ORIGINAL RESEARCH

Role of the Aryl Hydrocarbon Receptor in Sugen 5416–induced Experimental Pulmonary Hypertension

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Abstract

Rats dosed with the vascular endothelial growth factor inhibitor Sugen 5416 (Su), subjected to hypoxia, and then restored to normoxia have become a widely used model of pulmonary arterial hypertension (PAH). However, the mechanism by which Su exacerbates pulmonary hypertension is unclear. We investigated Su activation of the aryl hydrocarbon receptor (AhR) in human pulmonary artery smooth muscle cells (hPASMCs) and blood outgrowth endothelial cells (BOECs) from female patients with PAH. We also examined the effect of AhR on aromatase and estrogen levels in the lung. Protein and mRNA analyses demonstrated that CYP1A1 was very highly induced in the lungs of Su/hypoxic (Su/Hx) rats. The AhR antagonist CH223191 (8 mg/kg/day) reversed the development of PAH in this model *in vivo* and normalized lung CYP1A1 expression. Increased lung aromatase and estrogen levels in Su/Hx rats were also normalized by CH223191, as was AhR nuclear translocator (ARNT [HIF-1β]), which is shared by HIF-1α and AhR. Su reduced HIF-1α expression in hPASMCs. Su induced proliferation in BOECs and increased apoptosis in human pulmonary microvascular ECs and also induced translocation of AhR to the nucleus in hPASMCs. Under normoxic conditions, hPASMCs did not proliferate to Su. However, when grown in hypoxia (1%), Su induced hPASMC proliferation. In combination with hypoxia, Su is proliferative in hPASMCs and BOECs from patients with PAH, and Su/Hx-induced PAH in rats may be facilitated by AhR-induced CYP1A1, ARNT, and aromatase. Inhibition of AhR may be a novel approach to the treatment of pulmonary hypertension.

Keywords: aryl hydrocarbon receptor; estrogen; pulmonary hypertension; Sugen; VEGF

Clinical Relevance

The Sugen 5416/hypoxic rat model is a commonly used model of pulmonary hypertension (PH). Sugen is a vascular endothelial growth factor inhibitor, but to date the mechanism by which it actually facilitates the development of PH is unclear. Here we demonstrate that, at least in part, the mechanism involves activation of the aryl hydrocarbon receptor (AhR) and subsequent increased expression of CYP1A1 in the lung and translocation of AhR from the cytoplasm to the nucleus in human pulmonary artery smooth muscle cells. This is accompanied by an increase in CYP1A1 and aromatase expression, and an increase in estrogen synthesis. We show that Sugen causes the proliferation of blood outgrowth endothelial cells from patients with pulmonary arterial hypertension, but only causes the proliferation of human pulmonary artery smooth muscle cells when grown in hypoxic conditions. Sugen can also cause apoptosis in human microvascular pulmonary endothelial cells. Inhibition of AhR can reverse Sugen/hypoxic experimental PH and may be a novel approach to the treatment of PH.

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Pulmonary arterial hypertension (PAH) is a progressive disease leading to right heart failure. This condition is defined by vascular remodeling and complex vascular lesion formation arising from accelerated proliferation in pulmonary endothelial, smooth muscle and fibroblast cells (1). Many vasoactive factors have been associated with the associated pathobiology including vascular endothelial growth factor (VEGF); however, VEGF can exert both angiogenic and antiangiogenic effects, and its role in PAH is still unclear (2). Curiously, one administration of Sugen 5416 (Su), a VEGF receptor (VEGFR) inhibitor (with affinity at VEGFR2 > VEGFR1) to rats combined with hypoxic exposure can cause severe experimental pulmonary hypertension (PH) that develops after a subsequent period of normoxia. These Su/hypoxic (Su/Hx) rats develop high pulmonary pressures, and in some animals, occlusive/plexogenic-like pulmonary vascular lesions are also observed (3). The mechanism by which the combination of Su and hypoxia causes severe PH is unclear, and clarification of this issue would have important translational value for understanding clinical PAH.

Su has been studied as a potential therapy for advance malignancies. Curiously, it has a long-lasting inhibitory effect in animal tumor models even though it has a very short half-life of ~30 minutes, and it is believed that this long-lasting effect results from its accumulation in cells (4, 5). Su is an agonist at the cytoplasmic aryl hydrocarbon receptor (AhR), which is cytoplasmic when it is unligated (6). AhR is a member of the basic helix-loop-helix/Per-ARNT-Sim family of heterodimeric transcriptional regulators, which are highly expressed in the lung (7) and influence the major stages of tumorigenesis as well as energy metabolism, lipid metabolism, and diet-induced obesity (8, 9). We recently demonstrated that AhR expression is elevated in human pulmonary artery smooth muscle cells (hPASMCs) from patients with PAH, and that AhR expression may be increased in small occluded pulmonary arteries from the Su/Hx rat model (10). PAH occurs up to fourfold more frequently in women (11), and dysfunctional estrogen synthesis and metabolism may play an important role in the pathobiology of PH, both clinically and experimentally (10, 12–18). AhR is a major regulator of the estrogen-metabolizing enzyme CYP1A1 and can also regulate aromatase, which is the major enzyme in estrogen synthesis (19). Therefore, the effects of Su on AhR activation in the pulmonary circulation of patients with PAH is of interest.

To understand how hypoxia may synergize with the effects of Su on AhR, we investigated possible interactions among Su, AhR, and hypoxia-inducible factor 1α (HIF-1α) signaling. This is of interest because the AhR nuclear translocator (ARNT/HIF-1β) is a common binding partner for AhR as well as HIF-1α, and there is reciprocal cross-talk between hypoxia and AhR activation both *in vivo* and *in vitro* (20). In addition, HIF-1α has been implicated in the development of PAH (21). Under normoxic conditions, HIF-1α and HIF-2α are hydroxylated by prolyl hydroxylase (PHD) and complex with von Hippel-Lindau protein (VHL), causing subsequent proteasomal degradation. Under hypoxic conditions, PHD is inhibited and HIFα is stabilized and translocated to dimerize with HIF-1β in the nucleus. The heterodimer binds to the hypoxia response element, causing the expression of target genes. Factor-inhibiting HIF-1 (FIH-1) binds to HIF-1α and inhibits its transactivation function (22).

In light of these observations, we tested the hypothesis that (at least in part) the effects of Su in experimental PH may be due to activation of AhR and subsequent alterations in estrogen synthesis and CYP1A1 expression. We assessed the interactions among Su, AhR, and the HIF-1α pathway, and translated our findings to clinically relevant cells from patients with PAH.

Methods

An expanded Methods section is available in the data supplement.

Animal Studies

See the data supplement for ethical considerations and housing details.

The rat model of Su/Hx is described in detail in the data supplement. Briefly, adult female Wistar rats were given a single subcutaneous injection of Su 20 mg/kg or 0.9% saline and exposed to hypoxia for 14 days, and then maintained in normoxia for 6 weeks. CH223191 (8 mg/kg/day; Tocris) was delivered to the animals in the final 2 weeks of the 6 weeks of normoxic exposure.

Hemodynamics

Heart rate, right ventricular systolic pressure (RVSP), systemic arterial pressure, and cardiac output were measured and analyzed as previously described (10, 13). See the data supplement for details.

RV Hypertrophy

RV hypertrophy (RVH) was assessed by weight measurements of the RV free wall and left ventricle plus septum (LV+S). The ratio was expressed as RV/(LV+S). See the data supplement for details.

Lung Histopathology

Pulmonary vascular remodeling was assessed as previously described (23, 24). First, 5-μm sagittal sections were obtained from the left lungs. Sections were stained with elastin/Picrosirius red and microscopically assessed in a blinded fashion. Pulmonary arteries (25–100 μm external diameter) were counted. The arteries were considered muscularized if they possessed a distinct double-elastic lamina that was visible for at least half the diameter in the vessel cross-section. The percentage of vessels that contained double-elastic lamina was calculated as the number of muscularized vessels/total number of vessels counted × 100.

hPASMCs and Proliferation Studies

Briefly, distal hPASMCs and human blood outgrowth endothelial cells (BOECs) derived from female patients with PAH were prepared and characterized as previously described (25, 26). See Table E1 in the data supplement for details regarding the patients. Proliferation studies were performed in charcoal-stripped media using manual cell counting and a Countess II FL Automated Cell Counter (Thermo Fisher Scientific) with 0.4% Trypan blue exclusion for assessment of viability. See the data supplement for further details.

hPASMCs and Proliferation Studies in Hypoxia

hPASMCs were maintained in charcoal-stripped media in hypoxic conditions for 48 hours in a hypoxia incubator chamber (1%; Bilrups Rothenburg). Proliferation studies were performed using manual cell counting. See the data supplement for further details.

