17β-Estradiol Attenuates Hypoxic Pulmonary Hypertension via Estrogen Receptor–mediated Effects

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***Rationale*: 17β-Estradiol (E2) attenuates hypoxic pulmonary vasoconstriction and hypoxic pulmonary hypertension (HPH) through an unknown mechanism that may involve estrogen receptors (ER) or E2 conversion to catecholestradiols and methoxyestradiols with previously unrecognized effects on cardiopulmonary vascular remodeling.**
***Objectives*: To determine the mechanism by which E2 exerts protective effects in HPH.**
***Methods*: Male rats were exposed to hypobaric hypoxia while treated with E2 (75 μg/kg/d) or vehicle. Subgroups were cotreated with pharmacologic ER-antagonist or with inhibitors of E2-metabolite conversion. Complementary studies were performed in rats cotreated with selective ERα- or ERβ-antagonist. Hemodynamic and pulmonary artery (PA) and right ventricular (RV) remodeling parameters, including cell proliferation, cell cycle, and autophagy, were measured *in vivo* and in cultured primary rat PA endothelial cells.**
***Measurements and Main Results*: E2 significantly attenuated HPH endpoints. Hypoxia increased ERβ but not ERα lung vascular expression. Co-treatment with nonselective ER inhibitor or ERα-specific antagonist rendered hypoxic animals resistant to the beneficial effects of E2 on cardiopulmonary hemodynamics, whereas ERα- and ERβ-specific antagonists opposed the remodeling effects of E2. In contrast, inhibition of E2-metabolite conversion did not abolish E2 protection. E2-treated hypoxic animals exhibited reduced ERK1/2 activation and increased expression of cell-cycle inhibitor $p27^{Kip1}$ in lungs and RV, with up-regulation of lung autophagy. E2-induced signaling was recapitulated in hypoxic but not normoxic endothelial cells, and was associated with decreased vascular endothelial growth factor secretion and cell proliferation.**
***Conclusions*: E2 attenuates hemodynamic and remodeling parameters in HPH in an ER-dependent manner, through direct antiproliferative mechanisms on vascular cells, which may provide novel nonhormonal therapeutic targets for HPH.**

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

17β-Estradiol (E2) attenuates hypoxic pulmonary hypertension (HPH). It is unknown if the protection of E2 is mediated by estrogen receptor (ER) activation, or by conversion to catecholestradiols and methoxyestradiols that exert ER-independent effects. Furthermore, it is unknown if E2 modulates proproliferative signaling pathways and inhibits pulmonary vascular remodeling in HPH.

What This Study Adds to the Field

We show that E2 attenuates hemodynamic changes and pulmonary vascular and right ventricle remodeling in male rats with HPH in an ER-dependent manner, and that E2 conversion to downstream metabolites is not necessary for E2 protection. ERα and ERβ seem necessary for E2 protection in HPH. This study identifies novel mechanisms of action of E2 in the pulmonary vasculature and the right ventricle that may allow for the development of targeted, nonhormonal therapies for patients with HPH, and that may improve the understanding of sex differences in pulmonary vascular disease.

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Hypoxia-induced pulmonary hypertension (HPH) is a devastating consequence of long-term exposure to a low alveolar oxygen tension (1, 2). Characterized by pulmonary artery (PA) vasoconstriction and hyperproliferative remodeling, HPH leads to right ventricular (RV) failure and death (3–5). Although more prevalent than pulmonary arterial hypertension (PAH) (1), no specific treatment for HPH exists. Pulmonary vasodilators used in PAH frequently worsen ventilation–perfusion mismatch and oxygenation in HPH (6–8). Novel pharmacologic treatments for HPH are therefore needed. Several studies suggest that 17β-estradiol (E2) exerts protective effects in HPH. For example, hypoxic pulmonary vasoconstriction (HPV) is decreased in pregnancy (9, 10), and menstrual cycle–related increases in circulating E2 attenuate PA contraction (11). Finally, ovariectomy exacerbates HPH, whereas E2 replacement in ovariectomized animals attenuates the disease (12, 13). Consequently, a better

understanding of the molecular mechanisms of E2-mediated protection in HPH could help identify the pathophysiologic basis of the disparate effects of sex observed in different types of pulmonary hypertension (PH) (14, 15); this may lead to nonhormonal therapy that benefits patients of either sex. We investigated the mechanisms by which E2 mediates protective effects on PA and RV remodeling in HPH.

Although very rapid (nongenomic) E2 effects may occur by binding to the orphan G-protein–coupled receptor GPR30 (16), most of E2 action occurs either by activation of estrogen receptor (ER)-α and ERβ, or by conversion to catecholestradiols and methoxyestradiols (14, 17–22), active metabolites with ER-independent antiproliferative effects (14, 22). Conversion of E2 to catecholestradiols is mediated by cytochrome P-450 (CYP1A1/2, CYP1B1) enzymes, whereas conversion of catecholestradiols to methoxyestradiols is catalyzed by catechol *O*-methyltransferase (COMT) (23, 24). Recent interest in E2 metabolites was provoked by finding a shift from putative protective catecholestradiols and methoxyestradiols to promitogenic 16α-hydroxyestrone in women with hereditary PAH (23), and by beneficial effects of 2-methoxyestradiol in monocrotaline-induced PH (22, 25). However, the protective effects of E2 could also be mediated by ER activation, because ERα and ERβ are expressed in PA endothelial cells, where they up-regulate endothelial nitric oxide synthase (eNOS) and prostacyclin synthase (19–21). This may explain why direct activation of ERα or ERβ attenuates phenylephrine-induced PA vasocontraction and HPV, respectively (26).

The aim of this study was to determine whether the protective E2 effects in HPH are mediated by ER activation or by conversion to catecholestradiols and methoxyestradiols. We hypothesized that E2 attenuates HPH by ER-dependent attenuation of hemodynamic alterations, and by inhibition of pulmonary vascular and RV remodeling. Furthermore, we investigated if E2, in addition to being a vasodilator (11, 13, 26), has beneficial effects on PA and RV remodeling in HPH, and investigated the mechanism by which this may occur. We focused on E2 effects on cell proliferation, cell-cycle regulation, and autophagy, key processes implicated in the pathogenesis of PA remodeling in HPH (27–29). We describe a novel mechanism of E2 protection in HPH that implicates ER-mediated inhibition of cell proliferation and activation of autophagy. Parts of this study have been published in abstract form (30, 31).

METHODS

Animal Experiments

Male Sprague-Dawley rats (250–275 g) received E2 (75 μg/kg/d) or vehicle (1,2-propanediol [99.5%]) via subcutaneous osmotic minipumps (12, 13) for 1 week before and for the entire 2 weeks of hypoxia exposure. This regimen results in E2 levels physiologic for adult female Sprague-Dawley rats (13). In a subset of animals, the nonselective ER-antagonist ICI182780 (fulvestrant [ICI]; 3 mg/kg/d) (32), the selective ERα-antagonist MPP (850 μg/kg/d), the selective ERβ-antagonist PHTPP (850 μg/kg/d) (33), or vehicle (EtOH 100%) were given daily subcutaneously concomitantly with E2 for the entire experiment. In different subgroups, the COMT inhibitor OR-486 (1.5 mg/kg intraperitoneally) (34), the CYP450 inhibitor 1-aminobenzotriazole (ABT; 50 mg/kg/d subcutaneously) (35), or their vehicles (EtOH; 10% in phosphate-buffered saline [PBS] or NaCl 0.9%, respectively) were administered daily with E2, using doses previously shown to block E2 conversion to methoxyestradiols or catecholestradiols *in vivo* (34, 35).

In Vivo Hypoxia

We used a model of chronic HPH, characterized by exposure to hypobaric hypoxia (P_{atm} = 362 mm Hg; equivalent to 10% FI_{O_2} at sea level) in a custom-made exposure chamber.

Cardiopulmonary Measurements

The left carotid artery and right internal jugular vein were cannulated with PE-50 tubing and a 2F Millar catheter (Millar Instruments, Houston, TX), respectively. A thoracotomy was made in the left second intercostal space. A flow probe was placed around the aortic arch for continuous cardiac output (CO) monitoring (2.5PSL probe and TS420 monitor; Transonic, Ithaca, NY). RV systolic pressure (RVSP) and CO were assessed at room air during normocapnia and normal pH.

Hypoxia-induced Pulmonary Vascular Remodeling

Lung sections (4 μm) were stained for PA smooth muscle cells (PASMCs) using a mouse monoclonal anti–smooth muscle-α actin (SMA) antibody (1/100; Abcam, Cambridge, MA) and examined by light microscopy (×40 objective). The degree of PA muscularization and size was assessed in a blinded fashion. As previously described by others (36), we focused on small PAs (<200 μm) associated with alveolar ducts. These were then divided into nonmuscularized (α-SMA staining <25% of vessel circumference), partially muscularized (α-SMA staining 25–74% of circumference), or fully muscularized (α-SMA staining ⩾75% of circumference) vessels. Analysis of PAs associated with alveolar ducts only avoids misclassification of veins as nonmuscularized arteries. At least 40 arteries per lung were assessed. The percentages of nonmuscularized and partially or fully muscularized vessels were calculated by dividing the number of vessels in each category by the total number of blood vessels counted per slide.

Hypoxia-induced RV Remodeling

RV capillary density was analyzed using quantitative immunofluorescence microscopy (37) after incubation with wheat germ agglutinin conjugated to Oregon Green-488 (5 μg/ml in PBS, 10 min; Invitrogen, Carlsbad, CA) and DAPI (1 μM in PBS; Invitrogen). Wheat germ agglutinin is a universal glycocalyx marker used for cell membrane staining (37). Because wheat germ agglutinin nonselectively stains membranes of RV myocytes and capillary endothelial cells, we further identified capillaries by blood autofluorescence surrounded by glycocalyx staining of adjacent endothelial cells. Myocytes were identified by their typical shape (identified by wheat germ agglutinin staining of the cell membrane), and by myoglobin autofluorescence within the cell membrane. Capillaries and myocytes were then manually counted by a blinded investigator. Because RV myocytes and capillaries run parallel to each other, and because a longitudinal cutting plane may miss capillaries (and therefore underestimate their number), capillaries and myocytes were counted in areas where they were only transversally sectioned (37). Capillary density was expressed as the ratio of the number of capillaries per number of myocytes per high power field, averaging at least three randomly chosen fields per RV.

Cell Culture and *In Vitro* Hypoxia

Primary rat PA endothelial cells (RPAECs) and lung microvascular endothelial cells were provided by Dr. Troy Stevens (University of South Alabama Tissue and Cell Culture Core). Cells were derived from male Sprague-Dawley rats (350–400 g; n = 3) and maintained up to passage 20 in Dulbecco's modified Eagle high-glucose medium (Gibco; Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C in 5% CO_2 and 95% air. Media was changed every 3–4 days. For all experiments, cells (70% confluent) were exposed to 21% or 1% O_2 for 48 hours. RPAEC homogeneity was confirmed by cell morphology and by Western blotting and immunofluorescence staining for von Willebrand factor. Hypoxia exposure occurred in a dedicated workstation (Ruskinn, Inc., Pencoed, UK). Oxygen concentration in the chamber was measured in real time and constantly adjusted to maintain the desired concentration. Control cells of identical passage and confluence were grown concomitantly in a regular incubator at 21% O_2. All drugs or their vehicles were added to the media at the beginning of hypoxia exposure.

