SELECTIVE ESTROGEN RECEPTOR-ALPHA AND ESTROGEN RECEPTOR-BETA AGONISTS RAPIDLY DECREASE PULMONARY ARTERY VASOCONSTRICTION BY A NITRIC OXIDE DEPENDENT MECHANISM

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Running Head: Estrogen receptor agonists and pulmonary artery tone

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ABSTRACT

Both endogenous and exogenous estrogen decrease pulmonary artery (PA) vasoconstriction. Whether these effects are mediated via estrogen receptor (ER)-alpha or ER-beta, and whether the contribution of ERs is stimulus-dependent, remains unknown. We hypothesized that administration of the selective ER-alpha agonist propylpyrazole triol (PPT) and/or the selective ER-beta agonist diarylpropionitrile (DPN) rapidly decreases PA vasoconstriction induced by pharmacologic and hypoxic stimuli via a nitric oxide (NO)-dependent mechanism. PA rings (n=3-10/group) from adult male Sprague-Dawley rats were suspended in physiologic organ baths. Force displacement was measured. Vasoconstrictor responses to phenylephrine (10^{-8}M – 10^{-5}M) and hypoxia (pO_2 35-45 mmHg) were determined. Endothelium-dependent and -independent vasorelaxation were measured by generating dose-response curves to acetylcholine (10^{-8}M – 10^{-4}M) and sodium nitroprusside (10^{-6}M – 10^{-5}M). PPT or DPN (10^{-9}M – 5x10^{-5}M) were added to the organ bath in the presence and absence of the NO-synthase inhibitor L-NAME (10^{-4}M). Selective ER-alpha activation (PPT, 5x10^{-5}M) rapidly (<20 min) decreased phenylephrine-induced vasoconstriction. This effect, as well as PPT’s effects on endothelium-dependent vasorelaxation, were neutralized by L-NAME. In contrast, selective ER-beta activation (DPN, 10^{-5}M) rapidly decreased phase II of hypoxic vasoconstriction (HPV). L-NAME eliminated this phenomenon. Lower PPT or DPN concentrations were less effective. We conclude that both, ER-alpha and ER-beta decrease PA vasoconstriction. The immediate onset of effect suggests a nongenomic mechanism. The contribution of specific ERs appears to be stimulus-specific, with ER-alpha primarily modulating phenylephrine-induced vasoconstriction, and ER-beta inhibiting HPV. NO inhibition eliminates these effects, suggesting a central role for NO in mediating the pulmonary vascular effects of both ER-alpha and ER-beta.
**Key words:** propylpyrazole triol, diarylpropionitrile, phenylephrine, hypoxic pulmonary vasoconstriction, nongenomic effects
INTRODUCTION

Female sex is increasingly recognized as a protective factor in patients suffering from trauma and sepsis (12, 16). The critical role of sex hormones has recently been recognized. In particular, 17β-estradiol (E2) has been shown to improve outcomes in experimental trauma-hemorrhage and shock (13, 14, 41), sepsis (5, 7, 12), myocardial ischemia/reperfusion (39, 40) and acute lung injury (30, 35, 42). The protective effects of 17β-estradiol are mediated via estrogen-receptor (ER)-α and ER-β. The effects of these estrogen receptor subtypes are tissue and compartment specific. For example, ER-α decreases proinflammatory cytokine production by splenic macrophages and Kupffer cells after trauma-hemorrhage (33, 34). On the other hand, ER-β decreases inflammatory markers and mortality in shock-induced lung injury (5, 42).

In this context, the recently discovered nongenomic effects of estrogen have gained much attention (21). In contrast to the well-described genomic effects, which occur at a transcriptional level and which take hours to days to develop, nongenomic mechanisms use existing proteins and signaling pathways, therefore taking only seconds to minutes to mediate their effects.

Estrogens have also been shown to affect the pulmonary vasculature (18). In fact, both endogenous and exogenous estrogen decrease pulmonary artery (PA) vasoconstriction under normoxic as well as hypoxic conditions (8, 19). We have recently demonstrated that exogenous 17β-estradiol acutely decreases PA vasoconstriction through a rapid and therefore most likely nongenomic mechanism (17).

