lahm, K. M. Patel, P. R. Crisostomo, T. A. Markel, M. Wang, C. Herring and D. R. Meldrum

Am J Physiol Endocrinol Metab, September 1, 2007; 293 (3): E865-E871.

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Estradiol-induced attenuation of pulmonary hypertension is not associated with altered eNOS expression

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Resta, Thomas C., Nancy L. Kanagy, and Benjimen R. Walker. Estradiol-induced attenuation of pulmonary hypertension is not associated with altered eNOS expression. *Am J Physiol Lung Cell Mol Physiol* 280: L88–L97, 2001.—Female rats develop less severe pulmonary hypertension (PH) in response to chronic hypoxia compared with males, thus implicating a potential role for ovarian hormones in mediating this gender difference. Considering that estrogen upregulates endothelial nitric oxide (NO) synthase (eNOS) in systemic vascular tissue, we hypothesized that estrogen inhibits hypoxic PH by increasing eNOS expression and activity. To test this hypothesis, we examined responses to the endothelium-derived NO-dependent dilator ionomycin and the NO donors *S*-nitroso-*N*-acetylpenicillamine and spermine NONOate in U-46619-constricted, isolated, saline-perfused lungs from the following groups: 1) normoxic rats with intact ovaries, 2) chronic hypoxic (CH) rats with intact ovaries, 3) CH ovariectomized rats given 17 β -estradiol (E₂ β), and 4) CH ovariectomized rats given vehicle. Additional experiments assessed pulmonary eNOS levels in each group by Western blotting. Our findings indicate that E₂ β attenuated chronic hypoxia-induced right ventricular hypertrophy, pulmonary arterial remodeling, and polycythemia. Furthermore, although CH augmented vasodilatory responsiveness to ionomycin and increased pulmonary eNOS expression, these responses were not potentiated by E₂ β . Finally, responses to *S*-nitroso-*N*-acetylpenicillamine and spermine NONOate were similarly attenuated in all CH groups compared with normoxic control groups. We conclude that the inhibitory influence of E₂ β on chronic hypoxia-induced PH is not associated with increased eNOS expression or activity.

chronic hypoxia; right ventricular hypertrophy; vascular remodeling; nitric oxide-dependent vasodilation; endothelial nitric oxide synthase

PULMONARY HYPERTENSION associated with chronic hypoxia is characteristic of chronic obstructive pulmonary diseases as well as of residence at high altitude. It has been noted that women with chronic obstructive pulmonary diseases exhibit a decreased risk of mortality compared with men (36), although insufficient comparisons have been made to determine the etiology of this sexual dimorphism. Consistent with this observation

are studies (22, 28) indicating that female rats and swine develop less severe pulmonary hypertension, right ventricular hypertrophy, arterial remodeling, and polycythemia in response to chronic hypoxia compared with males. Isolated ovine lung studies (15, 38) have further demonstrated inhibitory effects of 17 β -estradiol (E₂ β) on hypoxic pulmonary vasoconstriction (HPV). These findings are consistent with a role for estrogen in attenuating both fixed (remodeling, polycythemia) and active (HPV) components of chronic hypoxia-induced pulmonary hypertension, although the mechanisms by which estrogen may exert such protective influences have not been examined.

Studies in other vascular beds suggest estrogen exhibits antiatherogenic properties that are mediated in part by alterations in endothelial function (1; reviewed in Ref. 17), including enhanced synthesis of the vasodilator and antimitogenic factor nitric oxide (NO) (4, 17, 18, 21). Sustained increases in NO synthesis in response to estrogen have been attributed to upregulation of endothelial NO synthase (eNOS) in both systemic and pulmonary vascular tissue (17, 18, 21). Consistent with these findings are preliminary reports from our laboratory suggesting that 1 wk of estrogen administration to normoxic ovariectomized (OVX) rats augments endothelium-derived NO (EDNO)-dependent pulmonary vasodilation (14) and elevates pulmonary vascular eNOS levels (13). We therefore hypothesized that the inhibitory influence of estrogen on the development of chronic hypoxia-induced pulmonary hypertension is a function of increased eNOS expression and activity. eNOS activity was assessed by examining responses to the EDNO-dependent vasodilator ionomycin in lungs isolated from chronically hypoxic (CH) rats with intact ovaries as well as from CH OVX rats receiving E₂ β or vehicle for the duration of hypoxic exposure. Pulmonary eNOS expression was determined for each group of rats by Western blotting. Our findings indicate that although E₂ β attenuates chronic hypoxia-induced right ventricular hypertrophy and pulmonary arterial remodeling, this protective influence of E₂ β is not associated with increased pulmo-

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nary eNOS expression or enhanced EDNO-dependent responsiveness.

METHODS

All protocols and surgical procedures employed in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico School of Medicine (Albuquerque, NM).

Experimental Groups

Four groups of female Sprague-Dawley rats (200–350 g; Harlan Industries) were used for all experiments: 1) normoxic rats with intact ovaries, 2) CH rats with intact ovaries, 3) CH OVX rats that received $E_2\beta$ (20 $\mu\text{g}/\text{day}$ via subcutaneous osmotic pumps), and 4) CH OVX rats that received vehicle. Animals designated for exposure to chronic hypoxia were housed in a hypobaric chamber with barometric pressure maintained at 380 mmHg for 4 wk. The chamber was opened 3 times/wk to provide animals with fresh food, water, and clean bedding. On the day of experimentation, animals were removed from the hypobaric chamber and immediately placed in Plexiglas chambers continuously flushed with a 12% O_2 -88% N_2 gas mixture to reproduce inspired PO_2 (~70 mmHg) within the hypobaric chamber. Age-matched control animals were housed at ambient barometric pressure (~630 mmHg). All animals were maintained on a 12:12-h light-dark cycle.

Surgical Procedures for Ovariectomy and Osmotic Pump Implantation

Rats designated for ovariectomy were anesthetized with a mixture of ketamine (90 mg/kg im) and acepromazine (0.9 mg/kg im). With sterile technique, ovaries were resected through bilateral flank incisions. Rats were allowed 2 wk to recover before implantation of the osmotic pumps (Alzet model 2ML4) for the 4-wk administration of $E_2\beta$ or vehicle (97% 1,2 propanediol-3% ethanol). Osmotic pumps were implanted subcutaneously via a midline incision between the scapulae in rats anesthetized with ketamine-acepromazine. All animals were administered systemic and topical antibiotics postoperatively. The rats were placed in the hypobaric chamber the morning after osmotic pump implantation.