Data and Statistical Analysis

Results are expressed as mean ± SEM. Experimental groups were compared by analysis of variance with *post hoc* Holm-Sidak test or Student *t* test (SigmaPlot 11, San Jose, CA). Where appropriate,

Kruskal-Wallis analysis of variance by ranks was performed on nonparametric data. Differences at α level of 0.05 ($P < 0.05$) were considered statistically significant.

Additional details on methods and measurements are provided in the online supplement.

RESULTS

E2 Attenuates HPH

To evaluate if E2 attenuates hemodynamic and remodeling parameters in male rats, animals received E2 for 1 week before and for the entire 2 weeks of hypoxia exposure. E2 treatment significantly decreased RVSP, RVSP/CO (a surrogate of pulmonary vascular resistance), and RV/(LV+S), and increased CO (*see* Figures E1A–E1D in the online supplement). Furthermore, E2 decreased erythrocytosis, a marker of global hypoxia (*see* Figure E1E). E2 was well tolerated by all animals. Pulmonary vasodilator therapy during hypoxia may worsen oxygenation by counteracting physiologic HPV (7). Notably, when compared with untreated hypoxic rats, E2 treatment did not worsen oxygenation in either hypoxic or normoxic conditions (*see* Figure E1F), suggesting there was no significant increase in ventilation–perfusion mismatch with E2. Furthermore, no significant E2 effects on mean arterial pressure (MAP) and MAP/CO (a surrogate of systemic vascular resistance) were observed during hypoxia (*see* Figure E2). Interestingly, E2 tended to increase MAP and MAP/CO during normoxia only, albeit to a nonstatistically significant degree (*see* Figure E2). Because administration of E2 vehicle alone did not affect any of the measured parameters during hypoxia (*see* Figure E1), for simplicity the untreated hypoxic rats served as the hypoxia reference group in subsequent experiments.

Conversion to Methoxyestradiols or Catecholestradiols Is Not Necessary for E2 Protection in HPH

We next investigated whether COMT-mediated E2 conversion to biologically active methoxyestradiols (14, 24) is necessary for E2 protection. We coadministered with E2 the COMT inhibitor OR-486 using a dose previously shown to effectively inhibit E2 conversion *in vivo* (34). Interestingly, OR-486 co-treatment did not attenuate the effects of E2 on hemodynamic parameters (Figures 1A–1E), suggesting that conversion of E2 to methoxyestradiol is not necessary for E2 protection in HPH.

COMT inhibition may cause upstream accumulation of catecholestrogens, which may exert ER-independent biologic effects (14, 23). We investigated the role of E2-catecholestradiol conversion (mediated by CYP450) in the protection of E2 against HPH. Rats were cotreated with E2 and the CYP450 inhibitor 1-ABT, administered at a concentration previously shown to block E2-to-catecholestradiol conversion *in vivo* (35). Inhibition of E2-to-catecholestradiol conversion did not attenuate the effects of E2 (Figures 1A–1E). On the contrary, 1-ABT co-treatment enhanced the effects of E2 on several hemodynamic targets, increasing CO to levels similar to those in normoxic animals. Neither OR-486 nor 1-ABT affected systemic blood pressure (*see* Figure E2), and vehicle controls for OR-486 or 1-ABT did not alter the effects of E2 (data not shown). Taken together, these results indicate that conversion to downstream metabolites is not necessary for E2 to mediate protective effects in HPH.

ER Blockade Attenuates the Protective Effects of E2 in HPH

We next determined whether the effects of E2 on cardiopulmonary hemodynamics in HPH require ER activation. We used the ER antagonist ICI, a pharmacologic inhibitor of ERα and ERβ. Interestingly, rats cotreated with E2 and ICI exhibited significant attenuation of E2-induced improvements in right ventricular hypertrophy (RVH) (Figure 2A), hemodynamics (Figures 2C and 2D), and hypoxia-induced erythrocytosis (Figure 2E). ICI failed, however, to block E2 effects on RVSP (Figure 2B). This lack of a robust effect on RVSP may be explained by the concomitant marked decrease in CO in ICI- and E2-treated hypoxic animals (Figure 2C), with decreased RV pump function preventing increases in RVSP (38). The ICI-associated decrease in CO in E2-treated hypoxic animals was associated with an overall increase in MAP/CO (*see* Figure E2), suggesting a concomitant increase in systemic vascular resistance. In contrast, ICI alone did not cause significant changes (Figures 2A–2E). Similarly, ICI vehicle did not alter the effects of E2 (data not shown). These results suggest an ER-mediated mechanism of E2 action on major hemodynamic, RV remodeling, and hematologic endpoints in HPH.

E2 Attenuates Hypoxic Pulmonary Vascular Remodeling

We next investigated if the effects of E2 extend to hypoxia-induced PA remodeling, reflected by the degree and number of muscularized small PAs associated with alveolar ducts (36). As expected, hypoxia markedly increased PA muscularization (Figure 3A), indicated by a significant decrease in nonmuscularized vessels, and by a significant increase in fully muscularized vessels (Figure 3B). Remodeling was attenuated by E2, which significantly increased nonmuscularized vessels by 59% versus the untreated hypoxia group (Figures 3A and 3B). No significant E2 effect on PA architecture was noted during normoxia (not shown). The beneficial effects of E2 were inhibited in animals cotreated with the ER inhibitor, reflected by a trend toward a decrease in nonmuscularized arteries, and by a significant increase in fully muscularized vessels (Figures 3A and 3B). In contrast, co-treatment with E2 conversion inhibitors did not significantly affect the effects of E2 on PA remodeling (Figures 3A and 3B).

Because attenuation of hypoxic PA remodeling may be caused by increased apoptotic cell death, we performed caspase-3-activity assays. We did not detect any increases in caspase-3 activity in the lungs of normoxic, untreated hypoxic, or hypoxic E2-treated rats (not shown).

E2 Attenuates Hypoxia-induced RV Remodeling

To investigate if E2 inhibits hypoxic RV remodeling, we evaluated RV capillarization (expressed as RV capillary/myocyte ratio), a marker of hypoxia-induced RV remodeling (39). Predictably, hypoxia increased RV capillarization (Figures 4A and 4B), whereas E2 treatment decreased hypoxia-induced RV capillarization by 25% (Figures 4A and 4B). Co-treatment with ER-antagonist attenuated the effect of E2 by 35% (Figures 4A and 4B), suggesting a mechanism that involves, at least in part, activation of ER.

E2 effects on RV capillarization may be caused by an overall decrease of RV hypoxic stress with reduced need for compensatory RV remodeling, or may reflect a direct action on RV angiogenesis or remodeling. We could not detect, however, any significant differences in RV collagen content (likely because of the relatively short duration of hypoxia exposure) or caspase-3 activity between normoxic, untreated hypoxic, or hypoxic E2-treated rats (data not shown).

ERα Blockade Attenuates the Protective Effects of E2 on RVH and Hemodynamic Endpoints in HPH Rats

To investigate which ER subtype is necessary for E2-mediated protection from HPH, we cotreated male E2-supplemented rats with the