AhR Translocation Studies

The REAP (Rapid, Efficient And Practical) method was applied to determine AhR protein translocation between the cytoplasm and nuclear fractions in hPASMCs and BOECs. See the data supplement for details.

Protein Analysis

Protein expression in whole lungs and hPASMCs was assessed by immunoblotting as described previously (13, 16) and in the data supplement.

qRT-PCR

mRNA expression was assessed in the lungs of rats and mice by qRT-PCR as described previously (13, 16) and in the data supplement.

Estrogen Immunoassay

The levels of 17β-estradiol (E2) were determined by competitive immunoassay in lung samples and plasma from female rats from each group. See the data supplement for details.

Apoptosis

Apoptosis assays were performed under normoxic conditions between passages 5 and 8 in human pulmonary microvascular ECs (PMECs; Promocell) from donors without PAH. See the data supplement for details.

Results

AhR and CYP1A1 Expression in Su/Hx Male and Female Rat Lungs

As discussed in the Introduction, we previously showed that AhR expression is increased in hPASMCs from patients with PAH and may be increased in small occluded arteries from Su/Hx rats (10). We therefore examined the protein and mRNA expression of AhR and CYP1A1 in the lungs from Su/Hx rats. Because there is sexual dimorphism in PAH, we compared male and female lungs to determine any sex differences in the expression of AhR or CYP1A1. Figure 1 demonstrates that total AhR was equally expressed in the lungs of control and Su/Hx male and female rats at both the protein and mRNA levels (Figures 1A, 1B, and 1E). CYP1A1 expression was the most sensitive marker of AhR activation, and Figure 1C shows that in Su/Hx male and female rat lungs, CYP1A1 mRNA expression was increased

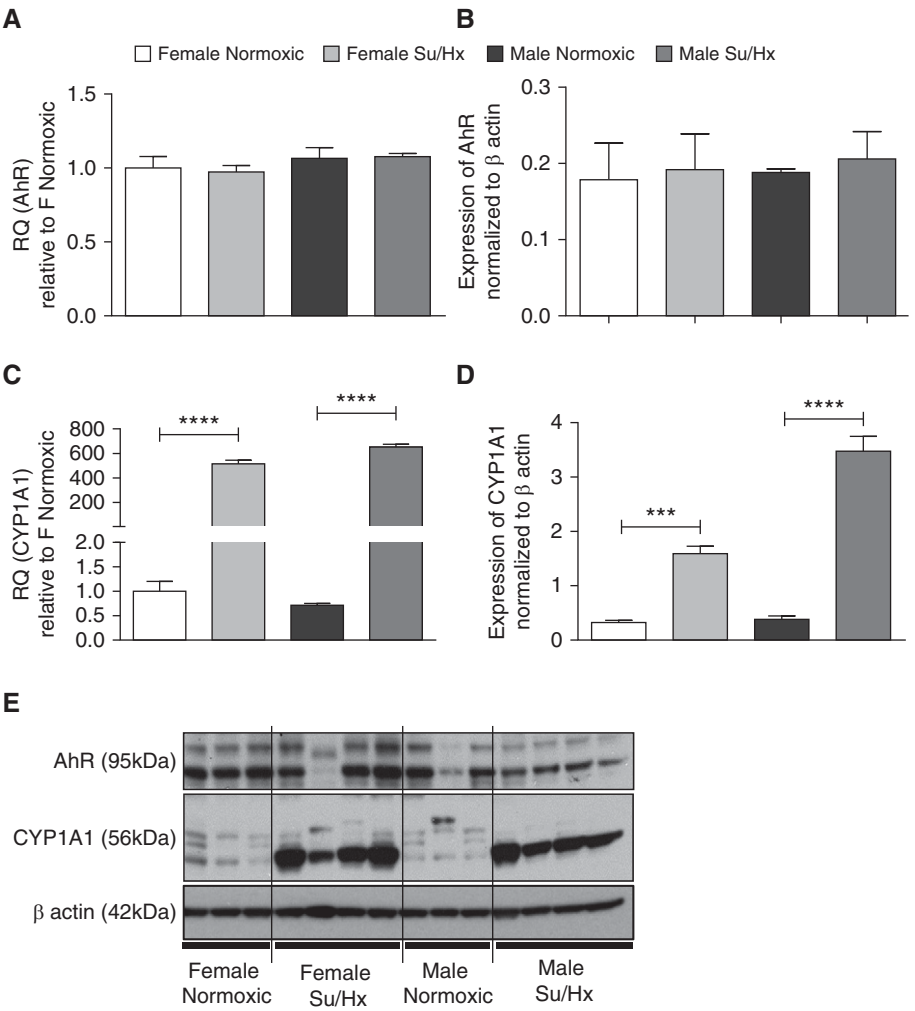

Figure 1. Expression of aryl hydrocarbon receptor (AhR) and CYP1A1 in lung tissue from the Sugen/hypoxic (Su/Hx) animal model. Expression of AhR mRNA (*A*) and protein (*B*), and expression of CYP1A1 mRNA (*C*) and protein (*D*) in whole lungs from male and female Su/Hx rats and their normoxic littermates (n = 3–4 per group, repeated in triplicate) are shown. (*E*) Representative immunoblots of AhR and CYP1A1 protein expression in whole lungs from male and female Su/Hx rats and their normoxic littermates. Vertical lines have been added to clarify the experimental groups. Data are displayed as mean ± SEM. $^{***}P < 0.001$, $^{****}P < 0.0001$ determined by one-way ANOVA with Tukey's *post hoc* test. CYP1A1 = cytochrome P450 enzyme 1A1; RQ = relative quantity.

some 400- to 600-fold (largely because CYP1A1 normally is not constitutively expressed [27]). This also resulted in an increase in the protein expression of CYP1A1 (Figures 1D and 1E).

To determine whether AhR activation was specific to the Su/Hx model, we investigated CYP1A1 expression in lungs from models that were either not exposed to Su or did not require hypoxia to induce the PH phenotype. We chose to examine hypoxic mice and the normoxic Smad1 knockout mouse model (28). In these models, lung CYP1A1 expression was actually reduced (Figures E1A and E1B), suggesting that Su is required for increased CYP1A1, and hypoxia alone does not increase CYP1A1.

Effect of an AhR Antagonist on the Development of PH in the Su/Hx Rat

Having determined that CYP1A1 is expressed in the Su/Hx rat lung, we sought to determine whether inhibition of AhR *in vivo* would reverse established PH in this model. The AhR antagonist CH223191 reversed the increase in RVSP, RVH, and pulmonary vascular remodeling in the

Su/Hx rats (Figures 2A–2D). CH223191 had no effect on systemic arterial pressure, heart rate, or cardiac output (Figure E2). LV weights did not change in the different treatment groups.

Effect of an AhR Antagonist on AhR, ARNT, and CYP1A1 Expression in the Su/Hx Rat

We confirmed that total AhR protein expression was not altered in whole lungs from Su/Hx rats, and found that CH223191 had no effect on total AhR lung expression (Figures 3A and 3D). CH223191 reduced the increase in CYP1A1 expression observed in the Su/Hx rats (Figures 3B and 3D). ARNT expression was elevated in the Su/Hx rats and normalized by CH223191 (Figures 3C and 3D). We assessed the expression of AhR and CYP1A1 by immunohistochemistry to determine localization in the pulmonary arteries. CYP1A1 under normoxic conditions was expressed mainly in the endothelium and the adventitial layers. In the Su/Hx rats, CYP1A1 expression was increased in line with the increase in vascular smooth muscle (Figure 3E). AhR expression was located in the medial layer, and whereas total lung AhR expression did not increase in the Su/Hx rat, AhR staining was clearly evident in all of the remodeled vascular smooth muscle cells of small pulmonary arteries from the Su/Hx rats (Figure 3F).