Assessment of Plasma $E_2\beta$, Uterine Weight, Polycythemia, and Right Ventricular Hypertrophy

Blood samples were obtained by direct cardiac puncture at the time of lung isolation for measurement of hematocrit and plasma levels of $E_2\beta$. Plasma $E_2\beta$ was determined by radioimmunoassay with a standard kit (DiaSorin). Uterine weight was measured as a further indication of $E_2\beta$ delivery.

Right ventricular hypertrophy was assessed as an index of chronic hypoxia-induced pulmonary hypertension with previously described methods (29–31). Briefly, after isolation of the heart, the atria and major vessels were removed from the ventricles. The right ventricle (RV) was dissected from the left ventricle and septum (LV+S), and each was weighed. The degree of right ventricular hypertrophy is expressed as the ratio of RV to total ventricle weight (T).

Vascular Morphometry

To determine whether the inhibitory influence of estrogen on chronic hypoxia-induced pulmonary hypertension was associated with decreased arterial remodeling, quantitative morphometric analyses of arterial cross sections were per-

formed on lungs from each group of rats as described previously by Resta and colleagues (29, 32). Rats from each group were anesthetized with intraperitoneal pentobarbital sodium (25 mg). After the trachea was cannulated with a 17-gauge needle stub, the lungs were ventilated with a Harvard positive-pressure rodent ventilator (model 683) at a frequency of 55 breaths/min and a tidal volume of 2.5 ml with a warmed and humidified gas mixture (6% CO_2 in room air). Inspiratory pressure was set at 9 cmH_2O , and positive end-expiratory pressure was set at 3 cmH_2O . After a median sternotomy, heparin (100 U) was injected directly into the RV, and the pulmonary artery was cannulated with a 13-gauge needle stub. The preparation was immediately perfused at 0.8 ml/min with a Masterflex microprocessor pump drive (model 7524–10) with a physiological saline solution (PSS) containing (in mM) 129.8 NaCl, 5.4 KCl, 0.83 MgSO_4 , 19 NaHCO_3 , 1.8 CaCl_2 , and 5.5 glucose with 4% (wt/vol) albumin, all from Sigma. Papaverine (10^{-4} M) was also added to the perfusate to maintain the vasculature in a dilated state during subsequent fixation. The LV was cannulated with a plastic tube (4-mm OD), and the heart and lungs were removed en bloc and suspended in a humidified chamber maintained at 38°C. The perfusion rate was gradually increased to 60 $\text{ml}\cdot\text{min}^{-1}\cdot\text{kg body wt}^{-1}$, and venous pressure (P_v) was maintained at 12 mmHg. Previous work from our laboratory (3) suggested that maximal recruitment, and thus maximal vascular surface area, is achieved at this flow and P_v . The vasculature was initially washed with 250 ml of PSS, followed by 250 ml of fixative (0.1 M phosphate buffer with 4% paraformaldehyde, 0.1% glutaraldehyde, and 10^{-4} M papaverine). Lungs were additionally inflated with fixative via the trachea to a pressure of 23 cmH_2O during perfusion. Arterial pressure (P_a) and P_v were measured with Spectramed model P23XL pressure transducers and monitored on separate channels of a Gould RS 3400 chart recorder. The trachea was ligated with 2-0 silk, the arterial and venous lines were simultaneously clamped, and the lungs were immersed in fixative. A transverse section (2- to 3-mm thick) of tissue from the hilar level of the left cranial lobe was removed, embedded in paraffin, sectioned at a thickness of 4 μm , and mounted on slides. Sections were stained for elastin, and arteries were identified by the presence of an internal elastic lamina (23). A total of 624 arteries from 15 rats were analyzed. Vessels were examined with a $\times 40$ objective on a Nikon Diaphot 300 microscope, and images were generated with a cooled digital CCD camera (Photometrics SenSys 1400). Vessel images were processed on a Dell Optiplex GXMT 5166 computer equipped with MetaMorph imaging system hardware and software (Universal Imaging). Measurements included medial circumference, assessed from the outer margin of the external elastic lamina, and luminal circumference. Vessels sectioned at oblique angles were excluded from analysis.

Isolated Lung Preparation

Separate sets of animals from each group were anesthetized with intraperitoneal pentobarbital sodium (25 mg). Lungs were isolated and perfused as described in *Vascular Morphometry* with the exceptions that papaverine was not included in the PSS and meclofenamate (10 $\mu\text{g}/\text{ml}$) was added to minimize the potential complicating influences of prostaglandins on vascular reactivity. This dose of meclofenamate is approximately threefold higher than that previously shown to provide effective inhibition of prostaglandin synthesis in this preparation (11). The perfusion rate was gradually increased to 30 $\text{ml}\cdot\text{min}^{-1}\cdot\text{kg body wt}^{-1}$ and maintained at this rate for the duration of the experiment. Twenty

milliliters of perfusate were washed through the lungs and discarded before recirculation was initiated with the remaining 40 ml. Lungs were allowed 30 min to equilibrate before experiments were begun. Experiments were performed with lungs in zone 3 conditions, achieved by elevating the perfusate reservoir until P_v was 3–4 mmHg. P_a and P_v were measured with Spectramed model P23XL pressure transducers and recorded on a Gould RS 3400 chart recorder. Data were stored and processed with a computer-based data acquisition/analysis system (AT-CODAS, Dataq Instruments).