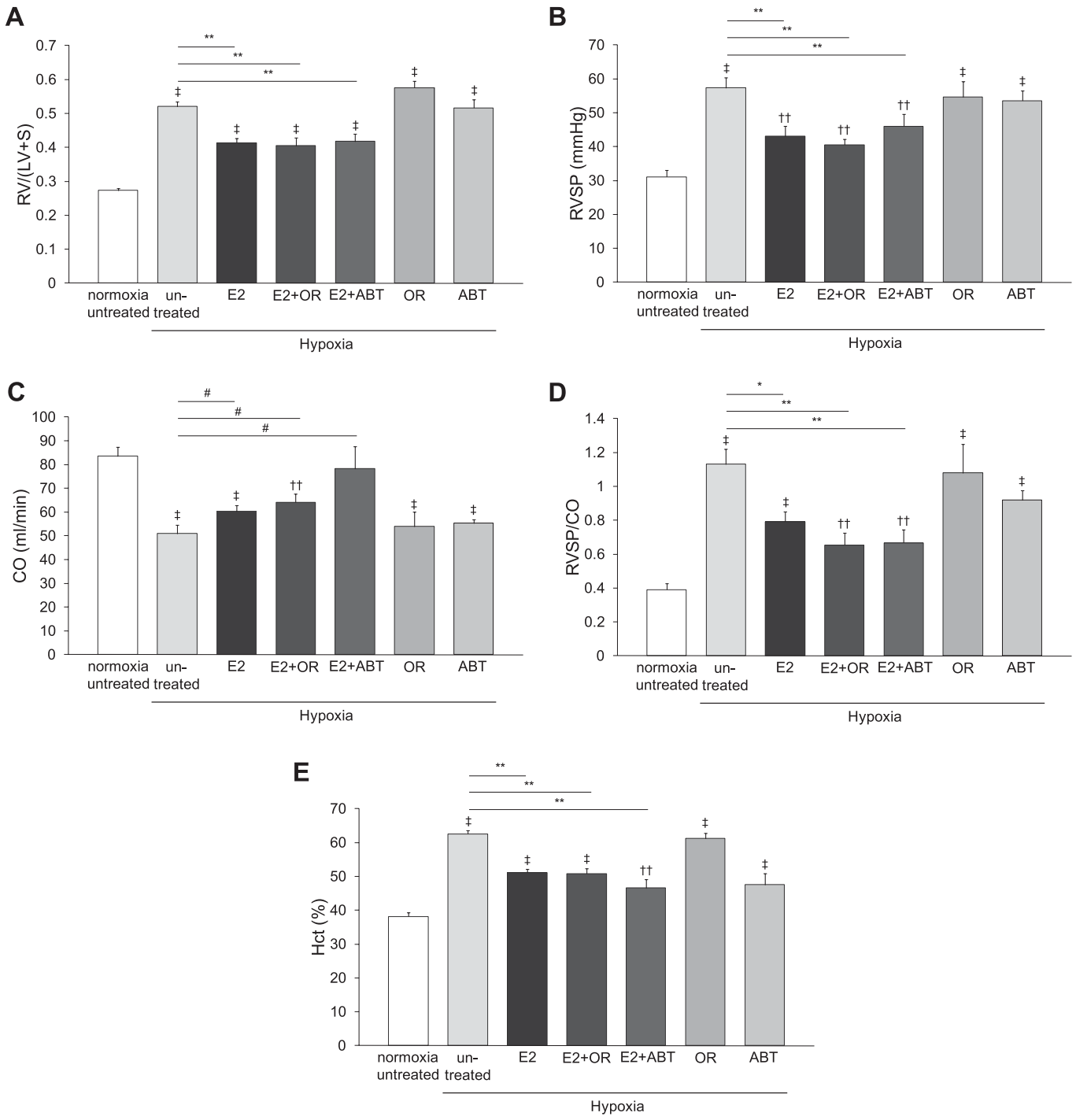

Figure 1. Treatment with 17β-estradiol (E2) conversion inhibitors does not attenuate E2 effects on hypoxia-induced pulmonary hypertension. (*A*) Right ventricular mass (RV/[LV+S]), (*B*) right ventricular systolic pressure (RVSP), (*C*) cardiac output (CO), (*D*) RVSP/CO, and (*E*) hematocrit (Hct) levels in male Sprague-Dawley rats exposed to normoxia ($F_{I_{O_2}}$ 21%) or chronic hypobaric hypoxia (P_{atm} = 362 mm Hg; equivalent to 10% $F_{I_{O_2}}$; 2 wk). Rats were either untreated; treated with E2 alone (75 μg/kg/d via subcutaneously implanted osmotic minipumps for 1 wk before and throughout hypoxia exposure); or cotreated with E2 and the catechol *O*-methyltransferase inhibitor OR-486 (1.5 mg/kg) or the CYP450 inhibitor 1-aminobenzotriazole (ABT; 50 mg/kg). Additional animals were treated with OR-486 or ABT alone. OR-486 and ABT were administered daily subcutaneously for 1 week before and for the entire 2 weeks of hypoxia exposure. Values are mean ± SEM ($^{\ddagger}P < 0.001$, $^{\dagger\dagger}P < 0.01$ vs. normoxia group; $^{**}P < 0.001$, $^{*}P < 0.01$, $^{\#}P < 0.05$ for all other comparisons; *A* and *E*, n = 13 for hypoxia E2, n = 6–12 per group for all other groups; *B–D*, n = 12 for hypoxia E2, n = 5–9 per group for all other groups).

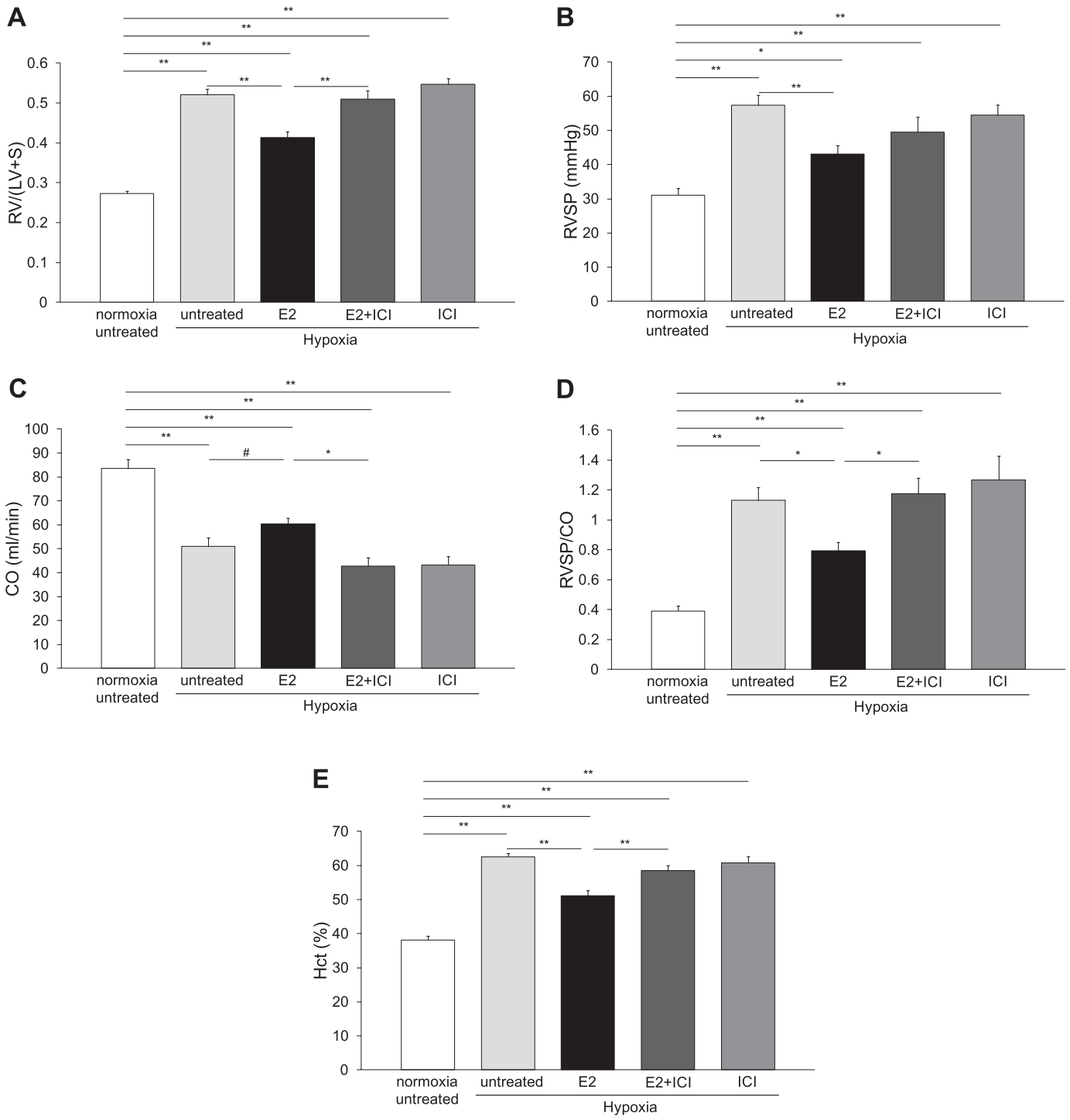

Figure 2. Estrogen receptor blockade with ICI182780 (fulvestrant [ICI]) attenuates protective effects of 17β-estradiol (E2) in hypoxia-induced pulmonary hypertension. (*A*) Right ventricular mass (RV/[LV+S]), (*B*) right ventricular systolic pressure (RVSP), (*C*) cardiac output (CO), (*D*) RVSP/CO, and (*E*) hematocrit (Hct) levels in normoxic and hypoxic (P_{atm} = 362 mm Hg; 10% F_{IO_2}; 2 wk) male rats treated with E2 (75 μg/kg/d; 1 wk before and during hypoxia) with or without ICI (3 mg/kg/d subcutaneously). Values are mean ± SEM (**$P < 0.001$, *$P < 0.01$, $^{\#}P < 0.05$; *A* and *E*, n = 13 for hypoxia E2, n = 6–12 per group for all other groups; *B–D*, n = 12 for hypoxia E2, n = 5–9 per group for all other groups).

selective ERα-antagonist MPP or the selective ERβ-antagonist PHTPP. Co-treatment with ERα- but not ERβ-antagonist abolished the effects of E2 on RVH, CO, and RVSP-CO (Figures 5A–5D). Of note, animals cotreated with E2 and ERα-antagonist exhibited a 50% lower CO and a 56% higher RVSP-CO than untreated hypoxia controls ($P < 0.001$ and $P < 0.01$, respectively). Even though there was a trend for both, neither ERα- nor ERβ-blockade significantly attenuated the effects of E2 on hypoxia-induced erythrocytosis (Figure 5E), whereas we noted a nonsignificant trend for ERβ-blockade to attenuate the effects of E2 on RVH.

Investigations of the effects of ERα- or ERβ-blockade on PA and RV remodeling in E2-treated HPH rats revealed that inhibition of either ER attenuated E2-induced improvements

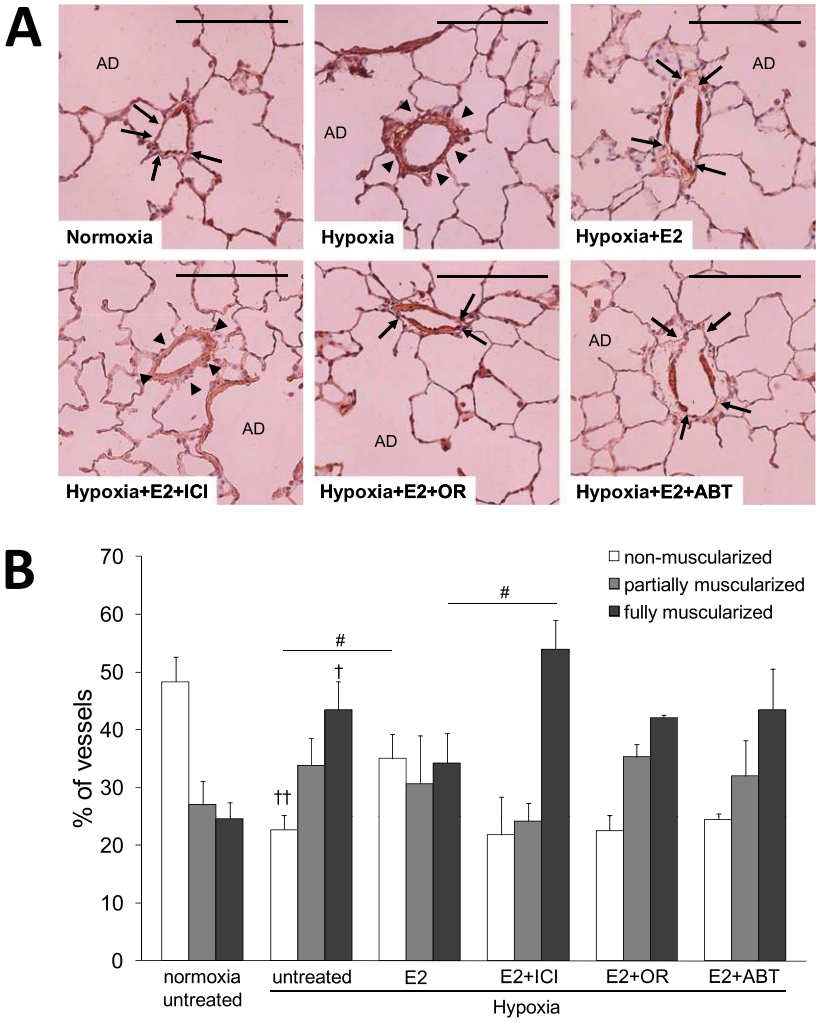

Figure 3. 17β-Estradiol (E2) attenuation of hypoxia-induced pulmonary artery (PA) muscularization is less pronounced after estrogen receptor (ER) blockade. (*A*) Representative images of α-smooth muscle actin (α-SMA) lung immunohistochemistry showing PAs associated with alveolar ducts (AD) of male rats exposed to 2 weeks of normoxia or hypobaric hypoxia. Hypoxia-exposed animals were either untreated, or treated with E2, E2 + ICI182780 (ICI), E2 + OR-486 (OR), or E2 + 1-aminobenzotriazole (ABT). Size bars = 100 μm. Note that hypoxia increased PA muscularization, resulting in homogenous thickening of the smooth muscle cell layer (*arrowheads*), whereas PAs of E2-treated rats with hypoxic pulmonary hypertension exhibited less complete muscularization (*arrows*). The E2-induced decrease in muscularization was attenuated after ER-antagonist (ICI) co-treatment, whereas treatment with E2 conversion inhibitors (OR-486 and 1-ABT) did not have a significant effect. (*B*) Quantification of PA muscularization. Alveolar duct–associated PAs (<200 μm) were divided into non-muscularized (α-SMA staining <25% of vessel circumference), partially muscularized (α-SMA staining 25–74% of vessel circumference), or fully muscularized (α-SMA staining ⩾75% of vessel circumference) vessels. Percentages of nonmuscularized (*white bars*), partially muscularized (*light gray bars*), or fully muscularized vessels (*dark gray bars*) were calculated. E2 treatment increased the percentage of nonmuscularized vessels compared with the untreated hypoxia group. In contrast, ER blockade in the presence of E2 treatment was associated with a significant increase in the percentage of fully muscularized vessels compared with E2 alone. Values are mean ± SEM ($^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$ vs. untreated normoxia group, $^{\#}P < 0.05$; n = 4–7 per group).

in PA muscularization (Figure 6A). In contrast, neither ERα- nor ERβ-selective inhibition significantly attenuated E2-induced changes in RV capillary/myocyte ratio (Figure 6B). Taken together, these data suggest that ERα seems predominantly responsible for mediating E2 effects on RVH and CO, whereas PA remodeling effects are mediated by ERα and ERβ.