Effect of Sugen on HIF-1α

We characterized the role of HIF-1α in hPASMCs. As a positive control, immunofluorescence demonstrated that 2-hour, but not 24-hour, stimulation with the PHD inhibitor $CoCl_2$ caused a significant increase in the stabilization of HIF-1α (Figures E3A–E3D). This was confirmed by immunoblotting in hPASMCs, where HIF-1α was observed in both the cytoplasmic and nuclear compartments of the cells after 2 hours of treatment with $CoCl_2$ (Figure E3E). Interestingly, Su treatment caused a significant reduction in both cytoplasmic and nuclear HIF-1α expression in hPASMCs as measured by immunofluorescence (Figures E4A–E4C). This reduction in HIF-1α by Su was confirmed by immunoblotting (Figures E4D and E4E). Su had no effect on the protein expression of other regulatory components of the HIF-1α pathway, such as PHD2, VHL, and FIH-1 (Figures E4F–E4I).

Effect of an AhR Antagonist on Aromatase Expression and Estrogen in the Su/Hx Rat Lung

Using the same protocols employed in this study, we previously showed that aromatase protein expression can be increased in lungs from Su/Hx rats (10). Here, we show that this was reversed after treatment with CH223191 (Figures 4A and 4B). We measured E2 levels in these lungs and found that they were elevated in the Su/Hx rat lungs and normalized by CH223191 (Figure 4C).

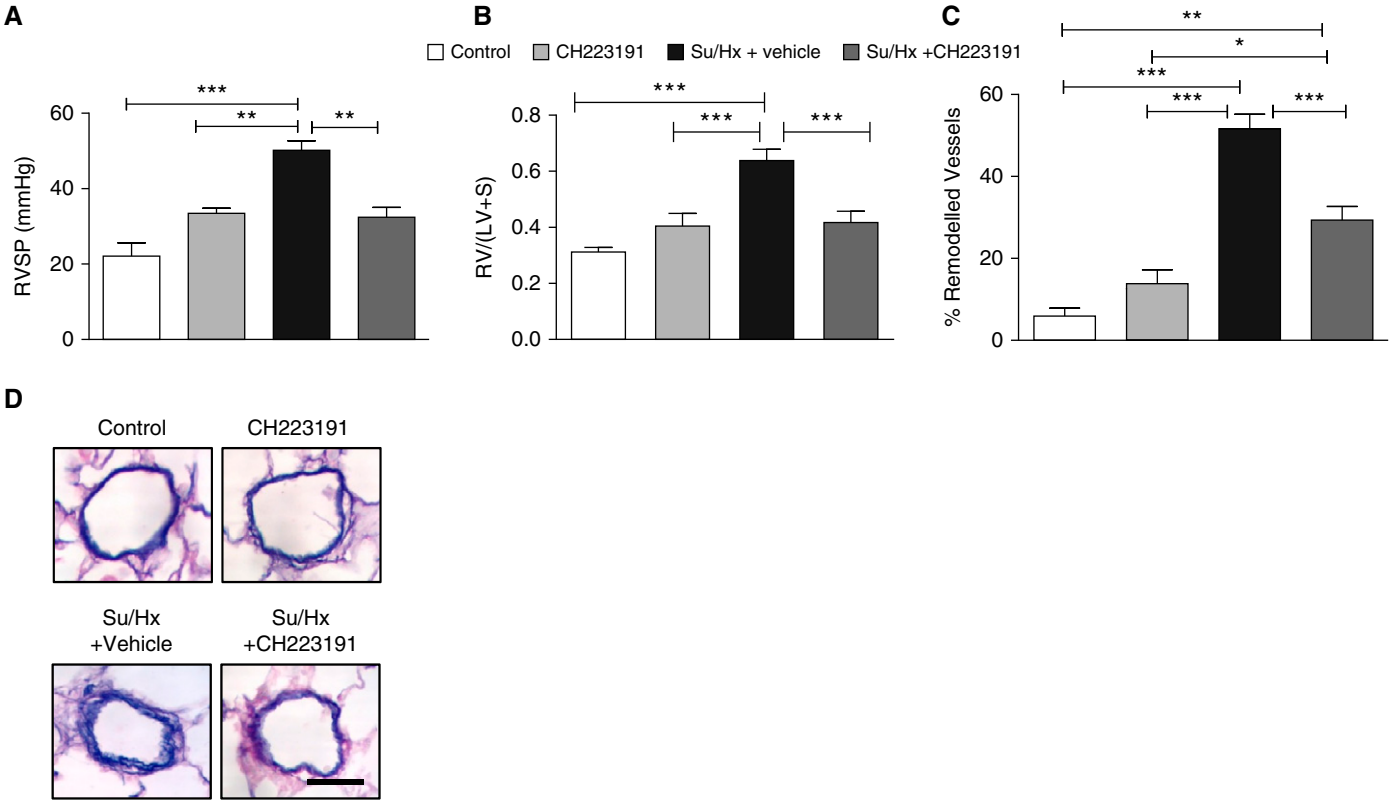

Figure 2. Effect of the AhR antagonist CH223191 on Su/Hx pulmonary hypertension in female rats. (*A*) Right ventricular systolic pressure (RVSP; n = 5–6 per group), (*B*) right ventricular hypertrophy (RV/[LV+S]; n = 8 per group), and (*C*) the percentage of remodeled arteries in lungs without treatment (control) or with CH223191 alone, Su/Hx treatment with vehicle, or CH223191; n = 5–6. (*D*) Representative images showing elastic laminae stained with elastin/Picrosirius red. Scale bar: 20 μm. Data represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as indicated, determined by one-way ANOVA followed by Bonferroni's *post hoc* test.

