Role of the aryl hydrocarbon receptor in Sugen 5416-induced experimental pulmonary hypertension

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Clinical Relevance: The Sugen 5416/hypoxic rat model is a commonly used model of pulmonary hypertension. Sugen is a vascular endothelial growth factor (VEGF) 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 cytoplasm to nucleus in human pulmonary artery smooth muscle cells (hPASMCs). This is accompanied by an increase in CYP1A1 and aromatase expression and an increase in estrogen synthesis. We show that sugen causes proliferation of blood outgrowth endothelial cells from PAH patients but only causes proliferation of hPASMCs when grown in hypoxic conditions. Sugen can also cause apoptosis in human microvascular pulmonary endothelial cells. Inhibition of AhR can reverse

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sugen/hypoxic experimental PH and may be a novel approach to the treatment of PH.

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Key words: pulmonary hypertension, VEGF, Sugen, hypoxia, aryl hydrocarbon receptor, estrogen

Abstract

Rationale: Rats dosed with the vascular endothelial growth factor (VEGF) inhibitor Sugen 5416 (Su), placed in hypoxia then restored to normoxia has become a widely used model of pulmonary arterial hypertension (PAH). The mechanism by which Su exaccerbates pulmonary hypertension is, however, unclear. **Objectives**: We investigated Su-activation of the aryl hydrocarbon receptor (AhR) in patient human pulmonary arterial smooth muscle cells (hPASMCs) and patient blood outgrowth endothelial cells (BOECs). We also examined the effect of AhR on aromatase and estrogen levels in the lung. **Methods, Measurements and Main Results:** Protein and mRNA analysis demonstrated that CYP1A1 was very highly induced in the lungs of Su/hypoxic (Su/Hx) rats. The AhR antagonist CH223191 (8mg/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 HIF1α expression in hPASMCs. Su induced proliferation in BOECs and increased apoptosis in human pulmonary microvascular endothelial cells (hPMECs) and also induced translocation of AhR to the nucleus in hPASMCs. Under normoxic conditions, hPASMCs do not proliferate to Su. However when grown in hypoxia (1%) Su induced hPASMC proliferation. **Conclusion:** In combination with hypoxia, Su is proliferative in patient hPASMCs and patient BOECs and Su/Hx-induced PAH in rats may be facilitated by AhR-induced CYP1A1, ARNT and aromatase. Inhibition of the AhR receptor may be a novel approach to the treatment of pulmonary hypertension.

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Introduction

Pulmonary arterial hypertension (PAH) is a progressive disease leading to right heart failure. This condition is defined by vascular remodelling 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 anti-angiogenic effects and its role in PAH is still unclear (2). Curiously, one administration of Sugen 5416 (Su), a 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. This 'Su/Hypoxic' (Su/Hx) rat model develops 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 still however, unclear but clarification of this would have important translational value in understanding clinical PAH.

Su has been studied as a potential therapy for advance malignancies. Curiously it has been observed that it has a long lasting inhibitory effect in animal tumor models despite it having a very short half-life of around 30 minutes; it is believed the long-lasting effects are due to it accumulating in cells (4, 5). Su is an agonist at the cytoplasmic aryl hydrocarbon receptor (AhR) which is cytoplasmic when unligated (6). The AhR is a member of the basic helix–loop–helix/Per–ARNT–Sim family of heterodimeric transcriptional regulators highly expressed in the lung (7) and influences 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 PAH patients and that AhR expression may be increased in small occluded pulmonary arteries from the Su/Hx rat model (10). PAH occurs up to four-fold more frequently in women (11) and dysfunctional estrogen synthesis and metabolism may play an important role in the pathobiology of pulmonary hypertension, both clinically and experimentally (10, 12-18). The AhR is a major regulator of the estrogen metabolising enzyme CYP1A1 and AhR 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 PAH patients is of interest.

To understand how hypoxia may synergise with the effects of Su on the AhR we investigated possible interactions between Su, AhR and hypoxia-inducible factor-1α (HIF-1α) signaling. This is of interest as the AhR nuclear translocator (ARNT/ HIF-1β) is a common binding partner for the AhR as well as HIF-1α and there is reciprocal crosstalk 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 (HRE) causing 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 test the hypothesis that (at least in part) the effects of Su in experimental pulmonary hypertension may be due to activation of AhR and subsequent alterations in estrogen synthesis and CYP1A1 expression. We

assess the interactions between Su, AhR and the HIF-1α pathway and translate our findings to clinically relevant cells from patients with PAH.

Materials and Methods

An expanded methods section is available in the online supplement.

Animal studies

See online supplement for ethical considerations and housing details.