Isolated Lung Protocols

Segmental vasodilatory responses to ionomycin. To examine whether estrogen increases pulmonary eNOS activity, we assessed total, arterial, and venous vasodilatory responses to the non-receptor-mediated, EDNO-dependent vasodilator ionomycin (calcium ionophore; Calbiochem) (33) in lungs from each group of rats. Lungs were isolated and allowed to equilibrate as described in *Isolated Lung Preparation*. Baseline capillary pressure (P_c) was assessed by a double-occlusion procedure at the end of the 30-min equilibration period to allow calculation of segmental resistances (see *Calculations and Statistics*) as described previously (29–33). After assessment of baseline P_c , the thromboxane analog 9,11-dideoxy-9 α ,11 α -epoxymethanoprostaglandin $F_{2\alpha}$ (U-46619; Cayman Chemical) was added to the perfusate reservoir until a stable arterial pressor response of ~10 mmHg was achieved. U-46619 provides consistent and stable pressor responses in this preparation and, unlike hypoxia, constricts both arterial and venous segments of the pulmonary vasculature (29–33). P_c was assessed at the plateau of the pressor response by double occlusion. The vasculature was then dilated with 350 nM ionomycin, and P_c was again determined at the point of maximal vasodilation. Ionomycin was chosen as a non-receptor-mediated, EDNO-dependent vasodilator in this study because interpretation of responses to receptor-mediated agonists would be complicated by possible changes in the number or affinity of their respective receptors in response to hypoxia, ovariectomy, or $E_{2\beta}$ replacement. Furthermore, our laboratory has shown that this concentration of ionomycin elicits submaximal vasodilatory responses in this preparation (33) that correlate in magnitude with vascular eNOS protein levels (29).

Inhibition of responses to ionomycin by N^{ω} -nitro-L-arginine. The following protocols were employed to document the contribution of endogenous NO in mediating vasodilatory responses to ionomycin in lungs from each group of rats. Lungs were isolated as described in *Isolated Lung Preparation* and perfused with PSS containing 300 μ M N^{ω} -nitro-L-arginine (L-NNA; Sigma). Resta and Walker (33) have previously demonstrated that this dose of L-NNA is effective in inhibiting EDNO-dependent pulmonary vasodilation in the isolated perfused rat lung. Segmental vasodilatory responses to ionomycin (350 nM) were assessed after precontraction with U-46619 as described in *Segmental vasodilatory responses to ionomycin*.

Segmental vasodilatory responses to exogenous NO. Because any differences in reactivity to ionomycin between groups could potentially result from altered vascular smooth muscle reactivity to NO, additional experiments examined segmental responses to the NO donors *S*-nitroso-*N*-acetylpenicillamine (SNAP; 1 μ M; Sigma) and spermine NONOate (2 μ M; Cayman Chemical) in U-46619-constricted lungs from each group. Resta and Walker (33) have previously employed these concentrations of each NO donor to elicit vasodilation in isolated rat lungs. Parallel experiments

were performed with 1 μ M *N*-acetylpenicillamine (Sigma) and 2 μ M spermine (Sigma) to test for possible nonspecific actions of SNAP and spermine NONOate, respectively.

Western Blotting for eNOS

To assess whether pulmonary eNOS levels are elevated by estrogen, separate sets of rats from each group were anesthetized with intraperitoneal pentobarbital sodium (25 mg) and their lungs were quickly snap-frozen in liquid nitrogen. Whole right lungs were fragmented with a mortar and pestle cooled in liquid nitrogen, then were homogenized on ice in 10 mM Tris·HCl buffer (pH 7.4) containing 255 mM sucrose, 2 mM EDTA, 12 μ M leupeptin, 4 μ M pepstatin A, 1 μ M aprotinin, and 2 mM phenylmethylsulfonyl fluoride (all from Sigma). Homogenates were centrifuged at 1,500 *g* at 4°C for 10 min to remove tissue debris. Protein concentrations of samples were determined by the Bradford method (Bio-Rad protein assay). Tissue sample proteins were resolved by SDS-PAGE with 7.5% acrylamide gels. In addition to the samples, each gel included both molecular mass (Bio-Rad) and eNOS (human endothelial lysate; Transduction Laboratories) standards. The separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) and blocked overnight at 4°C with 5% nonfat milk, 3% BSA (Sigma), and 0.05% Tween 20 (Bio-Rad) in a Tris-buffered saline solution (TBS) containing 10 mM Tris·HCl and 50 mM NaCl (pH 7.5). Blots were incubated for 4 h at room temperature with a mouse monoclonal antibody raised against human eNOS (1:2,500; Transduction Laboratories) in TBS. Immunochemical labeling was achieved by incubation for 1 h at room temperature with a horseradish peroxidase-conjugated goat anti-mouse IgG (1:5,000; Bio-Rad) in TBS followed by chemiluminescence labeling (Amersham ECL). eNOS protein bands were detected by exposure to chemiluminescence-sensitive film (Kodak). Membranes were stained with Coomassie brilliant blue to confirm equal protein loading per lane.

Calculations and Statistics

Vascular morphometry. External and luminal arterial diameters were calculated from the medial and luminal circumferences, respectively. Arterial wall thickness was assessed by subtracting the luminal radius from the external radius and is expressed as a percent of external diameter according to the formula [(2 × wall thickness)/external diameter] × 100.

Isolated lung experiments. Total pulmonary vascular resistance in isolated perfused lungs was calculated as the difference between P_a and P_v divided by flow (30 ml·min⁻¹·kg body wt⁻¹). Pulmonary arterial resistance was calculated as the difference between P_a and P_c divided by flow. Similarly, pulmonary venous resistance was calculated as the difference between P_c and P_v divided by flow. Vasodilatory responses were calculated as percent reversal of U-46619-induced vasoconstriction for the total pulmonary vasculature as well as for arterial and venous segments.

Western blotting. eNOS protein bands from samples were quantitated by densitometric analysis (SigmaGel, SPSS) and normalized to those of the eNOS standard to allow statistical comparisons between blots.

All data are expressed as means ± SE. Values of *n* are the number of vessels in each group for statistical comparisons of vessel wall area; for all other comparisons, *n* is the number of animals in each group. Where appropriate, a one-way or two-way ANOVA was used to make comparisons. If differences were detected by ANOVA, individual groups were com-

Table 1. Plasma $E_2\beta$ concentrations and uterine weights

	<i>n</i>	Plasma $E_2\beta$, pg/ml	<i>n</i>	Uterine Weight, g/kg body wt
Normoxic intact	6	5.0 ± 1.8	46	1.968 ± 0.077
CH intact	6	6.8 ± 1.4	34	1.803 ± 0.071
CH OVX vehicle	4	4.0 ± 0.8	29	0.406 ± 0.028*
CH OVX $E_2\beta$	5	45.6 ± 9.0*	29	1.727 ± 0.093

Values are means ± SE; *n*, no. of rats. CH, chronically hypoxic; OVX, ovariectomized; $E_2\beta$, 17 β -estradiol. * P < 0.05 vs. all other groups.

pared with the Student-Newman-Keuls test. A probability of P < 0.05 was accepted as significant for all comparisons.