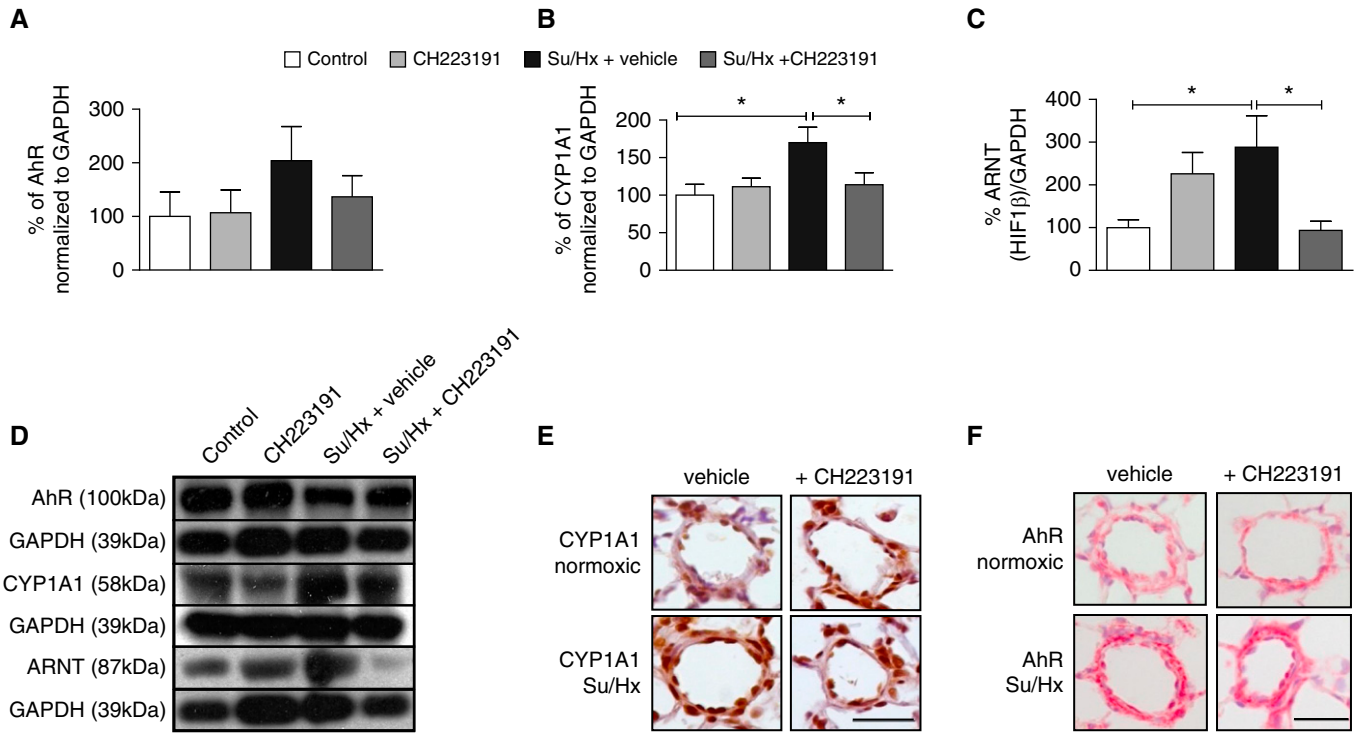

Figure 3. Effect of the AhR antagonist CH223191 on the protein expression of AhR, CYP1A1, and AhR nuclear translocator (ARNT) in female rat lungs. (*A*) AhR expression (n = 4), (*B*) CYP1A1 expression, and (*C*) ARNT expression, with (*D*) representative immunoblots (n = 4–6). (*E*) Representative CYP1A1 immunostaining in pulmonary arteries from rats. Scale bar: 50 μm. (*F*) Representative AhR immunostaining in pulmonary arteries from rats. Scale bar: 50 μm. Data are shown as mean ± SEM. *$P < 0.05$ as indicated, determined by one-way ANOVA followed by Bonferroni's *post hoc* test. HIF = hypoxia-inducible factor.

Effect of Su on the AhR/CYP1A1 Axis in hPASMCs

To examine the possibility that Su may activate AhR in hPASMCs, we examined the effects of Su on AhR and CYP1A1 expression in hPASMCs. Neither 1 nor 5 μM of Su affected the total protein expression of AhR (Figures 5A and 5B). However, indicative of activation of AhR, Su increased expression of CYP1A1 (Figures 5C and 5D). During activation, AhR is translocated into the nucleus; therefore, the total expression of AhR may not change. We therefore investigated whether Su (1 μM) could activate AhR and increase its translocation into the nucleus, and demonstrated that by 90 minutes there was an increase in translocation of AhR from the cytoplasmic to the nuclear fraction (Figure 5E).

Effect of Su on hPASMC Proliferation in Normoxia and Hypoxia

We investigated the combined effects of hypoxia and Su in hPASMCs. Su on its own did not cause significant proliferation of hPASMCs (Figure 6A). When grown in a hypoxic environment, however, Su caused significant hPASMC proliferation (Figure 6B). We demonstrated that serum starvation caused apoptosis/reduction in

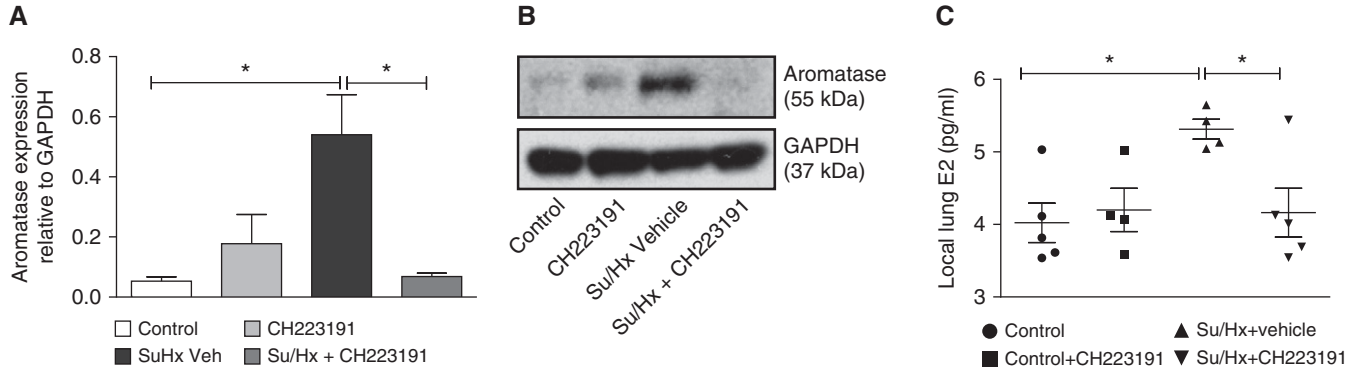

Figure 4. Effect of an AhR antagonist on aromatase expression and estrogen in Su/Hx rat lungs. (*A*) Aromatase expression in female rat lungs (n = 3) and (*B*) representative immunoblots. (*C*) Local estrogen levels in female rat lungs (n = 4–5). Data are shown as mean ± SEM. *$P < 0.05$ as indicated, determined by one-way ANOVA followed by Bonferroni's *post hoc* test. veh = vehicle.

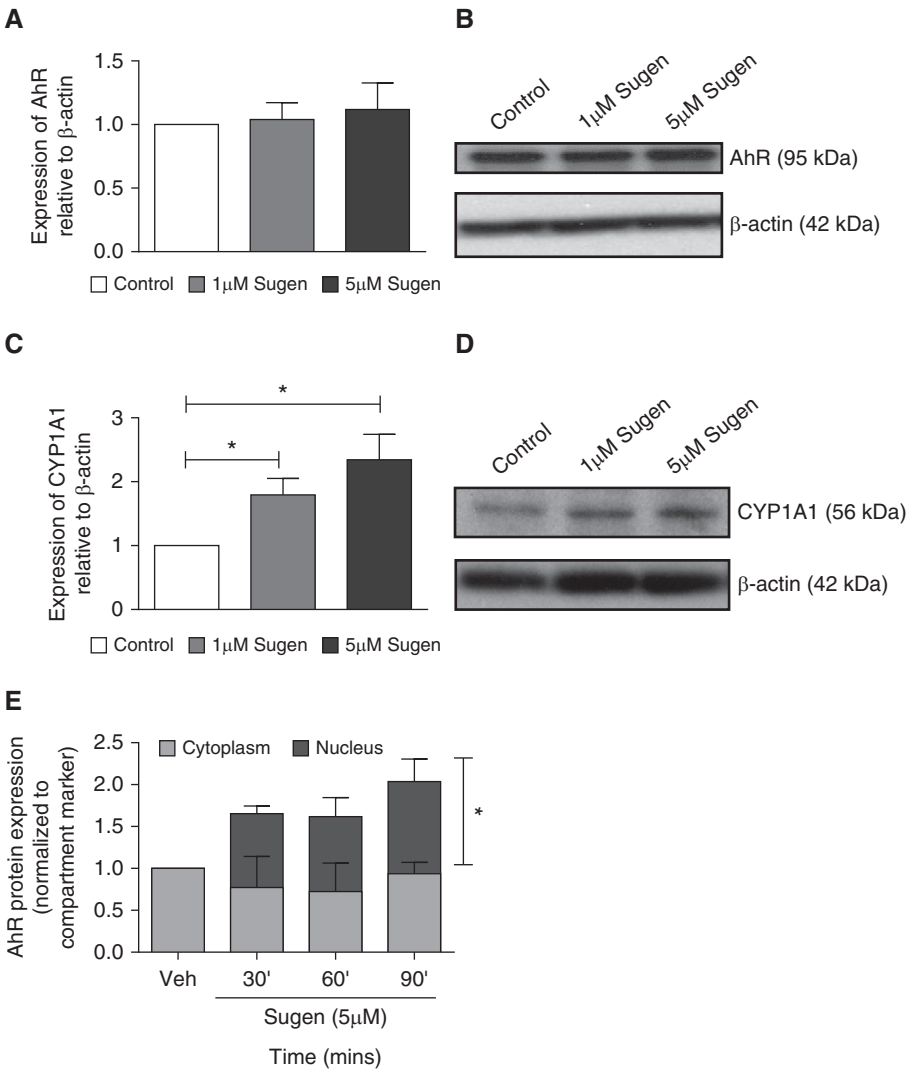

Figure 5. Effect of Sugen on AhR and CYP1A1 expression in hPASMCs. (*A* and *B*) AhR and (*C* and *D*) CYP1A1 protein levels in PASMCs from female patients with PAH stimulated with 1 or 5 μM of Sugen 5416 for 24 hours (n = 3–4 different cell lines). (*B* and *D*) Representative western blots, with irrelevant lanes removed on the right-hand side. (*E*) Sugen caused nuclear translocation of AhR after 30, 60, and 90 minutes (n = 3 for all groups, $^*P < 0.05$ as indicated, determined by area under the curve). AhR protein expression was normalized to α-tubulin and nucleoporin as markers for cytosolic and nuclear enrichment, respectively. Data are displayed as mean ± SEM. $^*P < 0.05$ in *C* as indicated, determined by one-way ANOVA followed by Bonferroni's *post hoc* test. hPASMCs = human pulmonary artery smooth muscle cells; PAH = pulmonary arterial hypertension.

cell numbers, as did the positive control resveratrol (Figure 6C). Both Su (1 μM) and the AhR agonist FICZ (5,11-dihydro-indolo[3,2-b]carbazole-6-carboxaldehyde, 6-formylindolo[3,2-b]carbazole; 50 nM) also caused apoptosis, as demonstrated by reduced cell numbers (Figure 6C).