However, it is unknown whether the acute pulmonary vascular effects of E2 are mediated by ER-α or ER-β. This is of importance, since a better mechanistic understanding of the pulmonary vascular effects of E2 may allow for future nonhormonal, targeted therapeutic interventions in pulmonary hypertension and acute lung injury. In addition, better insights into pulmonary vascular estrogen receptor signaling may improve our current understanding of why
acute and chronic hypoxic pulmonary hypertension are less common and less pronounced in females, while idiopathic pulmonary arterial hypertension is more prevalent in the female population (18, 20, 27).

In the systemic vasculature, both ER-α and ER-β have been demonstrated to mediate the vasodilator effects of estrogen (26, 43). This effect is mediated by genomic as well as nongenomic mechanisms, some of which include increases in local nitric oxide (NO) release (28). While the exact roles of ER-α and ER-β in the systemic vasculature have not been entirely characterized, data regarding the pulmonary vasculature are sparse and conflicting (3, 4, 11). For example, Hisamoto et al. (11) suggested that 17β-estradiol activates eNOS through ER-α and not through ER-β, while Chambliss et al. (3) demonstrated that ER-β is indeed capable of activating eNOS. This discrepancy may be due to the use of different cell populations and differences in experimental settings. Furthermore, the contribution of ER-α and ER-β to the regulation of PA tone during hypoxia remains unknown. Only recently have selective estrogen receptor agonists become available, allowing for detailed analysis of the role of ER-α and ER-β in the pulmonary vasculature. In particular, the effects of selective estrogen receptor agonists on acute hypoxic pulmonary vasoconstriction (HPV) have not yet been investigated. It also remains unknown whether the contribution of ER-α and ER-β differs between hypoxic and non-hypoxic stimuli.

We therefore hypothesized that the administration of selective ER-α and/or ER-β agonists acutely decreases PA vasoconstriction induced by pharmacologic and hypoxic stimuli. In addition, we hypothesized that selective ER agonists exert their effects through a nitric oxide-dependent mechanism.

To test our hypotheses, we measured isometric force displacement in isolated PA rings from adult male Sprague-Dawley rats. Our objectives were 1) to determine whether the selective ER-α agonist propylpyrazole triol (PPT) rapidly decreases phenylephrine- and hypoxia-
induced PA vasoconstriction, 2) to investigate whether the selective ER-β agonist diarylpropionitrile (DPN) rapidly decreases phenylephrine- and hypoxia-induced PA vasoconstriction, and 3) to measure whether the effects of PPT and DPN are attenuated in the presence of the nitric oxide synthase inhibitor N(omega)-nitro-l-arginine methyl ester (L-NAME).

MATERIALS AND METHODS

Animals

All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals [National Institutes of Health (NIH) publication no. 85-23, revised 1985]. All of the animal protocols were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine. Adult age matched male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 225–350 g were allowed ad libitum access to food and water up to the time of experimentation. All animals were cared for in a non-stressful environment for at least one week prior to experimentation.

Isolated pulmonary artery ring preparation

Rats were anesthetized with intraperitoneal injections of pentobarbital (150 mg/kg). Median sternotomy was performed and the heart and lungs were removed en bloc and placed in modified Krebs-Henseleit (KH) solution at 4°C. Under a dissecting microscope, extralobar pulmonary artery (PA) branches were dissected out and cleared of surrounding tissue. The right and left main branches were cut into 2- to 3-mm wide rings and suspended on steel hooks connected to force transducers (ADInstruments, Colorado Springs, CO) for isometric force measurement. Care was taken during the entire process to avoid injury to the endothelium. PA rings were immersed in individual water-jacketed organ chambers containing modified Krebs-Henseleit solution bubbled with 95% O₂/5% CO₂ at 37°C. Force displacement was recorded
using a PowerLab (ADInstruments) eight-channel data recorder on an Apple iMac PowerPC G4 Computer (Apple Computer, Cupertino, CA).