RESULTS

Plasma $E_2\beta$ and Uterine Weight

Plasma $E_2\beta$ levels and uterine weight were not different between normoxic and CH intact groups (Table 1). Levels of $E_2\beta$ in these groups were within the range expected for those phases of the 4-day estrus cycle when circulating $E_2\beta$ concentrations are low (8). Consistent with this observation, ovariectomy did not significantly decrease $E_2\beta$ concentrations in vehicle-treated CH rats but dramatically lowered uterine weight as expected. Administration of $E_2\beta$ to CH OVX rats resulted in plasma concentrations of the hormone similar to those previously reported (8) for the proestrus phase of the estrus cycle during which $E_2\beta$ levels are elevated over an ~12-h period. $E_2\beta$ replacement additionally maintained uterine weight similar to that of animals with intact ovaries.

Chronic Hypoxia-Induced Right Ventricular Hypertrophy, Polycythemia, and Arterial Remodeling

Greater RV-to-T ratios were observed for CH intact rats compared with normoxic control rats (Fig. 1), thus demonstrating right ventricular hypertrophy indica-

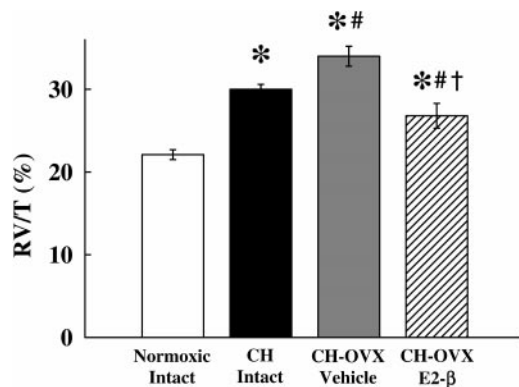


Fig. 1. Ratios of right ventricle (RV) to total ventricle (T) weight for normoxic rats with intact ovaries (normoxic intact; n = 6), chronically hypoxic (CH) rats with intact ovaries (CH intact; n = 10), CH ovariectomized (OVX) rats administered vehicle (CH OVX vehicle; n = 4), and CH OVX rats administered 17 β -estradiol ($E_2\beta$) (CH OVX $E_2\beta$; n = 4). Values are means ± SE. * P < 0.05 vs. normoxic intact. # P < 0.05 vs. CH intact. † P < 0.05 vs. CH OVX vehicle.

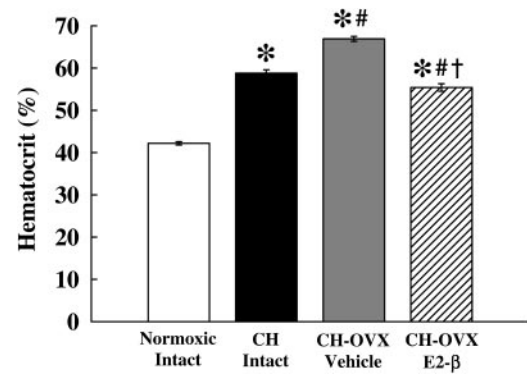


Fig. 2. Hematocrit for normoxic intact (n = 47), CH intact (n = 28), CH OVX vehicle-treated (n = 27), and CH OVX $E_2\beta$ -treated (n = 29) rats. Values are means ± SE. * P < 0.05 vs. normoxic intact. # P < 0.05 vs. CH intact. † P < 0.05 vs. CH OVX vehicle.

tive of pulmonary hypertension. Furthermore, whereas ovariectomy exacerbated the development of right ventricular hypertrophy in vehicle-treated animals, $E_2\beta$ replacement significantly lowered RV-to-T ratios compared with both CH intact and CH OVX vehicle groups. Although the LV+S-to-body weight ratio was not different between CH intact (2.253 ± 0.040 g/kg), CH OVX vehicle (2.045 ± 0.128 g/kg), and CH OVX $E_2\beta$ groups (2.155 ± 0.072 g/kg), all hypoxic groups demonstrated slightly but significantly lower LV+S-to-body weight ratios compared with normotensive control animals (2.457 ± 0.075 g/kg).

CH intact rats exhibited polycythemia as evidenced by a significantly greater hematocrit compared with normoxic intact animals (Fig. 2). This polycythemic response was potentiated by ovariectomy in vehicle-treated rats but was significantly lower in OVX rats receiving $E_2\beta$ relative to both CH intact and CH OVX vehicle groups.

Figure 3 illustrates percent wall thickness for small pulmonary arteries with external diameters of 20–50 μ m (Fig. 3A) and 51–100 μ m (Fig. 3B) from each group of rats. Chronic hypoxia was associated with increased wall thickness in all groups compared with normoxic control vessels. Furthermore, wall thickness was greater in OVX vehicle-treated animals compared with CH intact rats, and $E_2\beta$ replacement largely attenuated arterial remodeling as indicated by the smaller percent wall thickness compared with both CH intact and CH OVX vehicle groups for each range of vessel diameters.