Effect of Su on BOECs

We wished to investigate the effect of Su in BOECs derived from female patients with PAH. Su (1 μM) increased the proliferation of BOECs (Figure 7A), and this was attenuated in the presence of the AhR antagonist CH223191 (Figure 7A). We demonstrated, however, that both Su and CH223191 reduced BOEC viability as assessed by Trypan blue exclusion (Figure 7B). To examine the possibility that Su may activate AhR in BOECs, we examined the effects of Su (1 μM) on AhR cytosolic-to-nuclear translocation in BOECs. We observed that after 60 minutes of stimulation with Su, there was a decrease in cytoplasmic AhR, whereas nuclear AhR expression was unchanged (Figure 7C).

Discussion

Here, we demonstrate that the long-term effects of Su in experimental PH may be due in part to its agonist effects on AhR and subsequent alterations in estrogen synthesis and CYP1A1 expression. Many compounds affect AhR activity, including xenobiotics, drugs, flavonoids, indoles, and tryptophan metabolites (7). Importantly, the lungs are exposed to many AhR activators in airborne particulate matter. Indeed, exposure to diesel exhaust can increase pulmonary vascular tone at high cardiac output (29). Functionally, AhR has been shown to play a critical role in vascular development, angiogenesis, and cancer (30, 31). Unligated AhR is cytoplasmic, forming a complex with heat shock protein 90 (HSP90) and the cochaperones p23 and protein X-associated protein 2 (XAP2). Upon ligand binding, the phosphorylation of two protein kinase C sites leads to nuclear translocation of AhR. The AhR-chaperone complex dissociates and forms a heterodimer with ARNT (HIF-1β) in the nucleus. ARNT binds both AhR and HIF-1α, and is shared between the two signaling pathways (20). This heterodimer binds to dioxin-responsive element (DRE), leading to the transcription of several genes, including CYP1A1 (7).

Metabolism of E2 is mediated by several cytochrome P450 (CYP) enzymes, including CYP1A1 and CYP1B1. CYP1B1 expression in pulmonary arteries is elevated in experimental and clinical PAH, and may influence the development of PAH (16). A SNP of *CYP1B1* (and increased 16α-hydroxyestrone [16α-OHE1] in urine) has been associated with RV function in female patients with PAH and could underpin the sexual dimorphism observed in RV failure (17). Indeed, other investigators and we have recently demonstrated that dysfunctional E2 synthesis and metabolism may be involved in the increased prevalence of PAH among women (12, 13, 16, 17, 28, 32, 33).

Su is an inhibitor of both VEGFR2 and VEGFR1, but it has the highest affinity at VEGFR2. It can induce lung cell apoptosis

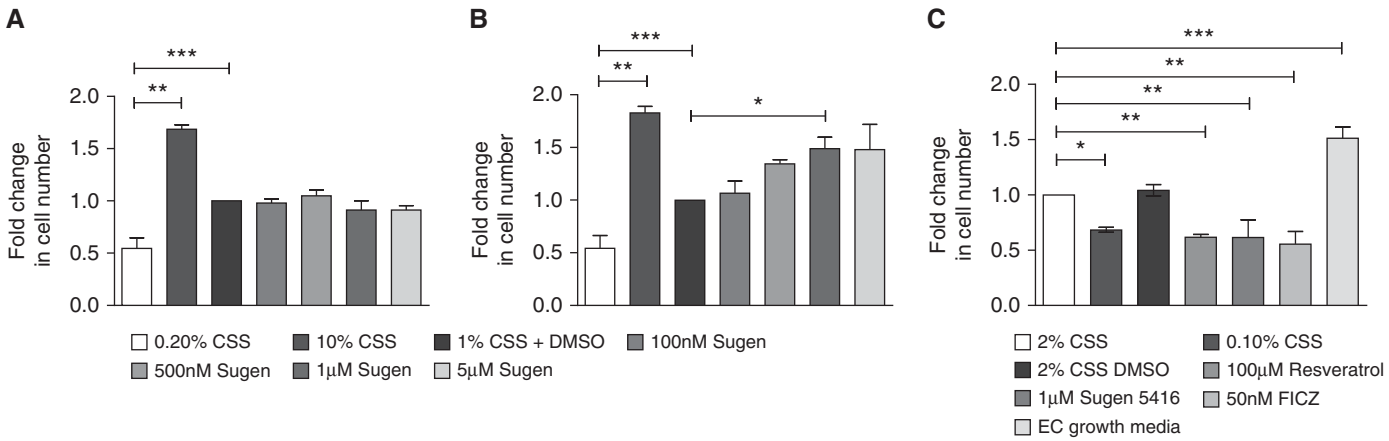

Figure 6. Sugen stimulates the proliferation of PASMCs from female patients with PAH under hypoxia but not under normoxia. Stimulation with Sugen had no effect on proliferation in female hPASMCs in normoxia (*A*); however, in hypoxia, 1 μM of Sugen 5416 induced cell proliferation (*B*); $n = 4$, repeated three times. (*C*) Stimulation with 1 μM of Sugen 5416 and the AhR agonist FICZ induced a decrease in the number of pulmonary microvascular endothelial cells. Resveratrol (100 μM) was used as the positive control, and endothelial cell growth media served as the negative control for apoptosis; $n = 4$, repeated three times. Data are displayed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.001$ as indicated, determined by one-way ANOVA followed by Bonferroni's *post hoc* test. CSS = charcoal stripped serum; EC = endothelial cell; FICZ = 5,11-dihydro-indolo[3,2-b]carbazole-6-carboxaldehyde, 6-formylindolo[3,2-b]carbazole.

and emphysema (34). When combined with chronic hypoxia in rats, Su causes PH and right heart failure, and in some animals it can induce obliterative vascular lesions (3). However, the mechanism by which the combined Su/Hx insult causes experimental PH is still unclear. It has been suggested that endogenous VEGFR inhibitors such as VEGF 165b, sVEGFR1 (s-Flt1), Decorin, TNFSF15, and CXCL4 may influence the development of PAH (35).