The rat model of Su/Hx is described in detail in the online supplement. Briefly, adult female Wistar rats were given a single injection of Su 20mg/kg (s.c.) or 0.9% (s.c.) saline and exposed to hypoxia for 14 days then retained in normoxia for six weeks. CH223191 (8 mg/kg/day, Tocris, UK) was delivered to the animals in the final two weeks of the six 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 online supplement for details.

Right Ventricular Hypertrophy

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

Lung Histopathology

Pulmonary vascular remodelling was assessed as previously described (23, 24). 5μm sagittal sections were obtained from left lungs. Sections were stained with Elastin/Picrosirius red and microscopically assessed in a blinded fashion. Pulmonary arteries (25 to 100 μm external diameter) were counted. The arteries were

considered muscularized if they possessed a distinct double-elastic lamina visible for at least half the diameter in the vessel cross-section. The percentage of vessels containing double-elastic lamina was calculated as number of muscularized vessels/total number of vessels counted×100.

Human Pulmonary Arterial Cells and proliferation studies

See online supplement for details. Briefly, distal hPASMCs derived from female patients with PAH and human blood outgrowth endothelial cells (BOECs) were prepared from female patients and characterized as previously described (25, 26). See online supplement Table 1 for PASMCs and BOECs patient details. Proliferation studies were carried out in charcoal-stripped media using manual cell counting and Countess II FL Automated Cell Counter (Thermo Fisher Scientific, UK) with 0.4% Trypan blue exclusion for assessment of viability.

Human Pulmonary Arterial Cells and proliferation studies in hypoxia

See online supplement for details. hPASMCs were maintained in charcoal-stripped media in hypoxic conditions for 48 hours in a hypoxia incubator chamber (1%; Bilrups Rothenburg, US). Proliferation studies were carried out using manual cell counting.

AhR translocation studies

The REAP method was applied to determine AhR protein translocation between the cytoplasm and nuclear fractions in hPASMCs and BOECs. See online supplement for details.

Protein Analysis

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

qRT-PCR

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

Estrogen Immunoassay

The levels of E2 were determined by competitive immunoassay in lung samples and plasma from female rats from each group. See online supplement for details.

Apoptosis

Apoptosis assays were performed under normoxic conditions between passages 5 and 8 in human pulmonary microvascular endothelial cells (PMECs) (Promocell, UK) from non-PAH donors. See online supplement for details.

Results

AhR and CYP1A1 expression in Su/Hx male and female rat lungs

As discussed in the Introduction, we have previously shown that AhR expression is increased in human pulmonary artery smooth muscle cells (hPASMCs) from PAH patients 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. As there is sexual dimorphism in PAH we compared male and female lungs to determine any sex differences in expression of AhR or CYP1A1. Figure 1 demonstrates that total AhR is equally expressed in the lungs of control and Su/Hx male and female rats at both protein and mRNA level (Figure 1A, B and E). CYP1A1 expression is the most sensitive marker of AhR activation and Figure 1C shows that, in Su/Hx male and female rat lungs, CYP1A1 mRNA expression is increased some 400-600 fold (largely due to the fact that CYP1A1 is not normally constitutively expressed (27)). This resulted in an increase in protein expression of CYP1A1 also (Figure 1D, E).

To determine if AhR activation was specific to the Su/Hx model therefore, we investigated CYP1A1 expression in the lungs from models which were either not exposed to Su or did not require hypoxia to induce the pulmonary hypertensive

phenotype. We chose to examine hypoxic mice and the normoxic Smad1 knockout mouse model (28). In these models, lung CYP1A1 expression was actually reduced (Figure E1, A and B) suggesting 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 wish to determine if 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 remodelling in the Su/Hx rats (Figure 2 A-D). 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 show that CH223191 had no effect on total AhR lung expression (Figure 3A, D). CH223191 reduced the increase in CYP1A1 expression observed in the Su/Hx rats (Figure 3B, D). ARNT expression was elevated in the Su/Hx rats and normalized by CH223191 (Figure 3C, D). We determined 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 whilst total lung AhR expression did not increase in the Su/Hx rat, AhR staining was clearly evident in all the remodelled

vascular smooth muscle cells of small pulmonary arteries from the Su/Hx rats (Figure 3F).

Effect of sugen on HIF1α

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 stabilisation of HIF-1α (Figure E3A-D). This was confirmed by immunoblotting in hPASMCs where HIF-1α was observed in both the cytoplasmic and nuclear compartments of the cell after 2 hours treatment with $CoCl_2$ (Figure E3E). Interestingly, Su treatment caused a significant reduction in both cytoplasmic and nuclear HIF-1α expression hPASMCs as measured by immunoflorescence (Figure E4A-C). This reduction in HIF-1α by Su was confirmed by immunoblotting (Figure E4D and E). Su had no effect on the protein expression of other regulatory components of the HIF-1α pathway such as PHD2, VHL or Factor inhibiting hypoxia inducible factor 1 (FIH1) (Figure E4F-I).