**Experimental protocol and groups**

Before starting experimental protocols, the PA rings were stretched to a predetermined optimal passive tension of 750 mg. The rings were allowed to equilibrate for 60 min, during which time the KH solution was changed every 15 min. Viability of PA rings was determined by measuring maximum contractile response to $8 \times 10^{-2}$ M of KCl. The dosage of KCl was determined to produce maximal contractile response in previous experiments. After KCl washout, the integrity of each PA endothelium was evaluated by dilation with acetylcholine (ACH, $10^{-6}$ M) after phenylephrine (PE, $10^{-6}$ M) precontraction. Rings demonstrating <200 mg contraction to PE were discarded. In endothelium-intact PA, rings demonstrating <50% vasorelaxation to ACh were discarded as well. After washout of ACh, PA rings were allowed to equilibrate. After having established viability and endothelial integrity, and following equilibration, PA rings were exposed to pharmacologically or hypoxia-induced vasoconstriction, respectively. Experimental groups consisted of PA rings treated with various concentrations ($10^{-9}$ M, $10^{-6}$ M, $5 \times 10^{-5}$ M) of the selective ER-α agonist PPT or the selective ER-β agonist DPN. To compare the rapid effects of the selective ER-α and ER-β agonists with those of E2, PA rings were also treated with $5 \times 10^{-4}$ M of E2. This concentration was shown to be effective in previous experiments (17). Control groups consisted of untreated and vehicle-treated animals.

**Pharmacologic vasoconstriction**

To investigate the rapid effects of selective ER-agonists on PE-induced vasoconstriction, ER-agonists were added to the organ bath 10 minutes prior to PE ($10^{-6}$ M). Dose-response curves for PPT and DPN ($10^{-9}$ M, $10^{-6}$ M, $5 \times 10^{-5}$ M) were generated. To investigate whether the pulmonary vascular effects of the selective ER-agonists are mediated by a NO-dependent
mechanism, separate vasoreactivity experiments were performed in the presence and absence of the nitric oxide synthase (NOS) inhibitor N(omega)-nitro-l-arginine methyl ester (L-NAME, 10^{-4} M). L-NAME was added to the organ bath 30 min prior to the ER-agonist. L-NAME concentration and timing of administration were based on previous experiments (37). PA vasoreactivity was investigated by generating dose-response curves to PE (10^{-8} M – 10^{-5} M). The ER-agonist was added to the organ bath 10 min prior to PE. After PE-precontraction, endothelium-dependent and -independent vasorelaxation were measured by generating dose-response curves to ACh (10^{-8} M – 10^{-4} M) and sodium nitroprusside (SNP, 10^{-9} M – 10^{-5} M), respectively, as described previously (38). Vasoreactivity experiments were terminated after the concentration of ACh or SNP, respectively.

**Hypoxic pulmonary vasoconstriction**

To measure the effect of hypoxia on PA rings, we gassed PE-precontracted PA rings with 95% N2/5% CO2 for 60 min. This produced a pO2 of 35–45 mm Hg in the organ bath, which was measured with a blood-gas analyzer (Synthesis 20; Instrumentation Laboratory, Lexington, MA). Dose-response curves for PPT and DPN (10^{-9} M, 10^{-6} M, 5x10^{-5} M) were generated. To investigate whether the vasoactive effects of ER-agonists are mediated by NO, additional experiments were performed in the presence and absence of L-NAME (10^{-4} M). L-NAME was added to the organ bath 30 min prior to the ER-agonist. As described in previous experiments, hypoxia caused a biphasic PA vasoconstriction: an early contraction (occurring 2-3 min after exposure to hypoxia) followed by a transient vasorelaxation and a late phase II (occurring 10–15 min after hypoxia exposure) contraction (Fig. 1). Due to its very brief and transient nature, phase I vasoconstriction was not measured. Maximum phase II vasoconstriction was measured as the difference between the highest and lowest force displacements during hypoxia and expressed as a percentage of maximum PE-precontraction. Maximum vasorelaxation was measured as the difference between PE-precontraction and the lowest force displacements.
during hypoxia and expressed as a percentage of maximum PE-precontraction. Hypoxia experiments were terminated after 60 min of hypoxia.

**Chemicals and reagents**

All chemical reagents were obtained from Sigma (St. Louis, MO) unless otherwise specified. PPT and DPN were obtained from Tocris Biosciences (Ellisville, MO). PPT and DPN were dissolved in ethanol (100%) according to the manufacturer’s instructions. All other reagents were dissolved in deionized distilled water. KH solution is a physiologically balanced salt solution containing (in mmol/l): 127 NaCl, 4.7 KCl, 17 NaHCO₃, 1.17 MgSO₄, 1.18 KH₂PO₄, 2.5 CaCl₂, and 5.5 D-glucose. The final pH of all solutions was 7.35–7.45.