Isolated Lung Experiments

Baseline segmental vascular resistances and responses to U-46619. Table 2 shows total, arterial, and venous baseline resistances in isolated perfused lungs from each group of animals. Total and arterial baseline resistances were greater in all hypoxic groups compared with normotensive control groups as previously demonstrated in lungs from male rats (29, 31–33). Because the pulmonary circulation of CH rats exhibits no detectable basal tone in this preparation (32), these data provide functional evidence for chronic hypoxia-

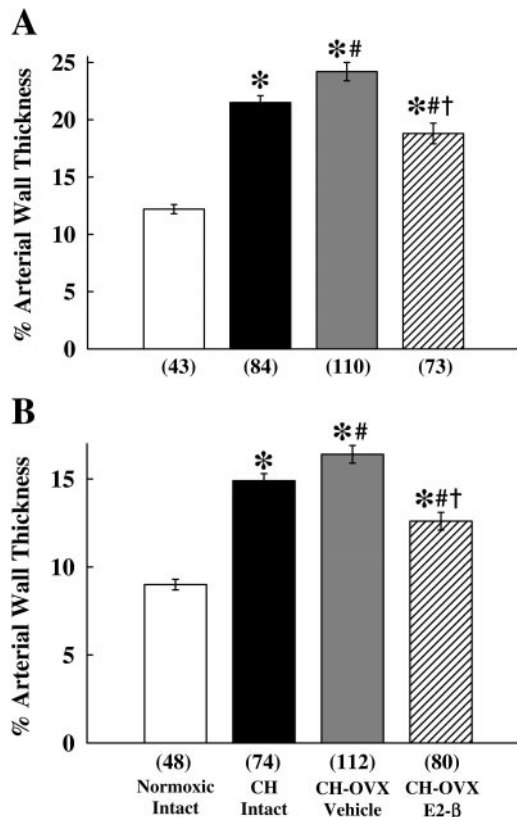


Fig. 3. Percent arterial wall thickness of arteries with external diameters of 20–50 μm (A) and 51–100 μm (B) for each group of rats. Values are means \pm SE; nos. in parentheses, no. of vessels. * $P < 0.05$ vs. normoxic intact. # $P < 0.05$ vs. CH intact. † $P < 0.05$ vs. CH OVX vehicle.

induced arterial remodeling. Additionally, total and arterial baseline resistances tended to be greater in lungs from CH OVX vehicle-treated rats compared with those from other CH groups, although significance was achieved only for total resistance between CH OVX vehicle and CH intact groups. These data are consistent with the more profound arterial remodeling observed in OVX rats treated with vehicle (Fig. 3). L-NNA was without effect on baseline resistances as Resta and colleagues (31, 33) have reported previously, suggesting that endogenous NO does not contribute to

the maintenance of low basal tone in this preparation. There were no differences in baseline venous resistance between groups.

Resistance changes to U-46619 in lungs from each group of rats are depicted in Table 3. Although U-46619 was administered to generate similar increases in resistance between groups, the following exceptions were noted: 1) venous constriction was significantly less in L-NNA-untreated lungs from CH OVX vehicle versus normoxic intact rats, 2) arterial responses were greater in L-NNA-treated lungs from CH OVX vehicle versus normoxic intact animals, 3) total and arterial constrictions to U-46619 were significantly greater in L-NNA-treated lungs from CH OVX vehicle rats compared with untreated lungs from the same group, and 4) arterial responses were similarly elevated in L-NNA-treated versus untreated lungs from normoxic intact rats.

The concentration of U-46619 required to constrict L-NNA-treated lungs was significantly less (130 ± 8 nM) than that required to constrict untreated lungs (201 ± 9 nM), indicating that endogenous NO buffers the vasoconstrictor response to U-46619. However, in contrast to previous studies from our laboratory (29, 33) that demonstrated that chronic hypoxia augments pulmonary vascular reactivity to U-46619 in lungs from male rats, we observed no differences in the concentration of U-46619 required to elicit comparable vasoconstriction in L-NNA-untreated groups (normoxic intact: 196 ± 11 nM; CH intact: 233 ± 15 nM; CH OVX vehicle: 181 ± 20 nM; CH OVX E₂β: 196 ± 26 nM) or in L-NNA-treated groups (normoxic intact: 122 ± 15 nM; CH intact: 150 ± 9 nM; CH OVX vehicle: 114 ± 13 nM; CH OVX E₂β: 137 ± 21 nM). These findings suggest that, unlike males, pulmonary vasoconstrictor responsiveness to thromboxane is unaltered by chronic hypoxia in female rats. The reason for this gender difference in vasoconstrictor reactivity is not clear but appears to be independent of ovarian hormones.

Segmental vasodilatory responses to ionomycin. Lungs isolated from CH intact and CH OVX vehicle rats exhibited greater total vasodilatory responses to the non-receptor-mediated, EDNO-dependent vasodilator ionomycin compared with normoxic control rats

Table 2. Segmental vascular resistances for baseline conditions in absence and presence of L-NNA

	n	Baseline Resistance		
		Total	Arterial	Venous
Without L-NNA				
Normoxic intact	26	0.070 \pm 0.003	0.034 \pm 0.003	0.037 \pm 0.002
CH intact	17	0.109 \pm 0.004*	0.071 \pm 0.004*	0.039 \pm 0.003
CH OVX vehicle	15	0.126 \pm 0.005*†	0.083 \pm 0.005*	0.043 \pm 0.003
CH OVX E ₂ β	14	0.115 \pm 0.004*	0.072 \pm 0.006*	0.043 \pm 0.004
With L-NNA				
Normoxic intact	7	0.071 \pm 0.005	0.038 \pm 0.005	0.033 \pm 0.006
CH intact	5	0.096 \pm 0.007*	0.059 \pm 0.009*	0.037 \pm 0.003
CH OVX vehicle	5	0.131 \pm 0.007*†	0.095 \pm 0.004*†	0.037 \pm 0.004
CH OVX E ₂ β	6	0.123 \pm 0.008*	0.079 \pm 0.007*	0.044 \pm 0.002

Values are means \pm SE in $\text{mmHg} \cdot \text{ml}^{-1} \cdot \text{min} \cdot \text{kg}$ body wt; n, no. of rats. L-NNA, N^o-nitro-L-arginine. * $P < 0.05$ vs. normoxic intact within respective group. † $P < 0.05$ vs. CH intact within respective group.