E2 Decreases ERK1/2 Activation in Lungs and RVs of HPH Rats in an ER-Dependent Manner

Given its inhibitory effect on PA remodeling and RVH, we evaluated whether E2 down-regulates signaling pathways associated with cell proliferation. We investigated the effects of E2 on ERK1/2, a key signaling pathway mediating proliferation in HPH (27). Intriguingly, E2 treatment was associated with decreased ERK1/2 activation in the lung and RV of HPH animals (Figures 7A and 7B). Of note, E2-induced attenuation of ERK1/2 activation in both organs was attenuated after co-treatment with ER antagonist, suggesting the E2 effects on hypoxia-induced ERK1/2 activation are ER dependent (Figures 7A and 7B). We were not able to detect reversal of E2 inhibition on ERK1/2 by coadministration of either ERα- or ERβ-selective antagonist in the whole lung or RV (*see* Figures E3A and E3B). These results indicate a redundant contribution of ERα or ERβ to E2-induced ERK1/2 inhibition in the lung vasculature and RV.

E2 Increases Cell-Cycle Inhibitor $p27^{Kip1}$ and Autophagy Marker LC3-II in HPH

We next evaluated if the effect of E2 on hypoxic remodeling extends beyond inhibition of proliferative signaling to that of concomitantly augmenting antiproliferative processes. First, we investigated whether E2 stimulates $p27^{Kip1}$, a cell-cycle inhibitor implicated in HPH pathogenesis (28). As previously reported (28), hypoxia decreased $p27^{Kip1}$ expression in the lung (Figure 8A). Interestingly, we also detected a previously unreported hypoxia-mediated decrease in $p27^{Kip1}$ expression in the RV (Figure 8B). Importantly, lung and RV decreases in $p27^{Kip1}$ were attenuated by E2 (Figures 8A and 8B). E2-induced increases in $p27^{Kip1}$ were not attenuated by ER inhibition (data not shown).

Based on these results, we speculated that E2 enhances autophagy, a catabolic nonproliferative state of adaptation to environmental stresses that protects from HPH (29). Indeed, E2 treatment was associated with a robust increase in the autophagy marker LC3-II in lungs of HPH rats (Figure 8C). Although a similar trend was seen in the RV of selected animals, this was not uniformly observed in all E2-treated rats, thereby not significantly altering the mean RV LC3-II changes in the E2 group as a whole (not shown). As seen with $p27^{Kip1}$, ICI co-treatment did not attenuate E2-induced changes in LC3-II expression (not shown).

HPH Is Associated with Increased Lung ERβ Expression

In light of the known sex differences in HPH and PAH, and given the prominent role of the ER in mediating the effects of E2

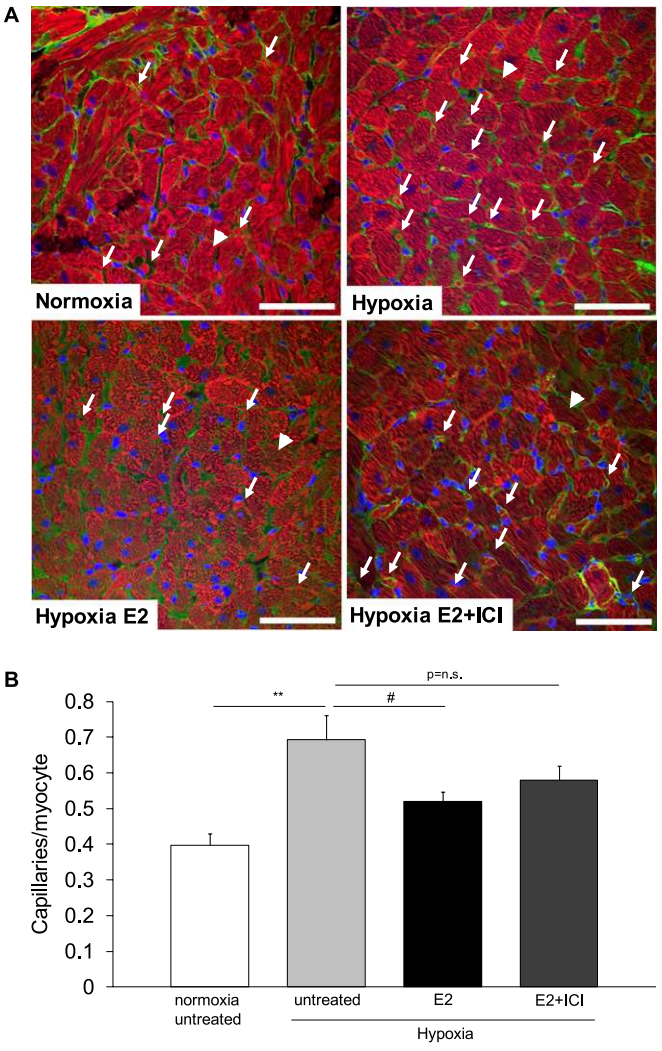

Figure 4. 17β-Estradiol (E2) decreases hypoxia-induced right ventricular (RV) remodeling. (*A*) Representative confocal microscopic images of RV sections from normoxic and hypoxic pulmonary hypertension rats. Hypoxic pulmonary hypertension animals were either untreated, or treated with E2 or E2 + ICI182780 (ICI). Glycocalyx is stained in *green* (wheat germ agglutinin conjugated to Oregon Green 488). Green staining therefore indicates cell membranes of myocytes or capillary endothelial cells. RV myocyte nuclei are stained in *blue* (DAPI). Capillaries (*arrows*) were identified by blood autofluorescence surrounded by glycocalyx staining of the endothelial cell membrane. Myocytes (*arrowheads*) were identified by size and shape (indicated by glycocalyx staining of the cell membrane), in conjunction with myoglobin autofluorescence surrounded by membrane glycocalyx staining. Size bars = 50 μm. Note that hypoxia increased RV capillarization, whereas E2 treatment attenuated it, but to a lesser extent in rats cotreated with estrogen receptor antagonist. (*B*) Quantification of capillary density, expressed as the ratio of capillaries to the number of myocytes per high-power field. Note that estrogen receptor blockade decreased E2 effect on hypoxia-induced RV remodeling. Values are mean ± SEM ($^{\#}P < 0.05$, $^{**}P < 0.001$; n.s. = not statistically significant; n = 4 animals per group).

during chronic hypoxia in our experiments, we sought to investigate if ER expression is altered in HPH. To assess localization of the ER, we performed IHC using antibodies for ERα or ERβ, respectively. Interestingly, 2 weeks of hypoxia exposure caused a robust increase in ERβ expression in PAs at the level of terminal bronchioles or alveolar ducts (Figure 9A). Of note, positive staining for ERβ mainly occurred in PA endothelial cells, whereas there was no significant staining of PASMCs (Figure 9A). Surprisingly, despite a working positive control, we did not detect immunostaining of ERα in normoxic or hypoxic lungs. We therefore investigated lung ERα expression via Western blotting in whole-lung homogenates, where there were no differences in lung ERα protein levels between normoxic and hypoxic lungs (Figure 9B).

E2 Decreases Proproliferative Signaling and Stimulates Cell-Cycle Inhibition and Autophagy in Hypoxic Primary RPAECs

The marked effect of E2 on PA remodeling and signaling *in vivo* was in large part mediated by ER. Because ERs are present throughout the pulmonary vasculature (17), we sought to identify the cellular target of E2 action in HPH. Because we previously demonstrated that E2 attenuates HPV in an endothelium-dependent fashion (26), and because PAECs significantly contribute to hypoxic PA remodeling via the release of proinflammatory and proproliferative mediators (3, 4, 40, 41), we focused our studies on primary RPAECs. Although E2 did not affect ERK1/2 activation in normoxic cells, it dose-dependently decreased ERK1/2 activation in hypoxic (1% O_2; 48 h) RPAECs (Figure 10A), suggesting opposite, oxygen tension-dependent E2 effects on ERK1/2 in RPAECs. Similar to *in vivo*, the inhibitory effect of E2 on ERK1/2 signaling during hypoxia was markedly less pronounced with ICI (100 nM [42, 43]) (Figure 10B), suggesting a mechanism of E2-inhibition of ERK signaling that is ER-mediated. Inhibitory E2 effects on hypoxia-induced ERK1/2 activation were generalized to primary PA and microvascular ECs isolated from different male rats (*see* Figures E4A and E4B). To investigate whether the inhibitory E2 effects on ERK1/2 signaling are mediated by ERα or ERβ, we cotreated RPAECs with E2 and selective ERα- (MPP; 1 nM to 1 μM) or ERβ-antagonist (PHTPP; 1 nM to 1 μM) for 48 hours during hypoxia exposure (Figure 10C). Either co-treatment opposed the inhibitory effect of E2 on ERK1/2, suggesting that either receptor is sufficient to mediate the inhibitory E2 effects on ERK1/2 during hypoxia in lung endothelial cells. MPP and PHTPP effects were replicated with a lower E2 concentration (100 nM; not shown).

E2 treatment, although decreasing $p27^{Kip1}$ or not affecting LC3-II expression during normoxia (Figures 10D and 10F), up-regulated $p27^{Kip1}$and LC3-II in RPAECs exposed to hypoxia only (Figures 10E and 10G). As noted *in vivo*, ER-inhibition did not alter E2 effects on $p27^{Kip1}$ or LC3-II during hypoxia (*see* Figure E5).