**Presentation of data and statistical analysis**

Force displacement after stimulation PE is expressed as percent change from baseline tension of 750 mg. Force displacement during hypoxia is expressed as percent change from the amount of PE-precontraction. All reported values are expressed as means ± SE. Experimental groups (n=3-10/group) were compared by two-way analysis of variance (ANOVA) with post hoc Bonferroni test or student’s t test (Prism 4; Graphpad Software, San Diego, CA). Differences at an alpha level of 0.05 (P < 0.05) were considered statistically significant.

**RESULTS**

**ER-α agonist PPT rapidly decreases pharmacologically-induced vasoconstriction**

The selective ER-α agonist PPT at a 10⁻⁹ M or a 10⁻⁶ M concentration did not significantly affect phenylephrine (PE)-induced vasoconstriction as compared to control or vehicle (PPT 10⁻⁹ M: 60.21±6.83%; PPT 10⁻⁶ M: 65.43±7.48%; control: 70.57±5.43%; vehicle: 57.34±4.16%) (Fig. 2). However, when given at a 5x10⁻⁵ M concentration, PPT significantly and rapidly (<20 min) decreased PE-mediated vasoconstriction similar to E2 (PPT 5x10⁻⁵ M: 45.67±5.23%).
ER-α agonist PPT rapidly decreases pharmacologically-induced vasoconstriction by a nitric oxide-dependent mechanism

To confirm the above described results, and to further investigate whether the observed PPT-related decrease in PE-induced vasoconstriction is mediated by a NO-dependent mechanism, we performed vasoreactivity studies by generating dose–response curves to PE. After PE-precontraction, endothelium-dependent and -independent vasorelaxation were measured by generating dose-response curves to ACh and SNP, respectively. The PPT concentration found to be effective in the above described experiments (5x10⁻⁵ M) was used for the vasoreactivity studies. PPT significantly decreased vasoconstriction at all PE concentrations (Fig. 3a). However, in the presence of the non-selective NOS inhibitor L-NAME, this effect was significantly attenuated, indicating that PPT decreases PE-induced vasoconstriction by a NO-dependent mechanism. The vasodilator effects of PPT in the presence of ACh were attenuated by L-NAME as well, again indicating that PPT exerts its vasodilator effects through NO (Fig. 3b). As expected, NOS-inhibition did not affect endothelium-independent vasorelaxation (Fig. 3c).

ER-α agonist PPT and ER-β agonist DPN do not affect vasorelaxation during hypoxia

Both, PPT and DPN in the highest concentrations enhanced vasorelaxation during hypoxia similar to E2 (PPT: -130.90±12.73%, DPN: -100.55±7.84%, E2: -130.90±13.70%, control: -72.94±4.16%). However, this effect was not different from vehicle (-108.13±6.91%). There were no significant differences in vasorelaxation between the highest concentrations of
PPT and DPN, as well as E2 and vehicle when measuring maximum vasorelaxation (Fig. 4a) or individual time points during the vasodilatory phase of hypoxia (Fig. 4b and c).

**ER-β agonist DPN rapidly decreases phase II hypoxic pulmonary vasoconstriction**

PPT at $10^{-6}$ M and $5 \times 10^{-5}$ M concentrations decreased maximal phase II of HPV (47.48±7.12% and 30.04±10.16%) as compared to control (80.79±8.61%). However, this effect was not different from vehicle (54.48±9.39%). In contrast, DPN ($5 \times 10^{-5}$ M) significantly and rapidly decreased maximal phase II HPV (6.20±6.47%) as compared to vehicle and control (Fig. 5a). This effect was significantly more pronounced than the effects of PPT. In addition, the DPN-induced decrease in HPV was readily detectable 25 min after induction of hypoxia (Fig. 5b and 5c).

**ER-β agonist DPN rapidly decreases phase II hypoxic pulmonary vasoconstriction by a nitric oxide-dependent mechanism**

To investigate whether the DPN-related decrease of HPV is mediated by NO, HPV was examined in the presence of the NOS inhibitor L-NAME. Interestingly, the effects of DPN on phase II HPV were neutralized by L-NAME (78.87±25.31%), indicating that the DPN-related decrease in HPV is mediated by NO (Fig. 6a and 6b). In contrast, vasorelaxation during hypoxia was not affected by NO-inhibition (Fig. 7).