Effect of an AhR antagonist on aromatase expression and estrogen in the Su/Hx rat lung

We have previously shown that aromatase protein expression can be increased in the lungs from Su/Hx rats using the protocols used in this study (10). Here we show this was reversed following treatment with CH223191 (Figure 4A, B). We measured E2 levels in these lungs and found these to be elevated in the Su/Hx rat lung and normalized by CH223191 (Figure 4C).

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 Su affected total protein expression of AhR (Figure 5A, B). However, indicative of

activation of AhR, Su increased expression of CYP1A1 (Figure 5C, D). During activation, AhR is translocated into the nucleus, therefore total AhR expression may not change. We therefore investigated if 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 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 cell number as did the positive control resveratrol (Figure 6C). Both Su (1 μM) and the AhR agonist FICZ (50 nM) also caused apoptosis as demonstrated by reduced cell number (Figure 6C).

Effect of Su on BOECs

We wished to investigate the effect of Su in BOECs derived from female PAH patients. Su (1μM) increased proliferation of BOECs (Figure 7A) and this was attenuated in the presence of AhR antagonist, CH223191 (Figure 7A). We demonstrated however, that both Su and CH223191 reduced BOEC viability assessed by trypan blue exclusion (Figure 7B). To examine the possibility that Su may activate AhR in BOECs, we examined the effects of Su (1μM) 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 while nuclear AhR expression was unchanged (Figure 7C).

Discussion

Here we demonstrate that the long-term effects of Su in experimental pulmonary hypertension may be due in part to its agonist effects on the AhR and subsequent alterations in estrogen synthesis and CYP1A1 expression. We translated our findings in cells from patients with PAH. Many compounds affect AhR activity including xenobiotics, drugs, flavonoids, indoles and tryptophan metabolites (7). Importantly the lung is 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 co-chaperones p23 and protein X-associated protein-2 (XAP2). Upon ligand binding 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 signalling pathways (20).This heterodimer binds to dioxin-responsive element (DRE), leading to transcription of several genes, including CYP1A1 (7).

Metabolism of 17β-estradiol (E2) is mediated by several cytochrome P450 enzymes (CYP) 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 single-nucleotide polymorphism (SNP) of *CYP1B1* (and increased 16α-OHE1 in urine) has been associated with right ventricular (RV) function in female PAH patients and could underpin the sexual dimorphism in RV failure (17). Indeed, we and others have recently demonstrated that dysfunctional E2 synthesis and metabolism may be involved in the increased prevalence of PAH in women (12, 13, 16, 28, 32-34).

Su is an inhibitor of both the VEGFR2 and VEGFR1 but has highest affinity at VEGFR2. It can induce lung cell apoptosis and emphysema (35). 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 (36).

As Su is a ligand for AhR (6) we have 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 firstly examined 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 reported in the literature is extremely variable. It can depend on the strain of rat, the sex of the rat, the protocol as well as the source of Sugen (37). Not all studies report obliterative vascular lesions in this model and in this study we saw too few of these to analyse. However, our degree of experimental PH in terms of RVSP, RVH and remodelling was commensurate with other studies using female rats (38). 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 as 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 the Su/Hx rats with a 600-fold increase in CYP1A1 being observed, due to the fact that CYP1A1 is not normally constitutively active and dependent on AhR for its activation (7). There were also increased levels of CYP1A1 protein in the lungs from the Su/Hx rats. To determine if

Su mediated the change in CYP1A1, we examined CYP1A1 mRNA expression in hypoxic rat lung 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 signalling and CYP1A1 expression in certain cell lines (39, 40). This suggests that the increase in CYP1A1 in the Su/Hx rats was mediated by Su. To investigate this further and whether AhR is a potential new target for the treatment of PAH, we examined the effects of the AhR antagonist CH223191 (41) in the Su/Hx rat. We chose to study female Su/Hx rats as we previously showed that (in female Wistar Kyoto rats) that AhR was increased in the lungs of female Su/Hx rats (10) and we have also demonstrated the importance of endogenous E2 to the development of PH in the female Su/Hx rat (13). We adopted CH223191 as a selective and potent AhR inhibitor with no reported off-target effects that would influence our results (42, 43). The results demonstrated that RVSP, RVH and pulmonary vascular remodelling were all markedly increased in the Su/Hx rats. There was no effect of CH223191 on systemic pressures, heart rate or cardiac output. The experimental PH was accompanied by increase 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 the AhR and hypoxia. Su decreased HIF-1α expression in hPASMCs whilst having no effect on nuclear translocation of HIF-1α or affecting other aspects of HIF-1α signalling. Whilst increased HIF-1α has been observed in lungs from patients with PAH (21), others have also reported a decreased expression of HIF-1α in hPASMCs from patients and suggested this may underlie increased pulmonary vascular contraction (44).