E2 Decreases Vascular Endothelial Growth Factor Secretion in Hypoxic but Not Normoxic RPAECs

Because vascular endothelial growth factor (VEGF) is a major mediator of hypoxia-induced PA remodeling (3), we determined whether E2-associated changes in signaling pathways modulating cell proliferation are associated with attenuation of hypoxia-induced increases in VEGF secretion. Indeed, hypoxia-induced VEGF secretion was significantly attenuated in E2-treated cells (*see* Figure E6). As seen with signaling parameters, there were disparate E2 effects on VEGF secretion in hypoxia and normoxia, with no significant alterations in VEGF concentration in the media of normoxic E2-treated cells. Intriguingly, VEGF levels were unaffected by ER-blockade, suggesting ER-independent E2 effects on RPAEC VEGF secretion (not shown).

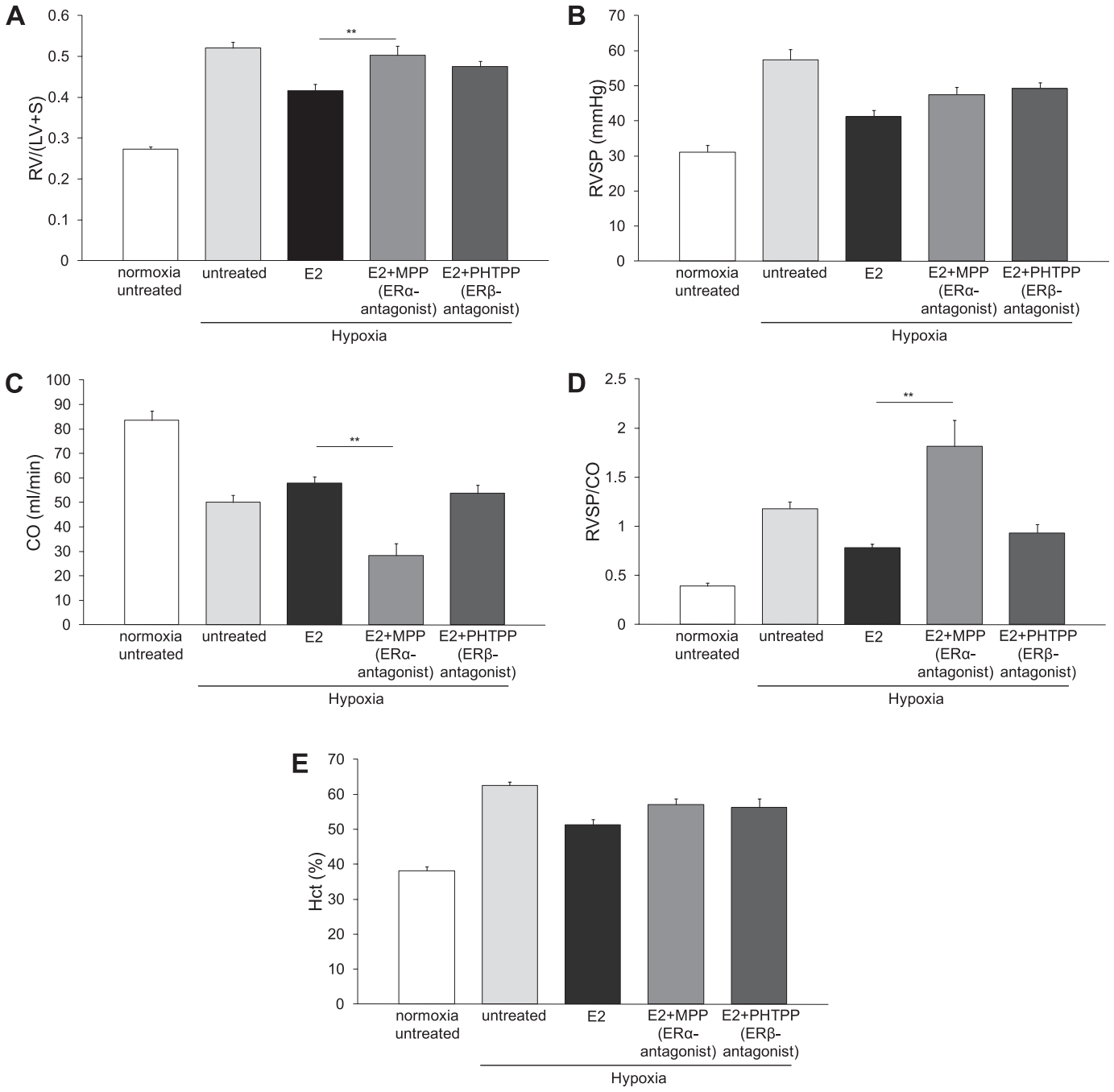

Figure 5. Estrogen receptor (ER)-α blockade attenuates 17β-estradiol (E2) protection in hypoxic pulmonary hypertension. (*A*) Right ventricular mass (RV/[LV+S]), (*B*) right ventricular systolic pressure (RVSP), (*C*) cardiac output (CO), (*D*) RVSP/CO, and (*E*) hematocrit (Hct) levels in normoxic and hypoxic male rats treated with E2 alone (75 μg/kg/d) or with E2 in combination with the selective ERα antagonist MPP (850 μg/kg/d) or the selective ERβ antagonist PHTPP (850 μg/kg/d). All drugs were started 1 week before hypoxia and continued throughout the entire 2-week hypoxia exposure. E2 was administered via subcutaneous osmotic minipumps; MPP and PHTPP were injected subcutaneously once per day. Values are mean ± SEM (**$P < 0.001$; n = 4–12 per group).

E2 Decreases Proliferation in Hypoxic but Not Normoxic RPAECs in an ER-Dependent Manner

To investigate the functional relevance of oxygen tension-dependent E2 effects on signaling pathways modulating cell proliferation, we studied the impact of E2 treatment on RPAEC proliferation using bromodeoxyuridine incorporation. E2 significantly increased RPAEC proliferation during normoxia (Figure 11A). In contrast, and consistent with its signaling effects, E2 decreased proliferation in hypoxic RPAECs (Figure 11A). This was attenuated by ER inhibition (Figure 11A). E2 treatment dose-dependently decreased the number of viable RPAECs during hypoxia, as assessed by automated cell counts (Figure 11B).

LC3-II Expression in Hypoxic RPAECs is ERK1/2 Dependent

Because E2 decreased ERK1/2 activation and increased LC3-II expression *in vivo* and *in vitro*, we investigated the link between ERK1/2 and autophagy. Intriguingly, ERK1/2-inhibition (PD98059, 50 μM) recapitulated the stimulatory effects of E2 on LC3-II

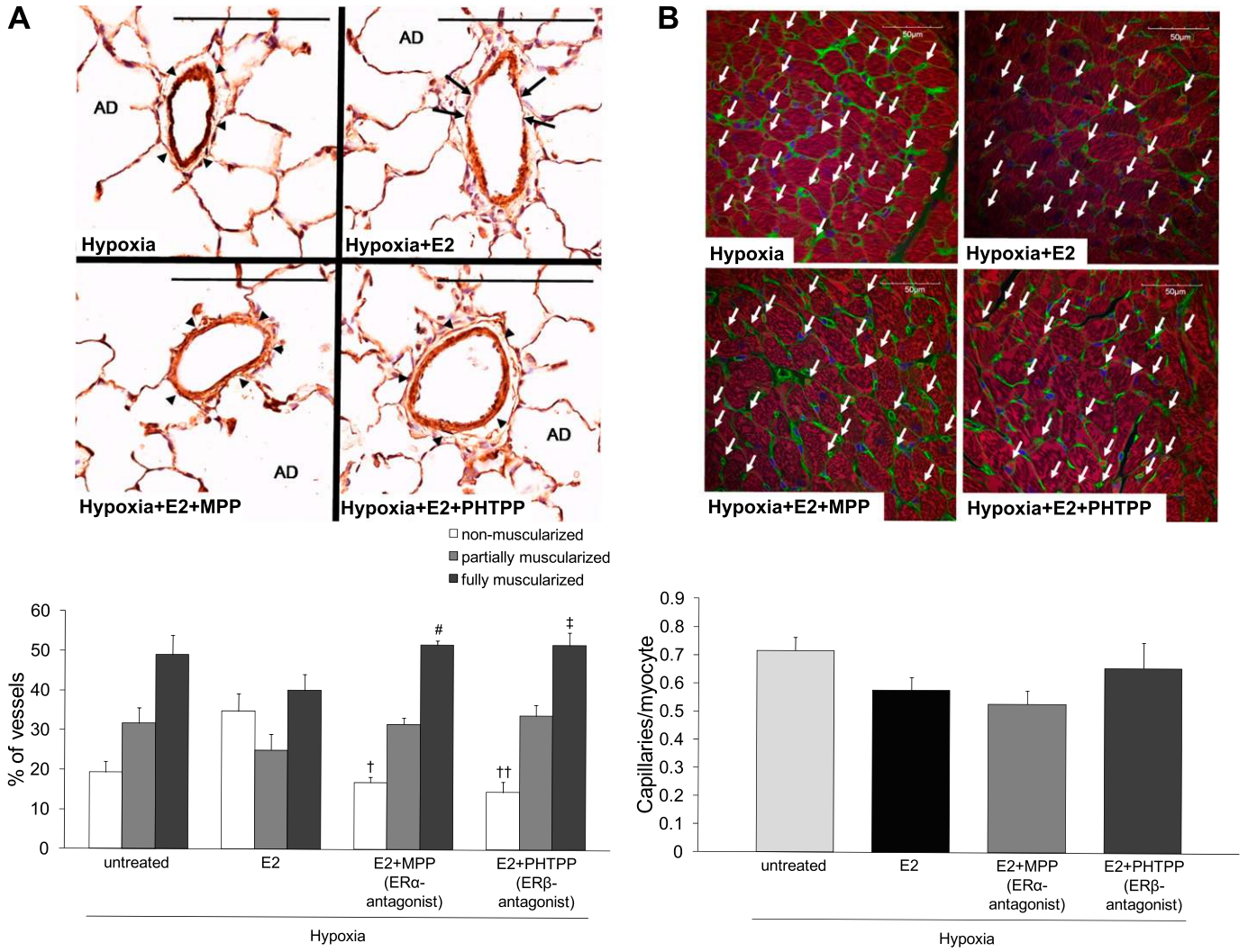

Figure 6. Selective estrogen receptor (ER)α- or ERβ-blockade attenuates protective 17β-estradiol (E2) effects on pulmonary artery (PA) remodeling (*A*), but does not significantly affect right ventricular (RV) capillarization (*B*) in E2-treated hypoxic pulmonary hypertension rats. (*A*) Representative images and quantification of muscularization of alveolar duct (AD)–associated PAs (<200 μm). Hypoxia-exposed animals were either untreated, or treated with E2, E2 + MPP (ERα antagonist; 850 μg/kg/d), or E2 + PHTPP (ERβ antagonist; 850 μg/kg/d). Immunohistochemistry for α-smooth muscle actin was performed, and degree of PA muscularization (nonmuscularized, partially muscularized, or fully muscularized) was determined as outlined in Figure 3. Size bars = 100 μm. Note that E2-associated decrease in hypoxia-induced PA muscularization (evidenced by areas of less complete muscularization [*arrows*]) was attenuated after co-treatment with ERα- or ERβ-antagonist, resulting in more complete PA muscularization (*arrowheads*). (*B*) Representative images and quantification of RV capillarization in untreated hypoxic pulmonary hypertension rats, or hypoxic rats treated with E2, E2 + MPP, or E2 + PHTPP. RV capillarization was determined as outlined in Figure 4. *Arrows* indicate representative capillaries; *arrowheads* indicate representative myocytes. Size bars = 50 μm. Note that neither coadministration of MPP nor PHTPP attenuated E2 effects on capillary/myocyte ratio. Values are mean ± SEM ($^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. hypoxia + E2 nonmuscularized; $^{\#}P < 0.05$, $^{\ddagger}P = 0.07$ vs. hypoxia + E2 fully muscularized; n = 4–6 animals per group).

expression during hypoxia (*see* Figure E7), suggesting the E2-mediated LC3-II increase may be a consequence of E2-induced ERK inhibition. In contrast, ERK1/2 inhibition did not affect $p27^{Kip1}$ expression.