**DISCUSSION**

Our data indicate that activation of ER-α or ER-β rapidly decreases pulmonary artery vasoconstriction. The selective ER-α agonist PPT rapidly attenuated phenylephrine-induced vasoconstriction, while the selective ER-β agonist DPN rapidly decreased phase II of acute HPV. These effects occurred within minutes of administration, suggesting a nongenomic mechanism. The observation that this decrease in PA vasoconstriction was attenuated by NOS-
inhibition indicates that the pulmonary vascular effects of ER-α and ER-β are mediated by a NO-dependent mechanism. These data are in concordance with previous cell culture data demonstrating that both estrogen receptors can rapidly activate eNOS (3, 4, 11). The novelty of our results is the finding that the contribution of specific estrogen receptors appears to be stimulus-specific, with ER-α primarily modulating phenylephrine-induced vasoconstriction, and ER-β mediating the inhibitory effect on acute HPV. To our knowledge, the effects of selective estrogen receptors on acute HPV have not been investigated yet.

Our findings of ER-α and ER-β mediating their effect by a NO-dependent mechanism provide a physiologic mechanism for the previously reported rapid vasodilator effects of E2 (8, 17). These rapid vasodilator effects of E2 were reproduced in the current study. As described and discussed in our previous paper (17), as well as by English et al. (8), E2 exerted its effects in isolated PA rings only at relatively high concentrations which may be difficult to achieve in vivo. In contrast, PPT and DPN exerted their vasoactive effects at 10-fold lower concentrations than E2, making these compounds a more feasible therapeutic option. A possible explanation for these findings is that only a small amount of E2 may interact with ER-α and ER-β to evoke acute, nongenomic effects, while a substantial amount of E2 may bind to cytosolic estrogen receptors and diffuse into the nucleus to exert slower, genomic effects. Alternatively, it is possible that PPT and DPN have higher affinities for estrogen receptors than E2 itself. In this context, it is of interest to note that PPT has a 410-fold higher binding affinity for ER-α than ER-β, while DPN has a 70-fold higher affinity for ER-β over ER-α (23, 31). The rapid onset of the ER-α and ER-β mediated decrease in PA vasoconstriction (<20 min for phenylephrine-induced vasoconstriction and <45 min for hypoxic vasoconstriction) is suggestive of a nongenomic increase in NO release. The concentrations required to mediate these effects were similar to those reported by other investigators demonstrating vasorelaxation in isolated systemic arteries (6, 36).
Nongenomic signaling by estrogens appear to be particularly important in “non-traditional” sex hormone target tissues (12). Interestingly, while estrogen receptors are present in almost all cells, they have tissue and compartment specific roles in mediating the protective effects of E2 on immune, metabolic and organ functions following trauma-hemorrhage, shock and sepsis. For example, the liver is rich in ER-α, while ER-β seems to more be predominant in the lung (12). In addition, even within the same organ system, ER-α and ER-β may have complementary, redundant and sometimes even antagonistic effects. For example, controversial data exist as to whether the protective effects of E2 during myocardial ischemia-reperfusion injury are mediated via ER-α, ER-β, or both (10, 39). Similarly, the specific roles of ER-α and ER-β in the systemic vasculature remain to be exactly determined (26, 43). Therefore, it is conceivable that the relative contribution of each receptor may depend on the clinical circumstance and the stimulus activating the receptor. The concept of stimulus-dependent estrogen receptor subtype activation may explain some of the apparently discordant results of previous studies evaluating the relative importance of ER-α and ER-β in various organ systems. The relative contributions of ER-α and ER-β in the pulmonary vasculature are even less well defined, and previous data have been equivocal. Cell culture experiments by Chen et al. (4) and Hisamoto et al. (11) suggested that ER-α activates eNOS via nongenomic mechanisms, while Chambliss et al. (3) demonstrated that ER-β is capable of exerting this effect as well. However, none of these studies evaluated whether the contribution of estrogen receptors is stimulus-dependent. In addition, these experiments were performed with different cell populations, and under normoxic conditions. Our data expand the current knowledge by evaluating the contribution of estrogen receptors during clinically relevant pharmacologic and hypoxic vasoconstrictor stimuli in a model of isolated PA rings. While the distal pulmonary arteries contribute to the majority of pulmonary vascular resistance, our results have clinically relevance since accumulating evidence suggests that altered physiological characteristics of the proximal
segments of the pulmonary vascular bed can also negatively affect right ventricular function (32).