By immunohistochemistry we determined that CYP1A1 was mainly expressed in the endothelium in the normoxic rat pulmonary arteries. This is consistent with the endothelium being the first line of defence, via the 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.

Aromatase (CYP19A1) is a member of the cytochrome P450 superfamily and synthesizes E2 through the aromatization of androgens, specifically testosterone and androstenedione. We have recently demonstrated that pulmonary artery smooth muscle cells express aromatase and that aromatase expression is increased in pulmonary arteries from PAH patients (13). We have shown previously that inhibition of aromatase with anastrozole has a therapeutic effect in animal models of PH (including the Su/Hx rat) (13). In addition, anastrozole has been shown to be clinically effective in PAH (12). It has previously been 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 the Su/Hx rats which was also normalized by the AhR antagonist. Interestingly the increase in lung E2 appears to be variable between species and studies; as in a previous study where 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 year there is variability in the metabolism of E2 in the lung which introduces variability in absolute E2 levels which

we need to consider as a potential limitation in the interpretation of 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 if this translates into humans, we examined the effect of Su in hPASMCs derived from females with PAH as well as human pulmonary endothelial cells and female PAH-derived BOECs. 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 effect of Su on AhR protein levels in the cytoplasm and the nucleus and showed that Su did cause an increase in nuclear AhR expression in hPASMCs. However, in BOECs, the cytoplasmic AhR expression decreased whilst the nuclear expression remained constant. Regulation of subcellular AhR localization is complex and dynamic involving mechanisms for 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 (45). Consistent with this activation of AhR, Su increased CYP1A1 protein expression in the hPASMCs. E2 can be converted 2-hydroxyestradiol (2-OHE2) by CYP1A1/2, CYP1B1 and CYP3A4. CYP1A1 also metabolizes estrone (E1) and E2 to 2-OHE2 and 16α-hydroxyestrone (16α-OHE1); these metabolites are mitogenic in hPASMCs and may contribute to the development of PAH (16, 46). 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 as major PAH registries report a greater incidence of PAH among women than men (47) and E2 metabolism is implicated in the increased penetrance of heritable PAH (HPAH) among female

patients harbouring 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 whilst culturing cells in either normoxia or hypoxia. We show that Su did not induce proliferation in normoxic cells but did in hypoxic cells. This demonstrates 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 thought that endothelial cell apoptosis may initiate vascular remodelling in experimental PAH. This could cause degeneration of pre-capillary arterioles or select apoptosis-resistant ECs that contribute to "angioproliferative" plexiform lesions (48). As Su can induce apoptosis we studied this in human pulmonary endothelial cells and showed that both Su and an AhR agonist could induce apoptosis in these cells. Apoptosis is providing a further potential role for the AhR in pulmonary vascular remodelling. Endothelial cells are more subject to contact inhibition in intact arteries than hPASMCs and do not normally proliferate. However it has been shown that BOECs from patients with PAH can exhibit increased proliferation (49). Indeed, it is considered that there is a key role for dysregulated endothelial proliferation in the development of clinical PAH (50). We demonstrate that Su can induce proliferation of BOECs from patients which could perhaps 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 to be a consequence of contact inhibition occurring in these cultures.

In summary, our data provides new insight 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, and 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 potential novel approach to the treatment of PAH should these results translate to the human situation. This is summarized in Figure 7.

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Figure legends

Figure 1. The expression of AhR and CYP1A1 in lung tissue of Sugen/Hypoxic (Su/Hx) animal model. The expression of AhR mRNA (A) and protein (B) and expression of CYP1A1 mRNA (C) and protein (D) in whole lung from male and female Su/Hx rats and their normoxic litter-mates ($n = 3–4$ per group, repeated in triplicate). Representative immunoblots of AhR and CYP1A1 protein expression in whole lung from male and female Su/Hx rats and their normoxic litter-mates (E). Vertical lines have been added to clarify experimental groups. Data displayed as mean ± SEM. ***$P < 0.001$, ****$P < 0.0001$ determined by one-way ANOVA with

Tukey's post-hoc test. AhR = Aryl hydrocarbon receptor; CYP1A1=Cytochrome P450 enzyme 1A1; CYP1B1=Cytochrome P450 enzyme 1B1; RQ = relative quantity.

Figure 2. Effect of the AhR antagonist CH223191 on Sugen/Hypoxic (Su/Hx) pulmonary hypertension in female rats. (A) Right ventricular systolic pressure (RVSP, n=5-6 per group), (B) Right ventricular hypertrophy (RVH, n=8 per group) and (C) percentage of remodelled arteries in lungs without treatment (control), 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 represents 20μm. Data represents mean ±SEM. * P<0.05, **P<0.01 ***P<0.001 as indicated, determined by one way ANOVA followed by Bonferroni post-hoc test.