Table 3. Segmental vascular resistance changes in response to U-46619 in absence and presence of L-NNA

	n	ΔResistance With U-46619		
		Total	Arterial	Venous
Without L-NNA				
Normoxic intact	26	0.305 ± 0.009	0.163 ± 0.009	0.142 ± 0.004
CH intact	17	0.312 ± 0.015	0.184 ± 0.015	0.128 ± 0.009
CH OVX vehicle	15	0.299 ± 0.014	0.193 ± 0.011	0.106 ± 0.007*
CH OVX E ₂ β	14	0.316 ± 0.017	0.186 ± 0.013	0.129 ± 0.007
With L-NNA				
Normoxic intact	7	0.342 ± 0.031	0.223 ± 0.030†	0.119 ± 0.006
CH intact	5	0.339 ± 0.037	0.198 ± 0.038	0.141 ± 0.006
CH OVX vehicle	5	0.398 ± 0.018†	0.296 ± 0.021*†	0.102 ± 0.004
CH OVX E ₂ β	6	0.342 ± 0.036	0.235 ± 0.029	0.108 ± 0.010

Values are means ± SE in mmHg·ml⁻¹·min·kg body wt; n, no. of rats. Δ, Change in. *P < 0.05 vs. normoxic intact within respective L-NNA group. †P < 0.05 vs. corresponding without L-NNA group.

(Fig. 4A) as Resta and colleagues (29, 33) have previously described for CH male rats. Although arterial reactivity to ionomycin also tended to be augmented after chronic hypoxia, significance was achieved only in the CH OVX vehicle group. Contrary to our hypothesis, E₂β replacement did not further enhance EDNO-de-

pendent responses but rather yielded total and arterial dilations that were not significantly different from those of the normoxic intact group. Furthermore, lungs isolated from CH OVX vehicle-treated rats showed a tendency for greater arterial responsiveness to ionomycin versus that in CH intact and CH OVX E₂β groups, although a significant difference was observed only versus the CH intact group. Venous responses were not different between groups.

Inhibition of responses to ionomycin by L-NNA. The NOS inhibitor L-NNA attenuated total and arterial reactivity to ionomycin in all groups (Fig. 4B), thus demonstrating a contribution of EDNO to ionomycin-induced vasodilation. Venous responsiveness was similarly inhibited by L-NNA, with statistical differences noted in CH intact and CH OVX E₂β groups. However, this L-NNA-induced attenuation was modest compared with that which Resta and Walker (33) have previously observed in lungs from male rats. Unexpectedly, lungs from CH OVX vehicle-treated rats exhibited greater total and arterial vasodilation to ionomycin in the presence of L-NNA compared with other groups.

Segmental vasodilatory responses to exogenous NO. Total and segmental reactivity to the NO donors SNAP (Fig. 5A) and spermine NONOate (Fig. 5B) was attenuated in all CH groups compared with that in normoxic control groups, suggesting that chronic hypoxia attenuates pulmonary vascular smooth muscle sensitivity to exogenous NO. However, no differences in reactivity to either SNAP or spermine NONOate were observed between CH groups, indicating that NO-dependent vasodilation is not further altered by either ovariectomy or E₂β replacement. N-acetylpenicillamine and spermine exhibited no apparent vasoactive properties in lungs from normoxic intact rats (data not shown; n = 2 for each compound).

eNOS Protein Levels

Immunoreactive eNOS was detected in lungs from all groups as a single band of ~140 kDa (Fig. 6). Greater quantities of eNOS were observed in lungs from CH versus normoxic control groups as previously reported for male rats (20, 29, 30, 35). Although eNOS

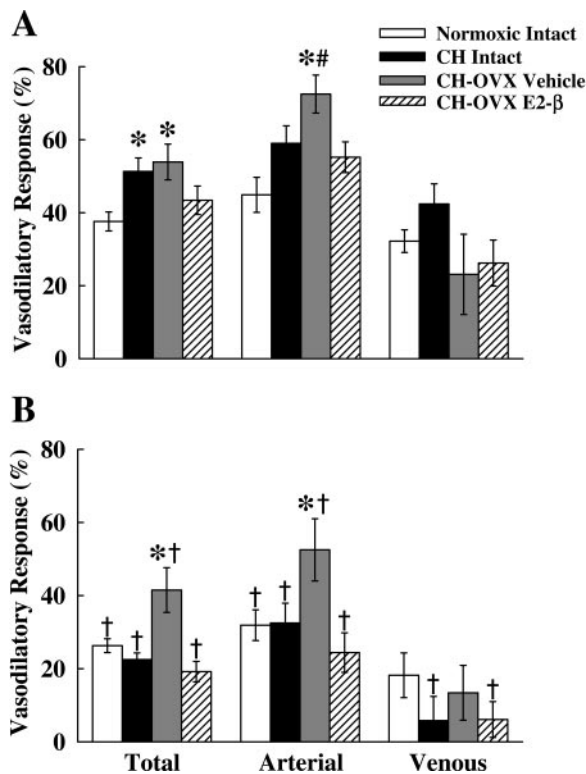


Fig. 4. Segmental vasodilatory responses (percent reversal of U-46619-induced vasoconstriction) to the endothelium-derived nitric oxide-dependent dilator ionomycin (350 nM) in untreated (A) and N^o-nitro-L-arginine (L-NNA; 300 μM)-treated (B) lungs from normoxic intact (untreated, n = 10; L-NNA-treated, n = 7), CH intact (untreated, n = 7; L-NNA-treated, n = 5), CH OVX vehicle-treated (untreated, n = 5; L-NNA-treated, n = 5), and CH OVX E₂β-treated (untreated, n = 6; L-NNA-treated, n = 6) rats. Values are means ± SE. *P < 0.05 vs. normoxic intact within untreated or L-NNA-treated groups. †P < 0.05 vs. CH intact within untreated or L-NNA-treated groups. ‡P < 0.05 vs. corresponding untreated group.