Interestingly, we observed an increase in vasorelaxation as well as a decrease in vasoconstriction during hypoxia with vehicle alone. The vehicle used in our experiments was ethanol, as this is the recommended carrier for both PPT and DPN according to the manufacturer due to solubility. While studies investigating the effect of ethanol on systemic vasomotor tone are conflicting (2, 29), we are not aware of any data demonstrating the effects of ethanol on PA vasomotor tone. However, when evaluating pharmacologically-induced vasoconstriction, we did not observe any significant effects of vehicle (Fig. 2), and the vasorelaxatory effects of the highest concentration of DPN during hypoxia were clearly more pronounced than those of the vehicle alone (Fig. 5), indicating that both, PPT and DPN have independent vasorelaxatory properties.

Our findings have potential implications for the treatment of pulmonary hypertension or acute lung injury and its more severe form, the acute respiratory distress syndrome. In these conditions, PA vasoconstriction can lead to right ventricular strain and decompensation (24). Since most of the currently available treatments for pulmonary hypertension and acute lung injury are limited by expense, side effects and lack of survival benefit (20, 24), new treatment alternatives are clearly needed. Improved understanding of the rapid and nongenomic signaling pathways used by sex and steroid hormone receptors represents a promising opportunity for the development of nonhormonal and targeted treatment alternatives for these conditions. The most impressive work by Dr. Chaudry’s group as well as other investigators in the areas of trauma-hemorrhagic shock (13, 14, 41), sepsis (5, 7, 12), myocardial ischemia-reperfusion injury (10, 25, 39, 40) and acute lung injury (30, 35, 42) highlights the significant therapeutic potential of sex hormones and selective estrogen receptor agonists in restoring the dysregulated immune response that characterizes these conditions (1, 22, 44). Along these lines, our laboratory has
recently been able to demonstrate that estradiol-treated mesenchymal stem cells improve myocardial recovery after global ischemia in an isolated heart model (9). Whether ER-α and ER-β are capable of decreasing the inflammatory response and remodeling within the vessel wall that are associated with hypoxic pulmonary hypertension as well as idiopathic pulmonary arterial hypertension (iPAH) (15, 32), will need to be explored in future experiments.

Further insights into sex hormone receptor pathways may also improve the current knowledge of why acute and chronic hypoxic pulmonary hypertension are less common in females (18, 20, 27), while iPAH, a disabling condition characterized by PA vasoconstriction and remodeling as well as in-situ thrombosis and eventually right heart failure, occurs twice as frequently in the female population (20). Furthermore, oral contraceptives have been considered to be a potential risk factor for iPAH (20). It is conceivable that a defect in estrogen-receptor pathways or intracellular signaling may contribute to these disparate findings. In this context, it is interesting to note that ER-α activation attenuated hypoxia-induced vasoconstriction, while ER-β activation decreased PA vasoconstriction induced by a non-hypoxic stimulus. This raises the question whether the previously described sex differences in hypoxic pulmonary hypertension and iPAH may at least in part be due to differences in ER-α and/or ER-β signaling.

**PERSPECTIVES AND SIGNIFICANCE**

Selective estrogen receptor agonists could provide a promising new strategy to further explore the role of sex differences and the roles of ER-α and ER-β in animal models of pulmonary hypertension. The findings of the present study advance the current knowledge of the pulmonary vascular effects of estrogen by demonstrating that both, ER-α and ER-β decrease vasoconstriction in a model of isolated pulmonary artery rings in a rapid and therefore most likely nongenomic manner. The contribution of specific ERs appears to be stimulus-
specific, with ER-α primarily modulating phenylephrine-induced vasoconstriction, and ER-β inhibiting acute hypoxic pulmonary vasoconstriction. The observation that NOS inhibition eliminates these effects suggests a central role for NO in mediating the rapid pulmonary vascular effects of both ER-α and ER-β. Since L-NAME is a nonspecific NOS inhibitor, the current study does not allow any conclusions as to which specific NOS isoform is mediating the vasorelaxatory effects of selective estrogen receptor agonists. However, data from the systemic vasculature and from experiments with isolated pulmonary artery endothelial cells suggest that eNOS most likely represents the responsible isoform. In future experiments, we will try to identify which NOS isoform is responsible for the ER-α and ER-β induced generation of NO observed in our model. Current research efforts in our laboratory aim to identify the mediators of ER-induced pulmonary vasorelaxation that act both upstream and downstream of NO, as this represents an area which holds promise to identify potential future therapeutic targets. Finally, the use of ER-α and ER-β specific antagonists in the presence and absence of PPT and DPN may allow for further characterization of the roles of ER-α and ER-β in the pulmonary vasculature.