Figure 3. Effect of the AhR antagonist CH223191 on protein expression of AhR, CYP1A1 and ARNT in female rat lung. AhR expression (n=4) (A), CYP1A1 expression (B) ARNT (C) expression with representative immunoblots (n=4-6) (D). C=Control, CH=CH223191, Su/Hx=Sugen/hypoxic rats, Su/Hx +CH= sugen/hypoxic rats treated with CH223191. Representative CYP1A1 immunostaining in pulmonary arteries from rats. Scale bar: 50μm. (E) Representative AhR immunostaining in pulmonary arteries from rats. Scale bar: 50μm (F). Data is shown as mean ±SEM. * P<0.05, as indicated, determined one way ANOVA followed by Bonferroni post-hoc test.

Figure 4. Effect of an AhR antagonist on aromatase expression and estrogen in the Su/Hx rat lung. Aromatase expression in female rat lung, (n=3) (A) and

representative immunoblot (B). Local estrogen levels within rat female lung (n=4-5). (C) C=Control, CH=CH223191, Su/Hx=Sugen/hypoxic rats, Su/Hx +CH= sugen/hypoxic rats treated with CH223191. Data is shown as mean ±SEM. * P<0.05 as indicated, determined one way ANOVA followed by Bonferroni post-hoc test.

Figure 5. Effect of Sugen on AhR and CYP1A1 expression in hPASMCs. AhR (A,B) and CYP1A1 (C,D) protein levels in PASMCs from female patients with PAH stimulated with 1 and 5μM SU5416 for 24 hours (n=3-4 different cell lines). Representative western blots (B,D) have had irrelevant lanes removed on the right hand side. Sugen caused nuclear translocation of aryl hydrocarbon receptor (AhR) after 30, 60 and 90 minutes (E) (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 is displayed as a mean ±SEM. * P<0.05 as indicated, determined by one way ANOVA followed by Bonferroni post-hoc test.

Figure 6. Sugen stimulates proliferation of female PAH patient PASMCs 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 Sugen 5416 induced cell proliferation (B), n=4, repeated 3 times. Stimulation with 1 μM Sugen 5416 and aryl hydrocarbon receptor (AhR) agonist FICZ induced a decrease in cell number of female pulmonary microvascular endothelial cells (C). 100 μM Resveratrol was used as positive control, while endothelial cell growth media served as negative control for apoptosis, n=4, repeated 3 times. Data is displayed as mean ±SEM. * P<0.05, **P<0.01 ***P<0.001 as indicated, determined one way ANOVA

671 followed by Bonferroni post-hoc test. FICZ=5,11-Dihydro-indolo[3,2-b]carbazole-6-
672 carboxaldehyde, 6-Formylindolo[3,2-b]carbazole.
673
674 **Figure 7.** Sugen stimulates proliferation of BOECs from female patients with PAH.
675 Stimulation with Sugen increased proliferation in female PAH-derived BOECs (A),
676 however, both aryl hydrocarbon receptor (AhR) antagonist 1 μM CH223191, and 1
677 μM Sugen 5416 reduced cell viability in BOECs by trypan blue exclusion (B), n=3,
678 repeated 3 times. Data is displayed as mean ±SEM. * P<0.05, **P<0.01 as indicated,
679 determined by one way ANOVA followed by Bonferroni post-hoc test. Sugen caused
680 nuclear translocation of AhR after 60 minutes (C) AhR protein expression was
681 normalized to α-tubulin and nucleoporin as markers for cytosolic and nuclear
682 enrichment, respectively. Data is displayed as a mean ±SEM. * P<0.05 as indicated,
683 determined by area under the curve. (D) Our data suggest that Su may activate AhR
684 nuclear translocation and subsequent activation of CYP1A1, apoptosis and
685 aromatase expression. The resulting increase in E2 synthesis and metabolism may
686 contribute to the experimental PH. We also demonstrate directly that Su and hypoxia
687 synergize, perhaps via ARNT, to cause hPASMC proliferation; suggesting inhibition
688 of AhR may be a potential new approach to the treatment of PAH. AhR, Aryl
689 Hydrocarbon Receptor; ARNT (HIF1β), Aryl Hydrocarbon Receptor Nuclear
690 Translocator; CYP1A1=Cytochrome P450 1A1; EC, Endothelial Cell; PAH,
691 Pulmonary Arterial Hypertension; PASMCs, Pulmonary Arterial Smooth Muscle Cell.
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Supplement text

Methods

Animal studies

Ethical information

All experimental procedures were carried out in accordance with the United Kingdom Animal Procedures Act (1986) and with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), and ethical approval was also granted by the University of Glasgow Ethics Committee. Experimental procedures using hPASMCs conform to the principles outlined in the Declaration of Helsinki. Experimental procedures using human pulmonary artery smooth muscle cells conform to the principles outlined in the Declaration of Helsinki. All non-PAH human lung biopsies were confirmed as macroscopically normal and collected from lung cancer patients undergoing pneumonectomy with no reported presence of PAH.