DISCUSSION

To our knowledge, this is the first study directly evaluating the role of the ER in HPH. Our work builds on previous studies of E2 protection in HPH, which demonstrated that in female rats, ovariectomy leads to a 10–20% worsening of hemodynamics, remodeling, and erythropoiesis, and that E2 replacement improves HPH endpoints by 20–30% (12, 13, 44). Previous work compared HPH outcomes in male and female rats (45) and evaluated the effects of E2 withdrawal on HPH (12, 13). Our complementary and distinctive investigation addressed the novel aspect of identifying previously unrecognized mechanisms by which E2 treatment exerts protective effects in HPH (*see* Figure E8). Our results indicate that the protection of E2 in HPH is independent of the subject's sex, which should further emphasize the recently recognized (46) need for studies of estrogen-dependent pathways in the hope of identifying non-hormonal therapeutic targets to benefit male and female HPH patients.

Our results indicate that important E2 effects on functional endpoints in HPH are predominantly mediated by ERα. In addition, both receptors are implicated in the effect of E2 on cardiopulmonary remodeling during HPH. This is not surprising

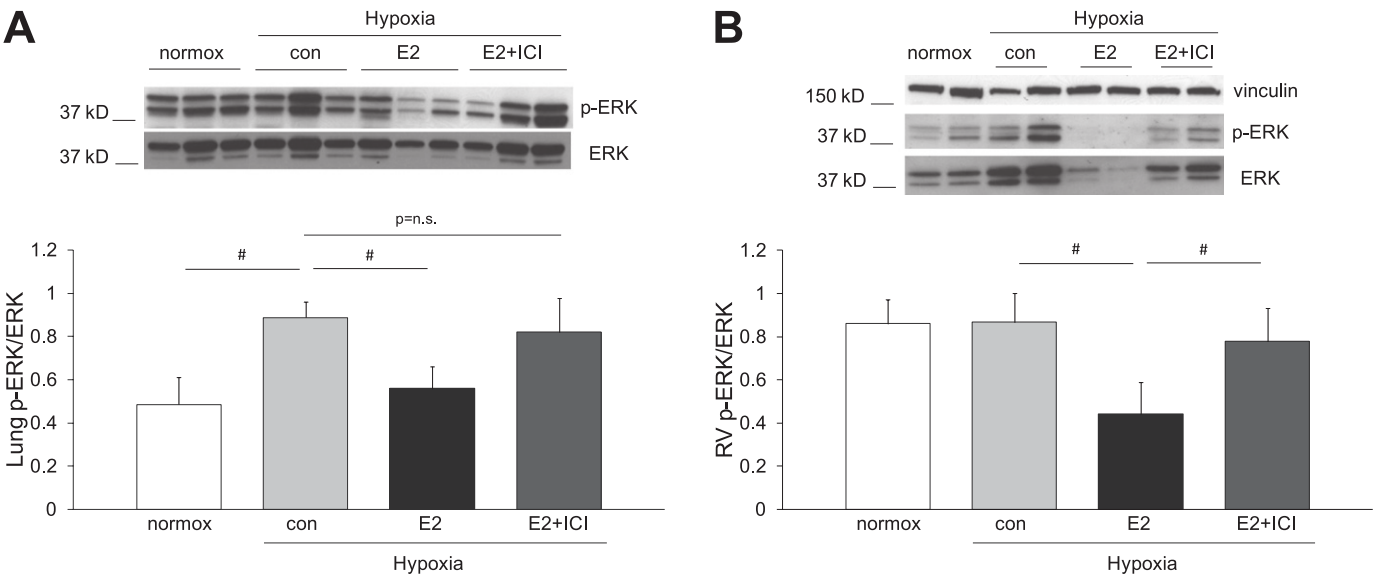

Figure 7. 17β-Estradiol (E2) decreases ERK1/2 activation in hypoxic pulmonary hypertension lungs and right ventricles (RVs) in an estrogen receptor (ER)–dependent manner. Activation of ERK1/2 in lungs (*A*) and RVs (*B*) of rats exposed to normoxia or hypobaric hypoxia (2 wk). Hypoxic rats were either untreated, treated with E2 alone, or cotreated with E2 and ICI182780 (ICI). Representative Western blots of phospho-ERK1/2, total ERK1/2, and vinculin (for RV) are shown in the *upper panels*. Bar graphs represent relative increase in phospho-ERK1/2 to total ERK1/2 measured by densitometry (mean ± SEM; $^{\#}P < 0.05$; n.s. = not statistically significant). (*A*) n = 6, (*B*) n = 4–5.

in the context of our previous work in PA rings, where ERα and ERβ mediated attenuation of PA vasocontraction (26). In contrast, production of E2 metabolites was not required for the protection of E2; moreover, inhibition of E2 conversion enhanced some of the effects of E2. Because measurements of E2 conversion enzyme activities are either not routinely available (47) or are notoriously unreliable (47), our study lacked measurements of CYP450- or COMT-activities or catecholestradiols or methoxyestradiols levels after respective inhibitor treatment. To address this limitation, we used inhibitor concentrations previously shown to be effective *in vivo* (34, 35) and *in vitro* (24), and we avoided the use of E2 conversion inhibitors with significant off-target effects. Similarly, we used E2 concentrations previously shown to be physiologically relevant and effective in adult female rats; this led to hemodynamic effects of similar magnitude as those in past studies (12, 13, 44).

The striking influence of sex on PH development and progression is perplexing, yet poorly understood. Female animals develop less severe HPH than males (45, 48). Women have a lower incidence of high-altitude pulmonary edema (a condition marked by profound HPV) (49, 50), and menopause represents a contributing factor for the development of chronic mountain sickness (51). However, female sex is a predisposing factor for idiopathic, hereditary, and certain associated forms of PAH (52–55). Despite this strong preference for the female sex, once affected by PAH women exhibit improved survival compared with men (53–55). In light of the important study by Austin and coworkers (23), it is conceivable that estrogens, although genuinely protective in the pulmonary vasculature, predispose to PAH development in the setting of altered metabolic pathways. In addition, estrogens may exert detrimental proproliferative and proinflammatory effects in a diseased and remodeled pulmonary vasculature, as has been demonstrated in systemic blood vessels (18). Lastly, in light of the ER-mediated E2 effects seen in our study, it is conceivable that alterations in ER signaling or tissue distribution may contribute to the female predominance in PAH. Such a scenario has been described in the systemic vasculature, where single nucleotide polymorphisms for ERα or ERβ predispose to cardiovascular disease (56–58). In this context, a recent study documented increased ESR1/ERα transcript in lungs of patients with PAH (59). However, it is not known if this represents a pathogenic mechanism of PAH development versus a consequence of the disease (e.g., a compensatory mechanism for impaired ERα or ERβ signaling). Our findings of ERα- and ERβ-mediated protection in HPH suggest that in human PAH, an increase in ERα transcript may be a compensatory process, and potentially an indicator of altered E2 signaling. Interestingly, HPH rats did not up-regulate ERα protein levels in the lung in response to hypoxia; rather it was ERβ that exhibited increased pulmonary vascular expression to several weeks of hypoxia. Future studies will have to determine if increased duration of hypoxia alters ERα levels and to measure the function of expressed ER during hypoxic conditions.

We describe for the first time marked E2 effects on RV function and remodeling in HPH, which were ER dependent. Similar to its RV cardioprotective effects in this study, E2 has been previously involved in the cardioprotection of the LV (60–63). It remains unknown if E2 induced a decrease in RV mass and RV capillary density indirectly (by reducing PA remodeling and therefore RV stress), or by directly affecting RV cardiomyocytes. We did not detect RV fibrosis or apoptosis within this short hypoxia time course and therefore could not implicate E2 in these two processes of RV remodeling, as previously described for the LV (60). However, given that RV systolic function in healthy and PAH-affected women is superior relative to men and correlates with E2 levels (64, 65), it is conceivable that E2 has direct RV cardioprotective effects. Such a notion is supported by our findings of significantly worsened CO after nonselective and selective ERα antagonism, suggesting a predominantly RV-directed mechanism of E2 action. Because RV function determines survival in PAH (53, 66), RV cardioprotective properties of E2 would partly explain the improved survival in female patients with PAH despite a higher prevalence of the disease, a concept supported by RV-protective E2 effects in rodents (33), and by a correlation between E2 levels and RV function described in humans (65).