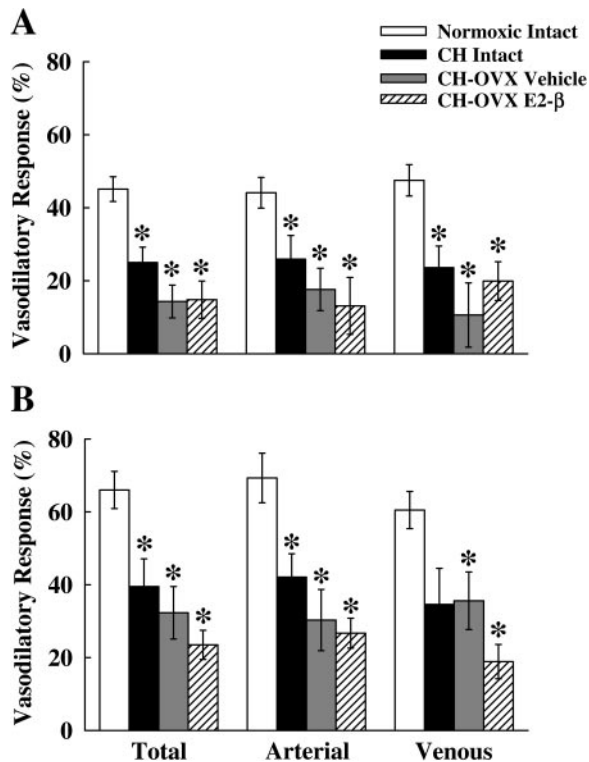


Fig. 5. Segmental vasodilatory responses to the NO donors *S*-nitroso-*N*-acetylpenicillamine (SNAP; 1 μ M; A) and spermine NONOate (2 μ M; B) in lungs from normoxic intact (SNAP, $n = 7$; spermine NONOate, $n = 5$), CH intact (SNAP, $n = 5$; spermine NONOate, $n = 5$), CH OVX vehicle-treated (SNAP, $n = 5$; spermine NONOate, $n = 5$), and CH OVX E₂β-treated (SNAP, $n = 4$; spermine NONOate, $n = 4$) rats. Values are means \pm SE. * $P < 0.05$ vs. normoxic intact.

levels tended to be greater in lungs from CH OVX vehicle-treated animals compared with both CH intact and CH OVX E₂β groups, these differences did not reach significance. These data appear to correlate with vasodilatory responsiveness to ionomycin in each group and suggest that E₂β does not upregulate pulmonary eNOS in chronic hypoxia.

DISCUSSION

The present study examined effects of estrogen on CH pulmonary hypertension and determined the ability of estrogen to regulate pulmonary eNOS expression and associated EDNO-dependent reactivity in this setting. The major findings from this study are that 1) ovariectomy exacerbated the right ventricular hypertrophy, polycythemia, and pulmonary arterial remodeling that result from long-term hypoxic exposure and E₂β replacement prevented these responses to ovariectomy; 2) whereas chronic hypoxia augmented pulmonary vasodilatory responsiveness to the EDNO-dependent dilator ionomycin, E₂β replacement did not potentiate this response; 3) E₂β was without effect on pulmonary vasodilatory responses to exogenous NO, although NO-dependent reactivity was attenuated after chronic hypoxia; and 4) chronic hypoxia-induced upregulation of pulmonary eNOS was unaltered by either ovariectomy or E₂β replacement. These findings

suggest that estrogen exerts a protective influence in the hypertensive pulmonary circulation but that this protection is not likely a function of increased eNOS expression.

The initial observation of a gender difference in the development of chronic hypoxia-induced pulmonary hypertension was made by Burton et al. (6), who noted that female chickens raised at 3,810 m developed less pulmonary hypertension and right ventricular hypertrophy than males. Subsequent studies (22, 28) have demonstrated a similar sexually dimorphic pattern in both swine and rats. Our present findings that ovariectomy augmented right ventricular hypertrophy, arterial remodeling, and polycythemic responses to long-term hypoxia agree with a previous report (26) of increased hematocrit and right ventricle weight after ovariectomy in CH rats and support a role for ovarian hormones in mediating the different susceptibility to hypoxic pulmonary hypertension between the sexes. Indeed, E₂β replacement prevented each of these responses to ovariectomy, suggesting that decreased levels of circulating E₂β account for the greater pulmonary hypertension that follows ovariectomy. Consistent with these data are previous studies demonstrating that pharmacological doses of E₂β administered to male rats suppress right ventricular hypertrophy and arterial remodeling associated with monocrotaline-induced pulmonary hypertension (10) as well as polycythemia resulting from chronic hypoxia (24). Chronic E₂β treatment in utero has further been shown to decrease pulmonary vascular resistance and remodeling in perinatal pulmonary hypertension (27). However, the mechanisms by which E₂β attenuates pulmonary hypertension are not presently understood.

Considering the known stimulatory effect of E₂β on vascular eNOS expression (17, 18, 21), we hypothesized that E₂β-induced attenuation of hypoxic pulmonary hypertension was associated with upregulation of pulmonary eNOS and enhanced EDNO-dependent re-

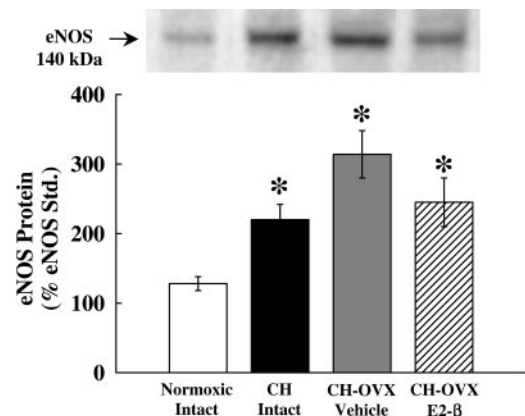


Fig. 6. Results from Western blot experiments (top) for endothelial nitric oxide synthase (eNOS) in lungs from normoxic intact ($n = 5$), CH intact ($n = 4$), CH OVX vehicle-treated ($n = 4$), and CH OVX E₂β-treated ($n = 4$) rats. Bottom: mean densitometric data for eNOS bands from each group. eNOS was identified as a single band at \sim 140 kDa. std, Standard. Values are means \pm SE. * $P < 0.05$ vs. normoxic intact.