Housing details

Rodents were housed in a 12-hour light dark cycle with access to food and water ad libitum. Wistar female rats, aged 9 weeks, were purchased from Harlan, UK. The animals were housed in a 12-hour light-dark cycle with access to food and water ad libitum. Animals were housed together to promote synchronisation of their estrous cycles. In order to ensure animals were synchronising together, a blunt, shortened tip of a Pasteur pipette was placed at the vaginal orifice. One drop of PBS was gently expelled into the vagina and aspirated back before being transferred to a microscope slide. Smears were examined microscopically and classified as to the stage of the cycle.

In vivo Hemodynamic Measurements

Animals were anaesthetically induced in 3% (v/v) isoflurane and then maintained at 1.5-2% (v/v) isoflurane supplemented with a constant flow of 5% (v/v) oxygen. Right ventricular systolic pressure (RVSP) measurements were taken using a Polyimide

Mikro-Tip pressure volume catheter (ADI instruments SPR-869NR); 12.5cm effective length, with four electrodes and a pressure sensor centered between E2 and E3. The catheter was used as per the manufacturer's instructions and attached to corresponding software (LabChart Pro). This catheter was inserted into the jugular vein and guided into the right ventricle of the heart to measure RVSP. After RVSP was determined, the carotid artery was isolated and the same catheter used to determine mean systemic arterial pressures. This pressure- volume (PV) loop system also generated the cardiac output (CO) data. Blood was collected immediately in a heparinised syringe for plasma analysis.

Right ventricular hypertrophy and tissue harvest

Immediately following hemodynamic assessment, the heart and lungs were flushed with ice-cold PBS at a low pressure to clear peripheral blood cells. The right lung was excised for molecular analysis. The left lung was inflated with 10% (v/v) neutral buffered formalin (NBF) and left in NBF solution for 48 hours before paraffin processing and embedding for immunohistological analysis. Right ventricular hypertrophy (RVH) was assessed by the Fulton Index (dry weight of the right ventricle/ (dry weight of the left ventricle + septum). Animals assigned to hypoxic conditions were placed in a hypobaric chamber (atmospheric pressure 550 mbar) for two weeks and then placed in normal room pressure (1013 mbar) for a further six weeks. CH223191 (8mg/kg/day, Tocris, UK) dosing was administered to the animals by oral gavage in the final two weeks of being at room pressure (reversal study).

Charcoal-Stripped Fetal Bovine Serum

Fetal bovine serum (FBS; Sera Labs, UK) was charcoal-stripped twice to remove estrogens. Dextran-coated charcoal (1% (w/v), Sigma-Aldrich, UK) in FBS was agitated gently overnight at 4ºC. Samples were centrifuged at 1811 g at 4ºC for 30 minutes. The stripped serum was decanted and filtered through a 0.22µm filter.

Human pulmonary arterial cells and proliferation studies

Human PAH patient PASMCs and BOECs were provided by Professor Nicholas W. Morrell (University of Cambridge). We chose to study cells from female patients as

these were most readily available and reproducible, since most PAH patients are female. Female BOECs were cultured from the peripheral blood of three PAH patients according to previous studies (1). BOECs were used between passages 4 and 5. Patient characteristics are shown in Table 1. Female hPASMCs were explanted from the distal pulmonary microvasculature from subjects with PAH. Assays were performed between passages 5 and 8. Cells were seeded in 24-well plates (for cell proliferation) and 6 well plates (for protein, RNA or analysis) at a density of 10,000 cells per cm^2. Cells were grown to 50-60% confluency and then synchronized by serum-deprivation (0.2% (v/v) charcoal-stripped FBS) in phenol-red free DMEM (Invitrogen, UK) for 24 hours for all experiments. Proliferation studies were carried out in charcoal-stripped media using either manual cell counting or Countess II FL Automated Cell Counter (Thermo Fisher Scientific, UK) with 0.4% Trypan blue exclusion for assessment of viability.

Human pulmonary arterial cells and proliferation studies in hypoxia

Female hPASMCs (as above) between passages 5 and 8 were used to assess the effects of Sugen on proliferation in hypoxic conditions. Cells were seeded in 24-well plates at a density of 10,000 cells per cm^2. Cells were grown to 50-60% confluency and then synchronized by serum-deprivation (0.2% (v/v) charcoal-stripped FBS) in phenol-red free DMEM (Invitrogen, UK) for 24 hours for all experiments. Cells were maintained in charcoal-stripped media in hypoxic conditions (1% O_2, 5% CO_2) for 48 hours in hypoxia incubator chamber (Bilrups Rothenburg, US). Proliferation studies were carried out using either manual cell counting or Countess II FL Automated Cell Counter (Thermo Fisher Scientific, UK) with 0.4% Trypan blue exclusion for assessment of viability.