As Su is a ligand for AhR (6), we investigated the hypothesis that activation of AhR may underpin Su/Hx experimental PH. As CYP1A1 gene expression is the most sensitive marker for AhR activation, we first examined the expression of AhR and CYP1A1 protein and mRNA in lungs removed from rats with PH induced by Su combined with hypoxia (3). The degree of experimental PH induced in this model as reported in the literature is extremely variable: it can depend on the strain of rat, the sex of the rat, the protocol used, and the source of the Sugen (36). Not all studies have reported obliterative vascular lesions in this model, and in this study we saw too few of these lesions to analyze. However, our degree of experimental PH in terms of RVSP, RVH, and remodeling was commensurate with other studies using female rats (37). AhR was expressed at low levels in whole lungs of normoxic and Su/Hx male and female rats. It was expected that total AhR expression would not change, because AhR activation normally follows translocation from the cytoplasm to the nucleus rather than an increase in expression. Activation of AhR was confirmed by the marked increase in CYP1A1 mRNA in lungs from Su/Hx rats, with a 600-fold increase in CYP1A1 being observed, given that CYP1A1 normally is not constitutively active and depends on AhR for its activation (7). There were also increased levels of CYP1A1 protein in the lungs from Su/Hx rats. To determine whether Su mediated the change in CYP1A1, we examined CYP1A1 mRNA expression in hypoxic rat lungs and demonstrated that CYP1A1 expression was decreased when rats were exposed to hypoxia alone. This is consistent with the observation that hypoxia can inhibit AhR signaling and CYP1A1 expression in certain cell lines (38, 39). This suggests that the increase in CYP1A1 in the Su/Hx rats was mediated by Su. To investigate this further and assess whether AhR is a potential new target for the treatment of PAH, we examined the effects of the AhR antagonist CH223191 (40) in Su/Hx rats. We chose to study female Su/Hx rats because we previously showed that AhR was increased in the lungs of female Wistar Kyoto, Su/Hx rats (10), and we have also demonstrated the importance of endogenous E2 for the development of PH in female Su/Hx rats (13). We adopted CH223191 as a selective and potent AhR inhibitor with no reported off-target effects that would influence our results (40, 41). The results demonstrated that RVSP, RVH, and pulmonary vascular remodeling were all markedly increased in the Su/Hx rats. CH223191 had no effect on systemic pressure, heart rate, or cardiac output. The experimental PH was accompanied by increased expression of CYP1A1 and ARNT, which was normalized by the AhR antagonist. These results suggest that Su/Hx-induced PH is associated with AhR activation of CYP1A1, as well as increased expression of ARNT/HIF-1β, providing a mechanism of cross-talk between AhR and hypoxia. Su decreased HIF-1α expression in hPASMCs while having no effect on the nuclear translocation of HIF-1α or affecting other aspects of HIF-1α signaling. Although Lei and colleagues observed increased HIF-1α in lungs from patients with PAH (21), others have reported a decreased expression of HIF-1α in hPASMCs from such patients and suggested that this may underlie the increased pulmonary vascular contraction observed in PH (42).

Using immunohistochemistry, we determined that CYP1A1 was mainly expressed in the endothelium in normoxic rat pulmonary arteries. This is consistent with the notion that the endothelium serves as the first line of defense, via AhR, between the arteries and circulating vasoactive/harmful substances. However, CYP1A1 expression was also observed in the medial layer of small pulmonary arteries from the Su/Hx rats, suggesting an effect of Su on CYP1A1 expression in PASMCs from these rats.

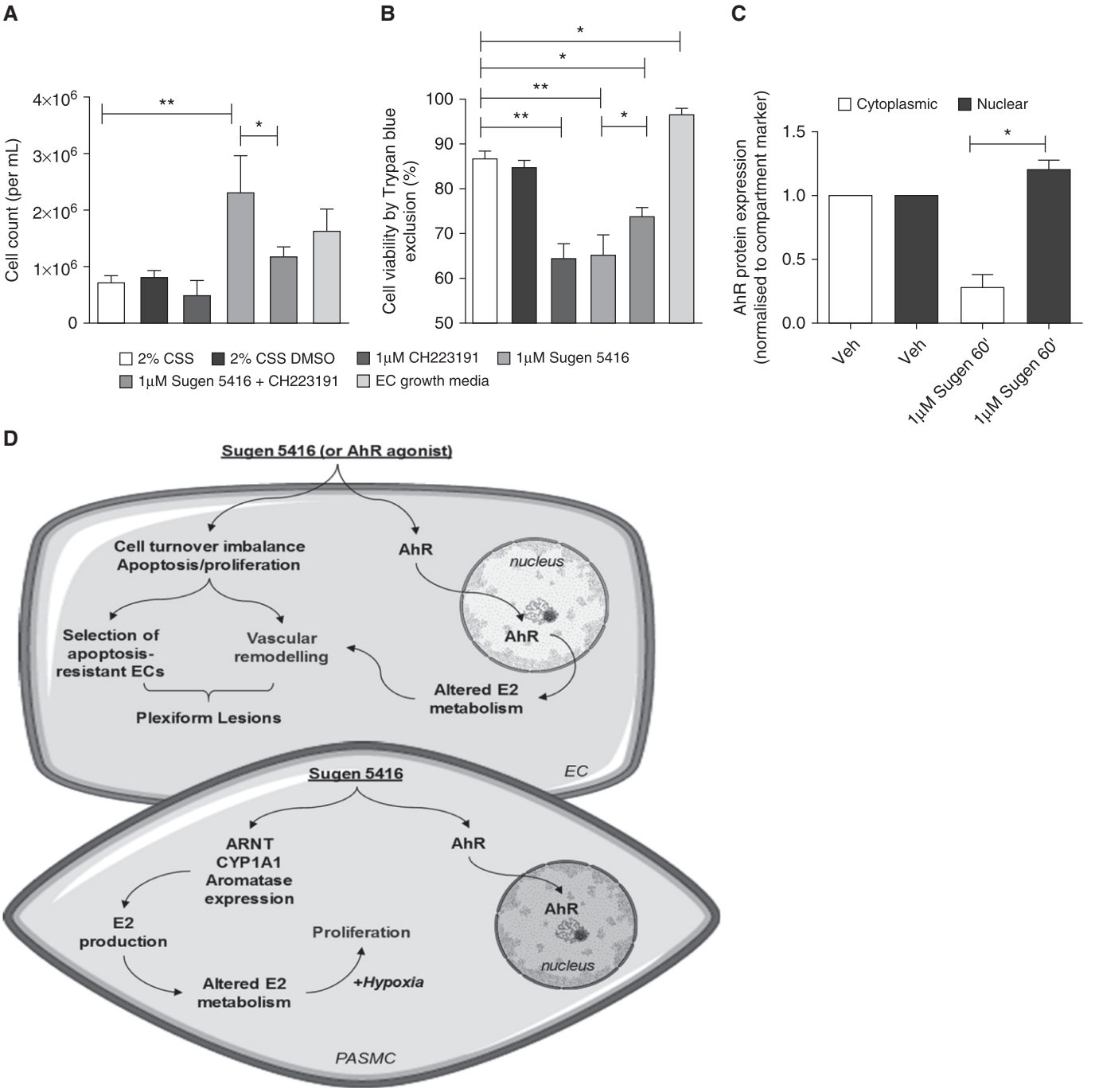

Figure 7. Sugen stimulates the proliferation of blood outgrowth endothelial cells (BOECs) from female patients with PAH. Stimulation with Sugen increased proliferation in BOECs (*A*); however, both the AhR antagonist CH223191 (1 μM) and Sugen 5416 (1 μM) reduced cell viability in BOECs by Trypan blue exclusion (*B*), $n = 3$, repeated three times. Data are displayed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ as indicated, determined by one-way ANOVA followed by Bonferroni's *post hoc* test. (*C*) Sugen caused nuclear translocation of AhR after 60 minutes. AhR protein expression was normalized to α-tubulin and nucleoporin as markers for cytosolic and nuclear enrichment, respectively. The data are displayed as mean ± SEM. $^{*}P < 0.05$ as indicated, determined by area under the curve. (*D*) Our data suggest that Su may activate AhR nuclear translocation and subsequent activation of CYP1A1, apoptosis, and aromatase expression. The resulting increase in E2 synthesis and metabolism may contribute to experimental PH. We also demonstrate directly that Su and hypoxia synergize, perhaps via ARNT, to cause hPASMC proliferation, suggesting that inhibition of AhR is a potential approach to the treatment of PAH.

Aromatase (CYP19A1) is a member of the cytochrome P450 superfamily and synthesizes E2 through the aromatization of androgens, specifically testosterone and androstenedione. In a recent study, we demonstrated that PASMCs express aromatase, and that aromatase expression is increased in pulmonary arteries from patients with PAH (13). We have also shown that inhibition of aromatase with anastrozole had a therapeutic effect in animal models of PH (including the Su/Hx rat). In addition, anastrozole has been shown to be clinically effective in PAH (12). It was previously shown that AhR can induce the CYP19 (aromatase) gene (19). We confirmed that aromatase protein expression was increased in the lungs from Su/Hx rats and that this was normalized by the AhR antagonist. Consistent with this, we also observed an increase in E2 in the lungs from Su/Hx rats, which was also normalized by the AhR antagonist. Interestingly, the increase in lung E2 appears to be variable among species and studies; for example, in a previous study in which we induced PH in Wistar Kyoto rats, there was no increase in lung E2 despite increased aromatase expression (10). It is possible that given different experimental animals at different times of the year, there is a variability in the metabolism of E2 in the lung that introduces variability into the absolute E2 levels. We need to consider this as a potential limitation when interpreting these studies in animal models.