GRANTS

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DISCLOSURES

None.
REFERENCES


FIGURE LEGENDS

Figure 1. Representative pressure tracing of hypoxic vasoconstriction (HPV) in an isolated pulmonary artery ring. Force in grams is depicted on the y-axis. Time in hours and minutes is represented on the x-axis. Pulmonary arteries precontracted using phenylephrine (10⁻⁶ M) were exposed to hypoxia (pO₂ = 35–45 mmHg) for 60 min. Maximum vasorelaxation was measured as the difference between the tension measured when hypoxia was induced (phenylephrine-precontraction) and the lowest force preceding phase II HPV. Maximum phase II HPV was measured as the difference between the lowest force preceding contraction and the highest force during 60 minutes of hypoxia. To investigate for rapid (and therefore most likely nongenomic effects), vehicle, 17β-estradiol, PPT or DPN were added to the organ bath 10 minutes prior to phenylephrine and 20 minutes prior to hypoxia. In the experiments investigating the effect of NOS-inhibition on ER signaling during HPV, L-NAME was added to the organ bath 30 min prior to the ER agonist.

Figure 2: Phenylephrine (PE)-induced vasoconstriction for PPT and DPN. Vehicle, control and 17β-estradiol (E2) are demonstrated for comparison. Force was measured immediately before the induction of hypoxia and is expressed as percent change from baseline contraction. The ER-α agonist at a 5x10⁻⁵ M concentration rapidly decreased PE-induced vasoconstriction similar to E2. N=4-10/group. † p<0.001 vs. control; # p<0.01 vs. vehicle; * p<0.05 vs. control, E2 and PPT 5x10⁻⁵ M.

Figure 3: Vasoreactivity studies for PPT with and without NOS-inhibition using L-NAME. PPT was added to the organ bath 10 min prior to PE. L-NAME was added 40 min prior to PE. Force in fig. 3a is expressed as percent change from baseline, while force in fig. 3b and 3c is expressed as change from PE-precontraction. PPT rapidly and significantly decreased PE-mediated vasoconstriction. This effect was attenuated in the presence of L-NAME (A). L-NAME
also attenuated PPT vasodilator effects in the presence of ACh (B). In contrast, endothelium-independent vasorelaxation was not affected by NOS-inhibition (C). N=3-7/group.

† p<0.05 vs. vehicle; †† p<0.001 vs. vehicle; * p<0.001 vs. PPT + L-NAME; # p<0.001 vs. L-NAME; ‡ p<0.05 vs. PPT + L-NAME.

**Figure 4:** Maximal vasorelaxation (A) and timeline for PPT (B) and DPN (C) during vasodilatory phase of hypoxia. Force is expressed as percent change from PE-precontraction. PPT and DPN in the highest concentrations enhanced vasorelaxation during hypoxia similar to E2. However, this effect was not different from vehicle. N=4-10/group.

(A) † p<0.001 for PPT 5x10⁻⁵ M, E2 and vehicle vs. control; # p<0.01 for DPN 5x10⁻⁵ M vs. control.

(B) * p<0.05 for PPT 5x10⁻⁵ M vs. control; ## p<0.05 for E2 vs. control; †† p<0.05 for vehicle vs. control.

(C) ‡ p<0.001 for DPN 5x10⁻⁵ M, E2 and vehicle vs. control.

**Figure 5:** Maximal phase II hypoxic pulmonary vasoconstriction (HPV) (A) and timeline for PPT (B) and DPN (C) during HPV. Force is expressed as percent change from PE-precontraction. While PPT (10⁻⁶ M and 5x10⁻⁵ M) decreased HPV as compared to control, this effect was not different from vehicle. In contrast, DPN (5x10⁻⁵ M) significantly and rapidly (<25 min after induction of hypoxia) decreased HPV as compared to vehicle and control. N=4-10/group.