AhR translocation in hPASMCs

The REAP method was applied to determine AhR protein translocation between the cytoplasm and nuclear fractions in PAH patient hPASMCs and BOECs (2). Cells were grown in monolayer in 10 cm dishes, washed with ice cold PBS, collected in PBS, and centrifuged for 10 seconds at 10,000 rpm. Then the supernatant was removed, and the pellet was re-suspended in 900 µl of 0.1% (v/v) NP-40 (Abcam ab142227), triturated and 300 µl removed as whole cell lysate and added to 100 µl of

4x NUPAGE sample buffer. Remaining material (600 µl) was centrifuged as before, and 330 µl of supernatant was removed as the cytosolic fraction and added to 100 µl of 4x NU sample buffer (ThermoFisher Scientific, UK). The remaining supernatant was removed and pellet re-suspended in 1 ml of 0.1% NP-40 (in PBS, Sigma, UK) and centrifuged. The supernatant was discarded and the pellet re-suspended in 90 µl of NUPAGE sample buffer as the nuclear fraction. Nuclear and cytoplasmic fractions were confirmed by western blotting for nucleoporin/lamin A and alpha tubulin respectively

Protein Analysis

Protein expression was assessed by immunoblotting in whole lung and hPASMCs. Whole lung rat samples were homogenized and hPASMCs were lysed in ice-cold 1% (v/v) lauryl maltoside (Abcam, UK) solution in PBS. Protein concentrations were determined by nanodrop (ND-1000 spectrophotometer (Thermo Scientific, UK). 20µg of protein was loaded for hPASMCs and whole lung homogenates, for protein identification by SDS-PAGE and immunoblotting. Protein expression was assessed using the following antibodies: anti-AhR (ab84833 1:500), anti-CYP1A1 (Abcam ab79819 1:1000), anti-ARNT (Cell signalling 3361 1:1000), anti-aromatase (Abbexa, abx13974 1:200), anti-HIF-1α (BD, 610958) anti-PHD2 (Cell Signaling #4835 1:1000), anti-VHL (Cell Signaling #2738 1:1000) and FIH1 (Novus NB100-428 1:500) by overnight incubation at 4°C. Membranes were then incubated with appropriate HRP-conjugated secondary antibodies. Immunoblots were developed using Pierce™ ECL Western Blotting Substrate (Life Technologies) or EMD Millipore Immobilon™ Western Chemiluminescent HRP Substrate (ECL) (Fisher Scientific), and normalized to either GAPDH (Abcam UK, ab8264; 0.2µg/ml) or beta actin (Sigma, A5441). Densitometrical analysis was performed using TotalLab TL100 software.

Immunoflorescence

Cellular localization of HIF-1α in hPASMCs was assessed by immunoflorescence. Briefly, cells were grown on glass coverslips until 50-60% confluent. Stimulated cells were then washed in PBS and fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature. Cells were then permeabilized with 0.2% Triton X-100/PBS (Sigma, UK) and blocked with 2%BSA/PBS with 0.05% Tween-20 (Sigma, UK). HIF-

1α primary antibody (1:250, Novus Biologicals, NB100-134) was added to the coverslips and incubated overnight at 4ºC. Cells were then washed in PBS and incubated with secondary antibody for 1 hour at room temperature (Alexa goat anti-mouse 488, ThermoFisher Scientific, UK). After a further PBS wash, ProLong™ Diamond antifade mountant with DAPI (ThermoFisher Scientific, UK) was applied to the coverslips and mounted on glass slides for imaging. Images were acquired using an LSM-510 laser-scanning confocal microscope (Zeiss, Germany). Images were then analysed using ImageJ and mean pixel intensity was used to determine relative florescence within each cell relative to cellular area.

qRT-PCR

Total RNA was extracted using miRNeasy mini kit (Qiagen, Germany) according to the manufacturer's instructions. RNA concentration was determined using Nanodrop 1000 spectrophotometer (Thermo Scientific). Expression of mRNA of CYP1A1 (Rn00487218_m1, Mm00487218_m1) and AhR (Rn00565750_m1) was assessed by Taqman qRT-PCR. To obtain a fold change mRNA expression data was normalised to β_2 microglobulin (Rn00560865_m1, Mm00437762_m1).

Lung immunolocalization

Additional sections were stained using anti-AhR (Abcam ab153744 1:200), anti-CYP1A1 (Abcam, ab79819 1:100) antibodies. An anti-rabbit IgG secondary antibody was used for each primary antibody (Vector Laboratories ImmPress kit) and protein immuno-localization was visualized with the DAB substrate kit (Vector labs UK (sk-4600) for CYP1A1 and Vector Red substrate kit (sk-5100).