These studies support the hypothesis that Su can regulate AhR and CYP1A1 in the lung, and this contributes to experimental PH. To examine whether this translates to humans, we examined the effects of Su in hPASMCs, human pulmonary ECs, and BOECs derived from female patients with PAH. Su had no effect on total AhR expression in hPASMCs. However, this might not be expected, as AhR is activated by translocation from the cytoplasm to the nucleus. We therefore examined the effects of Su on AhR protein levels in the cytoplasm and the nucleus, and found that Su caused an increase in nuclear AhR expression in hPASMCs. However, in BOECs, the cytoplasmic AhR expression decreased and the nuclear expression remained constant. The regulation of subcellular AhR localization is complex and dynamic, involving mechanisms for the retention and stabilization of AhR in the cytosol via XAP2 and continuous nuclear export. Also, binding of ligand can increase the rate of nuclear AhR import without stopping nuclear AhR export (43). Consistent with this activation of AhR, Su increased CYP1A1 protein expression in the hPASMCs. E2 can be converted to 2-hydroxyestradiol (2-OHE2) by CYP1A1/2, CYP1B1, and CYP3A4. CYP1A1 also metabolizes estrone (E1) and E2 to 2-OHE2 and 16α-OHE1. These metabolites are mitogenic in hPASMCs and may contribute to the development of PAH (16, 44). Unfortunately, at this time we are still developing accurate methods for measuring low concentrations of E2 metabolites from cells, so we were unable to measure these directly. The effects of AhR on E2 synthesis and metabolism are of interest because major PAH registries report a greater incidence of PAH among women than among men (45), and E2 metabolism is implicated in the increased penetrance of heritable PAH among female patients harboring a mutation in the gene encoding bone morphogenetic protein receptor II (BMPRII) (18).

Su requires a "second hit" of hypoxia followed by a period of normoxia to induce experimental PH, although this can be strain dependent (37). We assessed whether hypoxia could influence the effect of Su in hPASMCs by determining proliferation while culturing cells in either normoxia or hypoxia. We found that Su did not induce proliferation in normoxic cells, but did in hypoxic cells. This demonstrates a synergy between Su and hypoxia in hPASMCs. Given that ARNT protein levels are normalized by the AhR antagonist, it is possible that hypoxia synergizes with AhR activation via ARNT.

It is believed that EC apoptosis may initiate vascular remodeling in experimental PAH. This could cause degeneration of precapillary arterioles or select apoptosis-resistant ECs that contribute to "angioproliferative" plexiform lesions (46). As Su can induce apoptosis, we studied this in human pulmonary ECs, and found that both Su and an AhR agonist could induce apoptosis in these cells. Apoptosis may also be involved in the pulmonary vascular remodelling by AhR. ECs are more subject to contact inhibition in intact arteries than hPASMCs and normally do not proliferate. However, it has been shown that BOECs from patients with PAH can exhibit increased proliferation (47). Indeed, it has suggested that dysregulated endothelial proliferation plays a key role in the development of clinical PAH (48). We demonstrate that Su can induce the proliferation of BOECs from patients with PAH, and such proliferation could precede and contribute to the ability of Su to increase the development of occlusive lesions in some animals. The slight decrease in cell viability caused by Su is likely a consequence of contact inhibition occurring in these cultures.

In summary, our data provide new insights into potential mechanisms behind the Su/Hx model. The results suggest that Su may activate AhR nuclear translocation and subsequent activation of CYP1A1, apoptosis, and aromatase expression. The resulting increase in E2 synthesis and metabolism, as well as apoptosis, may contribute to experimental PH. We also demonstrate directly that Su and hypoxia synergize, perhaps via ARNT, to cause hPASMC proliferation. Our study also suggests that inhibition of AhR may be a novel approach to the treatment of PAH should these results translate to the human situation. This is summarized in Figure 7. ■

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

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References

1. Humbert M, Morrell NW, Archer SL, Stenmark KR, MacLean MR, Lang IM, *et al*. Cellular and molecular pathobiology of pulmonary arterial hypertension. *J Am Coll Cardiol* 2004;43(12, Suppl S) 13S–24S.
2. Tuder RM, Yun JH. Vascular endothelial growth factor of the lung: friend or foe. *Curr Opin Pharmacol* 2008;8:255–260.
3. Taraseviciene-Stewart L, Kasahara Y, Alger L, Hirth P, McMahon G, Waltenberger J, *et al*. Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. *FASEB J* 2001;15:427–438.