(A) * p<0.05 vs. control; # p<0.01 vs. control; ‡ p<0.0001 vs. control; † p<0.01 vs. vehicle, E2 and PPT 10⁻⁶ M.

(B) †† p<0.05 for PPT 5x10⁻⁵ M vs. control.

(C) ** p<0.001 for DPN 5x10⁻⁵ M and E2 vs. control; ##, ### p<0.05, p<0.001 for DPN 5x10⁻⁵ M vs. vehicle.
Figure 6: Effects of NOS inhibition on maximal phase II HPV (A) and timeline of hypoxic vasoconstriction (B). Force is expressed as percent change from PE-precontraction. The decrease of phase II HPV by DPN was neutralized in the presence of the NOS inhibitor L-NAME, indicating that DPN decreases HPV by a nitric oxide-dependent mechanism. L-NAME was added to the organ bath 50 min prior to the onset of hypoxia. N=3-6/group.

(A) * p<0.05 vs. vehicle, # p<0.05 vs. DPN + L-NAME, † p<0.001 vs. L-NAME.

(B) ‡, ‡‡, ‡‡‡ p<0.05, p<0.01, p<0.001 for DPN vs. L-NAME; **, ***, **** p<0.05, p<0.01, p<0.001 for DPN vs. vehicle; †† p<0.05 for DPN vs. DPN + L-NAME.

Figure 7: Vasorelaxation during hypoxia is not affected by NOS inhibition. L-NAME was added to the organ bath 50 min prior to the onset of hypoxia. Force is expressed as percent change from PE-precontraction. N=3-6/group.
Figure 1

- Hypoxia
- Maximum Phase II Hypoxic Vasoconstriction
- Maximum Vasoconstriction
- Vehicle, 17β-Estradiol PPT or DPN
- Phenylephrine
- Phenylephrine-induced vasoconstriction
- 10 min
- 10 min
- 60 min
- Maximum Phase II Hypoxic Vasoconstriction
Figure 2

![Bar graph showing force (%)](image)

- Control
- Vehicle
- E2 5x10^-4 M
- 10^-9 M
- 10^-8 M
- 5x10^-5 M

**ER-α agonist (PPT)**
- 10^-9 M
- 10^-8 M
- 5x10^-5 M

**ER-β agonist (DPN)**
- 10^-9 M
- 10^-8 M
- 5x10^-5 M

Legend:
- # indicates statistical significance compared to control
- † indicates statistical significance compared to vehicle
- * indicates statistical significance compared to E2 5x10^-4 M
Figure 3

A

B

C

### A

**Figure 3A**

- X-axis: PE (-log mM)
- Y-axis: Δ Force (%)
- Curves:
  - Vehicle
  - PPT 5x10^{-5} M
  - L-NAME 10^{-4} M
  - PPT 5x10^{-5} M + L-NAME 10^{-4} M

### B

**Figure 3B**

- X-axis: ACh (-log mM)
- Y-axis: Δ Force (%)
- Curves:
  - Vehicle
  - PPT 5x10^{-5} M
  - L-NAME 10^{-4} M
  - PPT 5x10^{-5} M + L-NAME 10^{-4} M

### C

**Figure 3C**

- X-axis: SNP (-log mM)
- Y-axis: Δ Force (%)
- Curves:
  - Vehicle
  - PPT 5x10^{-5} M
  - L-NAME 10^{-4} M
  - PPT 5x10^{-5} M + L-NAME 10^{-4} M
Figure 4

A

B

C
Figure 6

A

![Bar graph showing the change in force for different treatments.]

B

![Line graph showing the change in force over time for different treatments.]

- Vehicle
- DPN 5x10^{-5} M
- DPN 5x10^{-5} M + L-NAME 10^{-4} M
- L-NAME 10^{-4} M

**Significance Levels**

- †: p < 0.05
- #: p < 0.01
- *: p < 0.001
- **: p < 0.0001

**Time (min)**

- 20, 30, 40, 50, 60
Figure 7

A

Force [%]

vehicle
DPN 5x10^-5 M
DPN 5x10^-5 M + L-NAME 10^-4 M
L-NAME 10^-4 M

DPN 5x10^-5 M

B

Force [%]

time (min)

vehicle
DPN 5x10^-5 M
DPN 5x10^-5 M + L-NAME 10^-4 M
L-NAME 10^-4 M