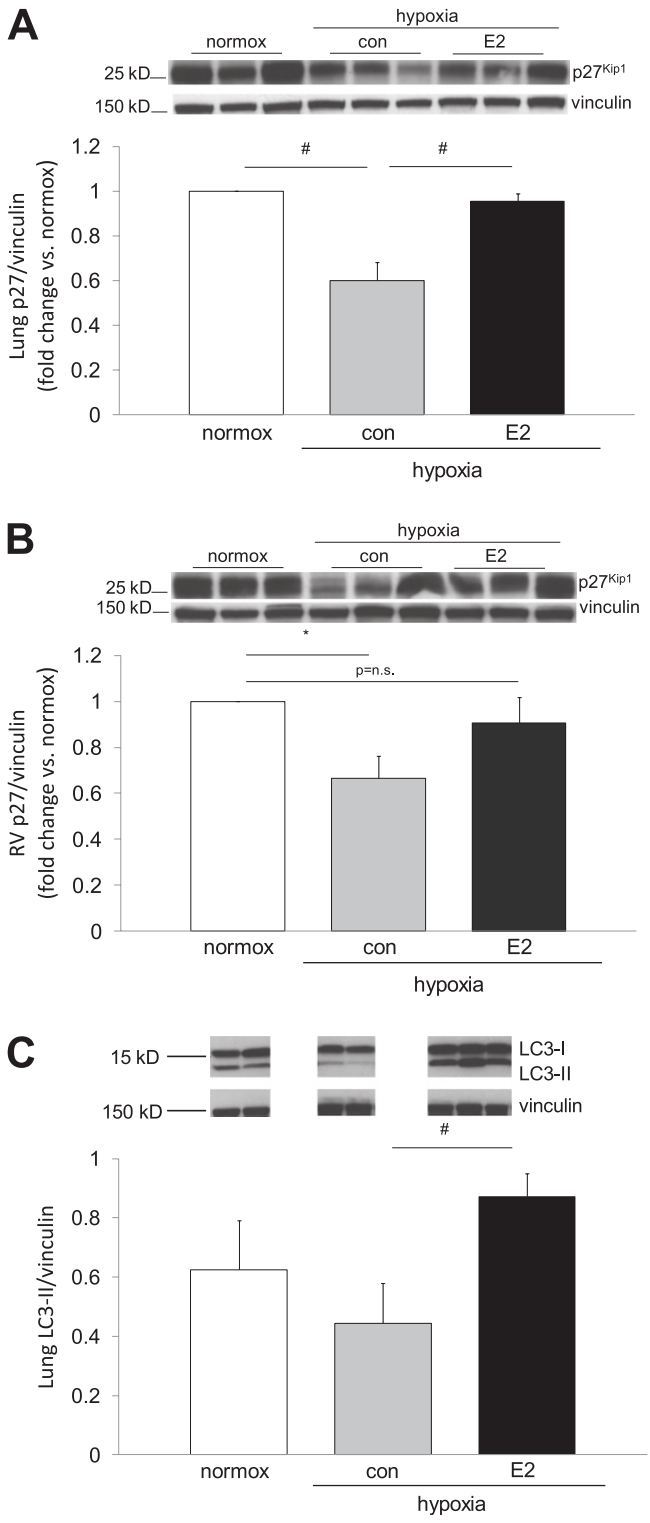

Figure 8. 17β-Estradiol (E2) increases expression of cell cycle inhibitor $p27^{Kip1}$ (*A* and *B*) and autophagy marker LC3-II (*C*) in hypoxic pulmonary hypertension rats. Expression of $p27^{Kip1}$ and LC3-II are demonstrated in lungs (*A* and *C*) and right ventricles (RVs) (*B*) of rats exposed to normoxia or hypobaric hypoxia (2 wk) and treated with E2. Representative Western blots of $p27^{Kip1}$ and LC3-II are shown in the *upper panels*. Bar graphs represent relative increase in $p27^{Kip1}$ and LC3-II, respectively, to vinculin measured by densitometry (mean ± SEM; normox = untreated normoxia group; con = untreated hypoxia control; $^{\#}P < 0.05$; $^{*}P < 0.01$; n.s. = not statistically significant). (*A*) n = 4, (*B*) n = 5, (*C*) n = 3–5.

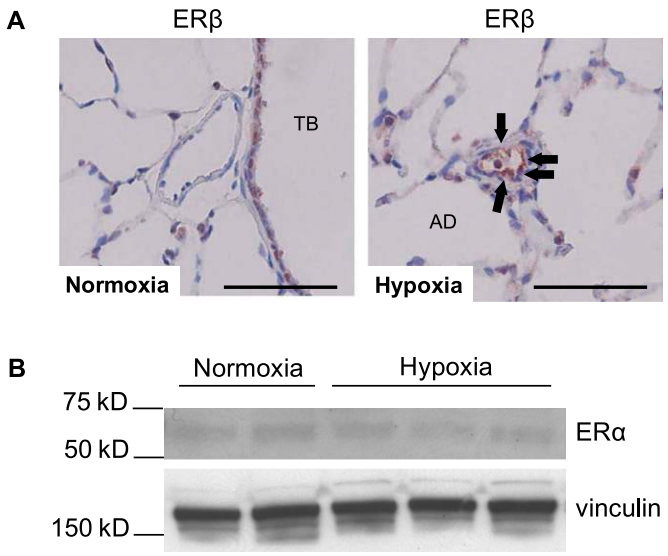

Figure 9. Hypoxic pulmonary hypertension is associated with increased lung estrogen receptor (ER)β-expression. Male Sprague-Dawley rats were exposed to 2 weeks of hypobaric hypoxia (P_{atm} = 362 mm Hg; equivalent to 10% $F_{I_{O_2}}$). Lungs were harvested, and immunohistochemistry (IHC) for ERβ (*A*) or Western blotting for ERα (*B*) was performed. ERα was not detectable by IHC. Representative IHC images (for ERβ; *A*) and Western blots (for ERα and vinculin loading control; *B*) are shown. Note the increase in ERβ-positive cells (*arrows*) in hypoxic pulmonary arteries (PA) at the level of terminal bronchioles (TB) or alveolar ducts (AD). Of note, positive staining for ERβ mainly occurred in PA endothelial cells, whereas there was no significant staining of PA smooth muscle cells. Size bars = 50 μm.

A decrease in blood viscosity and shear stress may be another mechanism by which E2 attenuates hemodynamic alterations in HPH. The effects of E2 on hypoxia-induced erythrocytosis (67) and hematopoiesis (68) have been long recognized. One question that still remains is whether the E2-associated decrease in hypoxia-induced erythrocytosis is compensated by an increase in CO to maintain oxygen delivery (DO_2). The degree of decrease in hematocrit and increase in CO with E2 were similar in our study. Given the strong impact of CO on DO_2, it seems likely that the latter was well maintained in the hypoxic animals. Cell culture studies suggest that the E2-mediated attenuation of hypoxia-induced erythrocytosis is mediated via decreased hypoxia-inducible factor (HIF)-1α signaling (69). Such an effect would also explain the E2-associated decrease in hypoxia-induced RV capillarization and the inhibitory E2 effect on VEGF secretion observed in our study, and warrants future investigations of the interaction between E2 and HIF-1α in HPH.

The striking effect of E2 on PA remodeling was assessed by measuring PA muscularization using a well-established method that analyzes α-SMA–stained vessels associated with alveolar ducts (36), and focusing on vessels in proximity to alveolar ducts. This method avoids misclassification of pulmonary veins as nonmuscularized arteries and may mitigate the limitation of omitting nonmuscularized vessels in sections lacking specific endothelial cell staining.

In contrast to a recently published report suggesting a role of ERβ in E2 protection in monocrotaline-PH (33), we provide mechanistic studies of E2 and ER signaling *in vivo* and *in vitro* that suggest a more prominent role for ERα in mediating E2 protection against HPH. This difference may reflect distinct mechanisms of action of E2 signaling in hypoxic animals compared with those exposed to monocrotaline. Similar to our

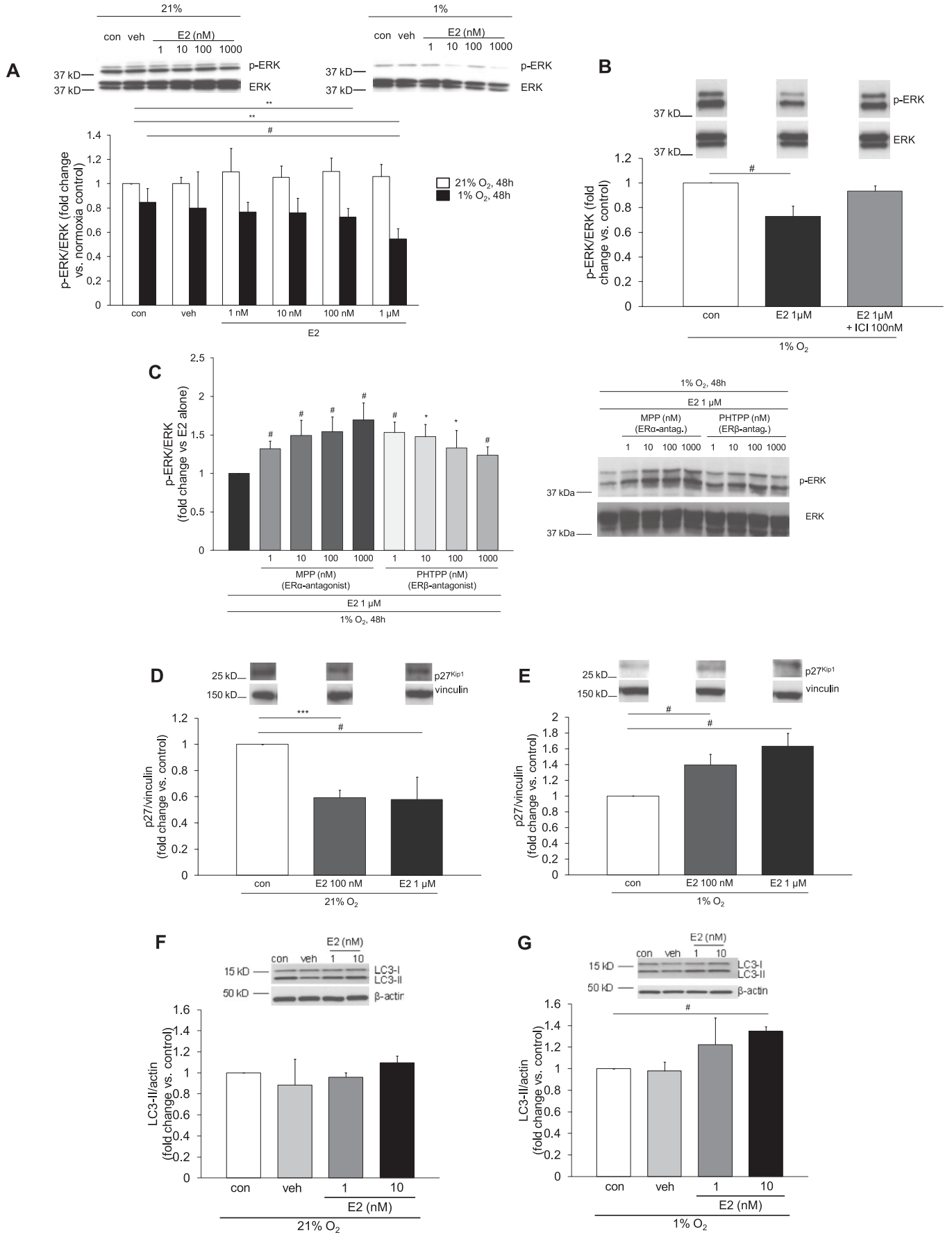
A
21%
1%
con veh E2 (nM) 1 10 100 1000
37 kD
p-ERK
ERK
p-ERK/ERK (fold change vs. normoxia control)
21% O2, 48h
1% O2, 48h
con
veh
1 nM
10 nM
100 nM
1 μM
E2
B
p-ERK/ERK (fold change vs. control)
con
E2 1μM
E2 1μM + ICI 100nM
1% O2
C
p-ERK/ERK (fold change vs E2 alone)
MPP (nM) (ERα-antagonist)
PHTPP (nM) (ERβ-antagonist)
E2 1 μM
1% O2, 48h
1% O2, 48h
E2 1 μM
MPP (nM) (ERα-antag.)
PHTPP (nM) (ERβ-antag.)
37 kDa
p-ERK
ERK
D
25 kD
150 kD
p27Kip1
vinculin
p27/vinculin (fold change vs. control)
con
E2 100 nM
E2 1 μM
21% O2
E
1% O2
F
15 kD
50 kD
LC3-I
LC3-II
β-actin
LC3-II/actin (fold change vs. control)
E2 (nM)
21% O2
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Figure 10. 17β-Estradiol (E2) decreases ERK1/2 activation and increases cell cycle inhibitor $p27^{Kip1}$ and autophagy parameter LC3-II in hypoxic, but not normoxic primary rat pulmonary artery cells (RPAECs). ERK1/2 activation (*A–C*), $p27^{Kip1}$ expression (*D* and *E*), and LC3-II expression (*F* and *G*) in RPAECs grown at 21% or 1% O_2 for 48 hours and either untreated (con) or treated with E2 or vehicle (veh). In (*B*) and (C), E2-treated cells were also cotreated with nonselective (ICI182780 [ICI]) or selective estrogen receptor (ER)α- (MPP) and ERβ-antagonists (PHTPP), respectively. Representative Western blots are shown in the *upper panels*. Bar graphs represent relative increase in phospho-ERK1/2 to total ERK1/2, in $p27^{Kip1}$ to vinculin, and in LC3-II to actin, all measured by densitometry. Values (mean ± SEM) are expressed as fold change compared with normoxic or hypoxic control group, respectively, except for *C*, where values are expressed as fold change compared with hypoxic E2 group (*A* and *B*, *D–G*, $^{\#}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$; *C*, $^{\#}P < 0.05$, $^{*}P < 0.01$ vs. E2; n = 3 independent experiments).