4. Mendel DB, Schreck RE, West DC, Li G, Strawn LM, Tanciongco SS, *et al*. The angiogenesis inhibitor SU5416 has long-lasting effects on vascular endothelial growth factor receptor phosphorylation and function. *Clin Cancer Res* 2000;6:4848–4858.
5. Sukbuntherng J, Cropp G, Hannah A, Wagner GS, Shawver LK, Antonian L. Pharmacokinetics and interspecies scaling of a novel VEGF receptor inhibitor, SU5416. *J Pharm Pharmacol* 2001;53: 1629–1636.
6. Mezrich JD, Nguyen LP, Kennedy G, Nukaya M, Fechner JH, Zhang X, *et al*. SU5416, a VEGF receptor inhibitor and ligand of the AHR, represents a new alternative for immunomodulation. *PLoS One* 2012; 7:e44547.
7. Murray IA, Patterson AD, Perdew GH. Aryl hydrocarbon receptor ligands in cancer: friend and foe. *Nat Rev Cancer* 2014;14:801–814.
8. Xu CX, Wang C, Zhang ZM, Jaeger CD, Krager SL, Bottum KM, *et al*. Aryl hydrocarbon receptor deficiency protects mice from diet-induced adiposity and metabolic disorders through increased energy expenditure. *Int J Obes* 2015;39:1300–1309.
9. Sato S, Shirakawa H, Tomita S, Ohsaki Y, Haketa K, Tooi O, *et al*. Low-dose dioxins alter gene expression related to cholesterol biosynthesis, lipogenesis, and glucose metabolism through the aryl hydrocarbon receptor-mediated pathway in mouse liver. *Toxicol Appl Pharmacol* 2008;229:10–19.
10. Dean A, Nilsen M, Loughlin L, Salt IP, MacLean MR. Metformin reverses development of pulmonary hypertension via aromatase inhibition. *Hypertension* 2016;68:446–454.
11. Frost AE, Badesch DB, Barst RJ, Benza RL, Elliott CG, Farber HW, *et al*. The changing picture of patients with pulmonary arterial hypertension in the United States: how REVEAL differs from historic and non-US contemporary registries. *Chest* 2011;139:128–137.
12. Kawut SM, Archer-Chicko CL, DeMichele A, Fritz JS, Klinger JR, Ky B, *et al*. Anastrozole in pulmonary arterial hypertension. a randomized, double-blind, placebo-controlled trial. *Am J Respir Crit Care Med* 2017;195:360–368.
13. Mair KM, Wright AF, Duggan N, Rowlands DJ, Hussey MJ, Roberts S, *et al*. Sex-dependent influence of endogenous estrogen in pulmonary hypertension. *Am J Respir Crit Care Med* 2014;190:456–467.
14. Dempsie Y, MacRitchie NA, White K, Morecroft I, Wright AF, Nilsen M, *et al*. Dexfenfluramine and the oestrogen-metabolizing enzyme CYP1B1 in the development of pulmonary arterial hypertension. *Cardiovasc Res* 2013;99:24–34.
15. Johansen AK, Dean A, Morecroft I, Hood K, Nilsen M, Loughlin L, *et al*. The serotonin transporter promotes a pathological estrogen metabolic pathway in pulmonary hypertension via cytochrome P450 1B1. *Pulm Circ* 2016;6:82–92.
16. White K, Johansen AK, Nilsen M, Ciuclan L, Wallace E, Paton L, *et al*. Activity of the estrogen-metabolizing enzyme cytochrome P450 1B1 influences the development of pulmonary arterial hypertension. *Circulation* 2012;126:1087–1098.
17. Ventetuolo CE, Mitra N, Wan F, Manichaikul A, Barr RG, Johnson C, *et al*. Oestradiol metabolism and androgen receptor genotypes are associated with right ventricular function. *Eur Respir J* 2016;47: 553–563.
18. Austin ED, Cogan JD, West JD, Hedges LK, Hamid R, Dawson EP, *et al*. Alterations in oestrogen metabolism: implications for higher penetrance of familial pulmonary arterial hypertension in females. *Eur Respir J* 2009;34:1093–1099.
19. Baba T, Mimura J, Nakamura N, Harada N, Yamamoto M, Morohashi K, *et al*. Intrinsic function of the aryl hydrocarbon (dioxin) receptor as a key factor in female reproduction. *Mol Cell Biol* 2005;25: 10040–10051.
20. Chan WK, Yao G, Gu YZ, Bradfield CA. Cross-talk between the aryl hydrocarbon receptor and hypoxia inducible factor signaling pathways. Demonstration of competition and compensation. *J Biol Chem* 1999;274:12115–12123.
21. Lei W, He Y, Shui X, Li G, Yan G, Zhang Y, *et al*. Expression and analyses of the HIF-1 pathway in the lungs of humans with pulmonary arterial hypertension. *Mol Med Rep* 2016;14: 4383–4390.
22. Mahon PC, Hirota K, Semenza GL. FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev* 2001;15:2675–2686.
23. Keegan A, Morecroft I, Smillie D, Hicks MN, MacLean MR. Contribution of the 5-HT(1B) receptor to hypoxia-induced pulmonary hypertension: converging evidence using 5-HT(1B)-receptor knockout mice and the 5-HT(1B/1D)-receptor antagonist GR127935. *Circ Res* 2001;89: 1231–1239.
24. Leach E, Howard P, Barer GR. Resolution of hypoxic changes in the heart and pulmonary arterioles of rats during intermittent correction of hypoxia. *Clin Sci Mol Med* 1977;52:153–162.
25. Ormiston ML, Toshner MR, Kiskin FN, Huang CJ, Groves E, Morrell NW, *et al*. Generation and culture of blood outgrowth endothelial cells from human peripheral blood. *J Vis Exp* 2015;(106)e53384.
26. Toshner M, Dunmore BJ, McKinney EF, Southwood M, Caruso P, Upton PD, *et al*. Transcript analysis reveals a specific HOX signature associated with positional identity of human endothelial cells. *PLoS One* 2014;9:e91334.
27. Choudhary D, Jansson I, Schenkman JB, Sarfarazi M, Stoilov I. Comparative expression profiling of 40 mouse cytochrome P450 genes in embryonic and adult tissues. *Arch Biochem Biophys* 2003; 414:91–100.
28. Mair KM, Yang XD, Long L, White K, Wallace E, Ewart MA, *et al*. Sex affects bone morphogenetic protein type II receptor signaling in pulmonary artery smooth muscle cells. *Am J Respir Crit Care Med* 2015;191:693–703.
29. Wauters A, Vicenzi M, De Becker B, Riga JP, Esmaeilzadeh F, Faoro V, *et al*. At high cardiac output, diesel exhaust exposure increases pulmonary vascular resistance and decreases distensibility of pulmonary resistive vessels. *Am J Physiol Heart Circ Physiol* 2015; 309:H2137–H2144.
30. Dietrich C, Kaina B. The aryl hydrocarbon receptor (AhR) in the regulation of cell-cell contact and tumor growth. *Carcinogenesis* 2010;31:1319–1328.
31. Gomez-Duran A, Carvajal-Gonzalez JM, Mulero-Navarro S, Santiago-Josefat B, Puga A, Fernandez-Salguero PM. Fitting a xenobiotic receptor into cell homeostasis: how the dioxin receptor interacts with TGFbeta signaling. *Biochem Pharmacol* 2009;77: 700–712.
32. Ventetuolo CE, Baird GL, Barr RG, Bluemke DA, Fritz JS, Hill NS, *et al*. Higher estradiol and lower dehydroepiandrosterone-sulfate levels are associated with pulmonary arterial hypertension in men. *Am J Respir Crit Care Med* 2016;193:1168–1175.
33. Wallace E, Morrell NW, Yang XD, Long L, Stevens H, Nilsen M, *et al*. A sex-specific microRNA-96/5-hydroxytryptamine 1B axis influences development of pulmonary hypertension. *Am J Respir Crit Care Med* 2015;191:1432–1442.
34. Kasahara Y, Tuder RM, Taraseviciene-Stewart L, Le Cras TD, Abman S, Hirth PK, *et al*. Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J Clin Invest* 2000;106: 1311–1319.
35. Voelkel NF, Gomez-Arroyo J. The role of vascular endothelial growth factor in pulmonary arterial hypertension. The angiogenesis paradox. *Am J Respir Cell Mol Biol* 2014;51:474–484.
36. Jiang B, Deng Y, Suen C, Taha M, Chaudhary KR, Courtman DW, *et al*. Marked strain-specific differences in the SU5416 rat model of severe pulmonary arterial hypertension. *Am J Respir Cell Mol Biol* 2016;54: 461–468.
37. Lahm T, Frump AL, Albrecht ME, Fisher AJ, Cook TG, Jones TJ, *et al*. 17β-Estradiol mediates superior adaptation of right ventricular function to acute strenuous exercise in female rats with severe pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 2016; 311:L375–L388.
38. Fradette C, Bleau AM, Pichette V, Chauret N, Du Souich P. Hypoxia-induced down-regulation of CYP1A1/1A2 and up-regulation of CYP3A6 involves serum mediators. *Br J Pharmacol* 2002;137: 881–891.
39. Vorrink SU, Severson PL, Kulak MV, Futscher BW, Domann FE. Hypoxia perturbs aryl hydrocarbon receptor signaling and CYP1A1 expression induced by PCB 126 in human skin and liver-derived cell lines. *Toxicol Appl Pharmacol* 2014;274:408–416.
40. Zhao B, Degroot DE, Hayashi A, He G, Denison MS. CH223191 is a ligand-selective antagonist of the Ah (dioxin) receptor. *Toxicol Sci* 2010;117:393–403.

41. Kim SH, Henry EC, Kim DK, Kim YH, Shin KJ, Han MS, *et al*. Novel compound 2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-o-tolylazo-phenyl)-amide (CH-223191) prevents 2,3,7,8-TCDD-induced toxicity by antagonizing the aryl hydrocarbon receptor. *Mol Pharmacol* 2006;69:1871–1878.
42. Barnes EA, Chen CH, Sedan O, Cornfield DN. Loss of smooth muscle cell hypoxia inducible factor-1α underlies increased vascular contractility in pulmonary hypertension. *FASEB J* 2017;31: 650–662.
43. Richter CA, Tillitt DE, Hannink M. Regulation of subcellular localization of the aryl hydrocarbon receptor (AhR). *Arch Biochem Biophys* 2001; 389:207–217.
44. Hood KY, Montezano AC, Harvey AP, Nilsen M, MacLean MR, Touyz RM. Nicotinamide adenine dinucleotide phosphate oxidase-mediated redox signaling and vascular remodeling by 16α-hydroxyestrone in human pulmonary artery cells: implications in pulmonary arterial hypertension. *Hypertension* 2016;68:796–808.
45. Shapiro S, Traiger GL, Turner M, McGoon MD, Wason P, Barst RJ. Sex differences in the diagnosis, treatment, and outcome of patients with pulmonary arterial hypertension enrolled in the registry to evaluate early and long-term pulmonary arterial hypertension disease management. *Chest* 2012;141: 363–373.
46. Jurasz P, Courtman D, Babaie S, Stewart DJ. Role of apoptosis in pulmonary hypertension: from experimental models to clinical trials. *Pharmacol Ther* 2010;126:1–8.
47. Toshner M, Voswinckel R, Southwood M, Al-Lamki R, Howard LS, Marchesan D, *et al*. Evidence of dysfunction of endothelial progenitors in pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2009;180:780–787.
48. Tuder RM, Groves B, Badesch DB, Voelkel NF. Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension. *Am J Pathol* 1994;144: 275–285.