activity. Contrary to our hypothesis, $E_2\beta$ -treatment in CH OVX rats neither increased pulmonary eNOS levels nor augmented vasodilatory responsiveness to the EDNO-mediated agonist ionomycin. In contrast, although reactivity to ionomycin was greater in both CH intact and CH OVX vehicle-treated groups compared with normoxic control groups, as Resta and colleagues (29, 33) have previously observed after chronic hypoxia in male rats, no such change in reactivity was observed in the CH OVX $E_2\beta$ group. The reason for the apparently reduced reactivity to ionomycin after administration of $E_2\beta$ is not clear. Previous studies from our laboratory (29, 33) suggest that the upregulation of pulmonary eNOS associated with chronic hypoxia in male rats is dependent on altered vascular mechanical forces associated with pulmonary hypertension as opposed to hypoxia per se. Therefore, it is possible that the lesser pulmonary hypertension and vascular remodeling observed in $E_2\beta$ -treated rats in the present study limited the induction of pulmonary eNOS by chronic hypoxia, which could potentially have masked any direct influence of $E_2\beta$ on eNOS expression. The correlation between accentuated right ventricular hypertrophy, arterial remodeling, and reactivity to ionomycin after ovariectomy alone is consistent with this possibility. Although it is additionally possible that any positive influence of $E_2\beta$ on eNOS activity was complicated by $E_2\beta$ -induced decreases in vascular smooth muscle sensitivity to NO, the similar responses to the NO donors SNAP and spermine NONOate observed between CH groups suggest that reactivity to exogenous NO was unaltered by either ovariectomy or $E_2\beta$ treatment.

Although our present findings do not support a role for altered eNOS expression in mediating the inhibitory effect of $E_2\beta$ on hypoxic pulmonary hypertension, they also do not preclude the possibility that this response is dependent on changes in endothelial function. Accumulating evidence suggests that decreased synthesis of the endothelium-derived vasoconstrictor and mitogenic factor endothelin-1 (ET-1) contributes to the antiatherosclerotic properties of $E_2\beta$ (2, 3, 39). ET-1 has additionally been implicated in mediating the vascular remodeling component of chronic hypoxia-induced pulmonary hypertension (5, 7) and may facilitate the pulmonary vasoconstrictor response to acute hypoxia via suppression of ATP-sensitive K^+ channel activity (34). Furthermore, ET-1 synthesis is elevated with chronic exposure to hypoxia and appears to contribute to increased basal tone in isolated lungs from CH rats after NO synthesis inhibition (25). Therefore, the inhibitory effect of estrogen on the development of HPV could potentially result from decreased ET-1 expression. Considering the apparent contribution of cyclooxygenase products to the inhibitory effects of $E_2\beta$ on HPV in isolated ovine lungs (15), an additional possibility is that the protective influence of estrogen is a consequence of increased prostacyclin production. It is also possible that basal production of NO is increased secondary to the acute stimulation of pulmonary eNOS by $E_2\beta$ (19). Alternatively, the lesser right

ventricular hypertrophy and arterial remodeling observed in $E_2\beta$ -treated animals may result from direct vasodilatory (12, 16) and antimitogenic effects of $E_2\beta$ on pulmonary vascular smooth muscle (9, 37) or, rather, to the upregulation of inducible NOS (iNOS) by $E_2\beta$ as previously reported in systemic vascular tissue (4). However, this latter possibility is unlikely considering that we observed no differences in pulmonary iNOS expression between groups as assessed by Western blotting (data not shown), whereas large increases in iNOS levels are apparent in lungs from rats treated with lipopolysaccharide (31).

It is noteworthy that the inhibitory effects of L-NNA on ionomycin-induced pulmonary vasodilation were rather modest compared with previously published observations from male rats (33). Whether the residual dilation to ionomycin in the presence of L-NNA is a result of incomplete NOS inhibition is not clear. This possibility seems unlikely, however, considering that a previous study from our laboratory (33) has demonstrated that the 300 μ M dose of L-NNA currently employed nearly abolishes arterial dilation to the EDNO-dependent pulmonary vasodilators arginine, vasopressin, and ET-1 in this preparation. Furthermore, preliminary studies indicate that this dose of L-NNA largely inhibits the accumulation of nitrite and nitrate in the perfusate of isolated saline-perfused rat lungs (Walker BR, Resta TC, and Nelin LD, unpublished observation), suggesting effective inhibition of NO synthesis. Alternatively, it is possible that an additional endothelium-dependent vasodilator contributes to ionomycin-induced vasodilation in this preparation. Because meclofenamate was added to the perfusate to inhibit prostaglandin synthesis, involvement of an endothelium-derived hyperpolarizing factor in the response to ionomycin is a likely possibility. A further unexpected finding was the greater total and arterial dilation to ionomycin in lungs from CH OVX vehicle-treated animals compared with other groups after NOS inhibition. Whether this greater reactivity after ovariectomy represented decreased responsiveness to L-NNA is not clear because the unpaired nature of the experiments precluded statistical comparisons of percent inhibition by L-NNA. An additional complicating factor is the greater constriction to U-46619 observed in L-NNA-treated versus untreated lungs in the CH OVX group. Finally, it is possible that the decreased endogenous synthesis of $E_2\beta$ associated with ovariectomy in vehicle-treated animals resulted in a compensatory increase in an alternative endothelium-dependent vasodilator. Nevertheless, the inhibitory effect of L-NNA on ionomycin-induced pulmonary vasodilation demonstrated a significant contribution of endogenous NO to this response, and the responses to ionomycin correlated in magnitude with pulmonary vascular eNOS levels, neither of which are potentiated by $E_2\beta$.

An interesting observation from these studies is the reduced reactivity observed to both SNAP and spermine NONOate in lungs isolated from CH rats compared with those from normoxic control rats. These findings suggest that pulmonary vascular smooth muscle sen-

sitivity to NO is reduced after exposure to chronic hypoxia, which is at odds with previously published observations (33) from our laboratory demonstrating no effect of chronic hypoxia on vasodilatory responsiveness to the same NO donors in lungs from male rats. The reason for this discrepancy is not presently clear but could represent a gender difference independent of $E_{2\beta}$.

In conclusion, we have demonstrated that $E_{2\beta}$ mediates an inhibitory influence on chronic hypoxia-induced right ventricular hypertrophy, pulmonary arterial remodeling, and polycythemia that is not associated with upregulation of pulmonary eNOS or enhanced EDNO-dependent responsiveness. Further investigation is required to determine whether this protective effect of $E_{2\beta}$ is attributable to decreased pulmonary ET-1 expression, to acute stimulation of eNOS activity, or to direct vasodilatory and antimitogenic properties of $E_{2\beta}$ in vascular smooth muscle.

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