findings in pulmonary vascular–RV injury from hypoxia, a requirement for ERα for the protective effect of E2 was also demonstrated in models of systemic vascular injury (70, 71). Furthermore, because model-dependent involvement of ERα and ERβ has also been shown in the protective effects of E2 on the LV (60, 62, 63), it is conceivable that in the pressure-overloaded RV, contributions of each ER subtype may be context-specific, with ERα having a more prominent protective role in HPH. However, because ERα-blockade attenuated RVH and CO without affecting RV capillary/myocyte ratio or ERK1/2 activation, such an effect would be independent of RV capillarization or ERK signaling. Alternatively, increased RV mass in the setting of unchanged capillarization may indicate a diffusion distance problem. Of note, although ERβ-blockade did not significantly attenuate E2 effects on RVH and hematocrit, there were trends that suggest a partial or minor contribution of ERβ to E2 signaling in the RV or hematopoietic system. In addition, there was a trend for the ERβ-antagonist to increase RV capillary/myocyte ratio, and treatment with ERβ-antagonist did attenuate inhibitory E2 effects on PA remodeling and ERK1/2 activation in hypoxic PAECs. These tissue and cell culture observations suggest that, despite the predominant role of ERα in mediating benefits on hemodynamics and RVH, the role of ERβ in HPH should not be dismissed. However, inhibition of ERα in E2-treated HPH rats actually worsened CO to values below those of the untreated hypoxia control group, suggesting that unopposed ERβ activation (or activation of non-ER pathways) may be detrimental for the overall RV function in this context. Because neither ERα- nor ERβ-inhibition alone significantly altered E2 effects on RV capillarization or ERK1/2 activation in whole lung or RV, this suggests potential redundant functions of each ER subtype in mediating E2 effects on these endpoints.

The lack of involvement of E2 metabolites was somewhat unexpected, because previous reports (22, 23, 25) suggested beneficial effects of these molecules in hereditary PAH and monocrotaline-PH. However, a recent study identified increased expression of E2 conversion enzyme CYP1B1 in serotonin transporter overexpression-induced PH, and in patients with PAH (72). It is therefore conceivable that E2 metabolites may be protective only in certain forms of PH (e.g., hereditary PAH or inflammatory PH), but not in HPH, a condition with distinct pathogenesis and phenotype (1).

A similar dichotomy of the effects of E2 was noted in cultured PAECs, where E2 inhibited proliferative mediators and signaling (VEGF and ERK1/2), while stimulating antiproliferative pathways and responses ($p27^{Kip1}$ and autophagy) during hypoxia, but not during normoxia. On the contrary, E2 stimulated cell proliferation during normoxia, possibly explaining the increased PAH prevalence in females, but decreased disease severity in those already affected. Although recent reports describe E2 effects on PASMCs (44, 73), its role in modulating hypoxic PAECs remained undefined. We focused our study of E2 effects on PAECs because their inflammatory and proliferative responses significantly contribute to hypoxic PA remodeling (3, 4, 40, 41). In addition, our previous studies demonstrated that E2 attenuates HPV in an endothelium-dependent fashion (26). Using a clinically relevant degree of moderate hypoxia (5), our studies indicate that hypoxic PAEC signaling and proliferation are significantly modulated by E2. Interestingly, relative to PASMC, PAECs seem to require a larger concentration of E2 to exhibit significant alterations in cell proliferation signaling (e.g., ERK1/2 inhibition). The

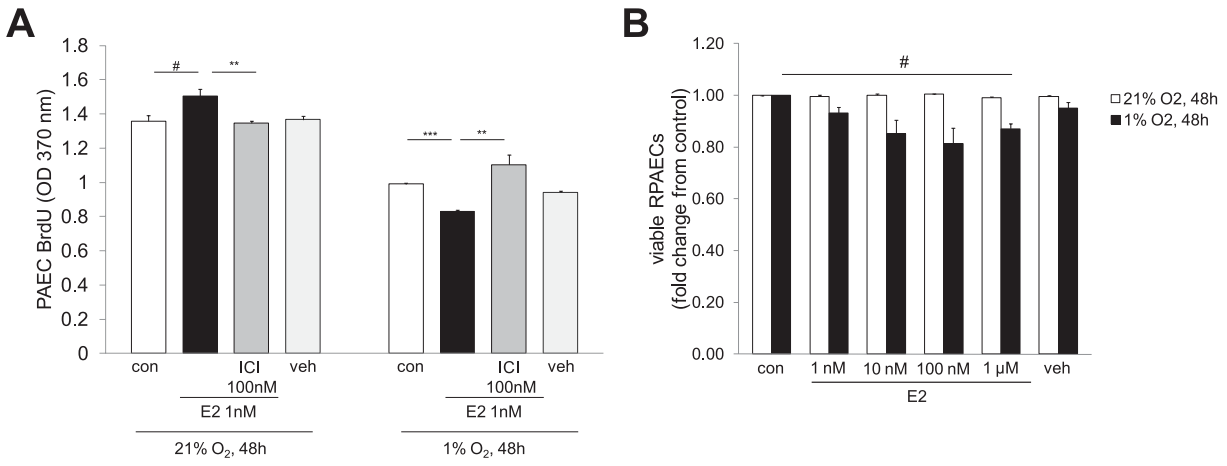

Figure 11. 17β-Estradiol (E2) decreases primary rat pulmonary artery cell (RPAEC) proliferation in hypoxia. (*A*) Bromodeoxyuridine (BrdU) uptake in RPAECs grown at 21% or 1% O_2 for 48 hours in the presence of E2, E2 ± ICI182780 (ICI), or E2 vehicle (veh). (*B*) Viable RPAECs (assessed by trypan blue uptake) exposed to 21% (*white bars*) or 1% O_2 (*black bars*) for 48 hours and treated with E2 or vehicle. Number of viable cells in E2- or vehicle-treated normoxia and hypoxia groups is expressed as fold change from their untreated control group, respectively. Values are mean ± SEM (con = control; OD = optical density; $^{\#}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$).

relevance of these cell type-specific differences to the PA hemodynamics and remodeling *in vivo* remains to be determined. The requirement for supraphysiologic E2 concentrations for *in vitro* effects is in concordance with our prior studies of E2 causing vasorelaxation in isolated hypoxic PA rings (26). Furthermore, it is well recognized that discrepancies can exist between the concentrations of agents that are required to produce effects *in vivo* and *in vitro*. For example, this has been shown for the potassium-channel opener cromakalim, which exerts vasodilatory effects *in vivo* at a lower dose than *ex vivo* (74, 75). Lastly, sex hormones may actively be adsorbed in the capillaries, so that the active hormone concentration at the endothelial cell membrane active site may be significantly higher than that estimated by serum samples (76). Therefore, it is conceivable that *in vivo* smaller E2 doses than the ones used *ex vivo* may be sufficient to exert similar effects. Importantly, the cell culture experiments are in concordance with the effect of pharmacologic administration of E2 *in vivo*. Of note, our *ex vivo* hypoxia exposure model was conducted in cell culture medium containing phenol red. Because phenol red can act as an ER agonist in breast cancer cells (77), we tested its effect on PAECs exposed to hypoxia. We report that phenol red presence did not affect the E2-induced signaling (e.g., ERK activation) in these cells (*see* Figure E9).

Mechanisms associated with the protection of E2 in hypoxic cardiopulmonary remodeling included inhibition of ERK1/2 activation, a previously unrecognized effect, with concomitant upregulation of the cell-cycle inhibitor $p27^{Kip1}$ not only in the lung, as shown previously (44), but also the RV. Because ERK1/2 contributes to HPH development (27) as a major signaling mediator of cell proliferation, we tested if its inhibition by E2 may cause autophagy or cell death during hypoxia. Although there was no increase in apoptosis, we did detect increased lung autophagy in response to E2. Although hypoxia was associated with minor increases in LC3-II, indicating autophagy (78), further enhancing this process of arrested cell proliferation may be beneficial in HPH (29). Interestingly, although pharmacologic ERK1/2-inhibition recapitulated the effects of E2 on autophagy, neither ER nor E2 conversion-inhibition blocked autophagy, suggesting that GPR30 activation (79), activation of an unidentified receptor responsive to E2, or E2 metabolites other than catecholestradiols or methoxyestradiols may be involved. Indeed, although ER pharmacologic blockade significantly decreased the magnitude of E2 protection, reversal of E2 effect was not complete. Given the use of pharmacologic ER inhibitor at concentrations previously documented to provide sufficient ER blockade *in vivo* (32) and *in vitro* (42, 43), suboptimal dosing seems unlikely, but we cannot completely rule out that hypoxia decreased ICI inhibitory efficiency. Alternatively, potential GPR30-agonistic effects of ICI (80) may have diluted the magnitude of its inhibitory action against E2 via ER blockade. Lastly, the lack of complete inhibition of the effects of E2 in ICI-treated rats may be caused by the relative short duration of hypoxia used in our model.

In conclusion, our report demonstrates that E2 administration attenuates hemodynamic and remodeling parameters in male HPH rats, and suggests that the protective effects of E2 are largely mediated by the ER, involving specific inhibition of hypoxia-induced mitogenic effects in the lung and RV. ERα is the predominant ER mediating E2 effects on cardiopulmonary hemodynamic parameters in HPH, whereas E2 effects on PA remodeling and endothelial cell activation are mediated by ERα and ERβ. Harnessing the signaling pathways engaged by E2 may provide nonhormonal therapeutic options in HPH.

Author disclosures are available with the text of this article at www.atsjournals.org.

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