Estrogen Immunoassay

The levels of 17β-estradiol were determined by competitive immunoassay in lung samples from female rats from each group. 20µg of protein was loaded and assayed in duplicate as per the manufacturer's instructions (Demeditec, USA). The plate was read at a wavelength of 405nm for kinetic and end point measurements (SpectraMax M2 plate reader, Molecular Devices, California, USA).

Apoptosis

Apoptosis assays were performed between passages 5 and 8 in pulmonary microvascular endothelial cells (PMECs) (Promocell, UK). Cells were grown to ~90% confluence and then synchronized by serum-deprivation in serum-free basal phenol-red MV endothelial cell media (Promocell, UK) for 2 hours for all experiments. Cells were then stimulated with Sugen (Novartis, UK), 50 nM 5,11-Dihydroindolo[3,2-b]carbazole-6-carboxaldehyde (FICZ, Sigma, UK) and 100 µM Resveratrol (Sigma, UK), which served as positive control for apoptosis, for 24 hours in 2% charcoal-stripped basal EC media. Changes in cell number were assessed with Countess II FL Automated Cell Counter (ThermoFisher Scientific, UK).

Figure E1

CYP1A1 expression in the lungs of female and male hypoxic mice compared with their normoxic controls. n=4-5 (A). CYP1A1 expression in the lungs of female and male mice heterogeneous for the Smad1 gene (Smad1+/-) compared with their wildtype (WT) controls. n=4-6. Data displayed as mean ± SEM. *P < 0.05, **P < 0.01 determined by one-way ANOVA with Tukey's post-hoc test. CYP1A1=Cytochrome P450 enzyme 1A1; RQ = relative quantity; F=female; M=male; WT=wildtype.

Figure E2

Mean Systemic Arterial Pressure (mSAP), cardiac output and heart rate in female rats exposed to CH223191, with and without Su/Hx. mSAP (A), heart rate (B), cardiac output (C). Data was analysed by one way ANOVA followed by Bonferroni post test, n=6-8. Su/Hx=sugen/hypoxic.

Figure E3

HIF-1α protein (green) localization in the cytoplasm and nuclei of hPASMCs before and after treatment with 200 μM $CoCl_2$ at 2 hour and 24 hour time points (A–D). Nuclei were counterstained with DAPI (blue). Scale bar=50 μm. Relative HIF-1α protein expression in cytoplasmic vs nuclear after addition of 200 μM $CoCl_2$ (E). Lamin-A and GAPDH were used as loading controls for nuclear and cytoplasmic compartments, respectively. n=3-4 biological replicates. Data is displayed mean ± SEM. *P < 0.05, determined by one-way ANOVA with Tukey's post-hoc test.

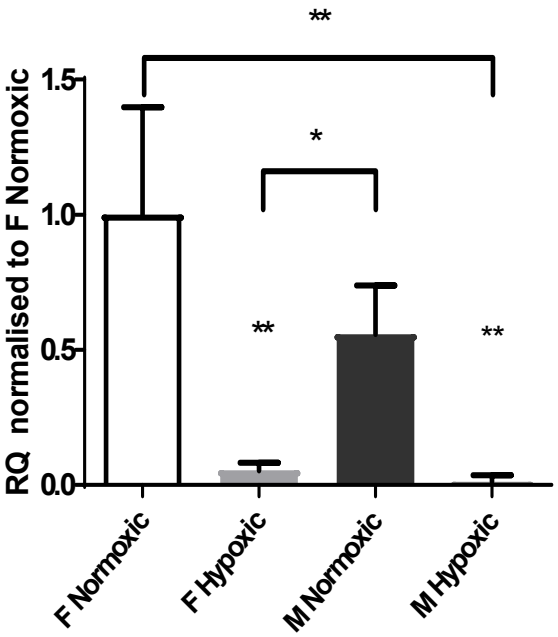
Figure E4

HIF-1α protein (green) localization in the cytoplasm and nuclei of hPASMCs before and after treatment with Sugen 5416 (5 μM) for 24 hours Nuclei were counterstained with DAPI (blue). Scale bar=50μm (A-C). Cytoplasmic and nuclear levels of HIF-1α protein before and after 24 hour treatment with Sugen 5416 (5 μM). Lamin-A and GAPDH were used as loading controls for nuclear and cytoplasmic compartments, respectively (D) and representative immunoblots (E). Whole cell protein levels of VHL (F), PHD2 (G), FIH1 (H) and representative immunoblots (I). n=3-4 biological

replicates. Data is displayed mean ± SEM. *P < 0.05, **P<0.01, ***P<0.001 determined by one-way ANOVA with Tukey's post-hoc test.

Figure E1

A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RQ normalised to F Normoxic
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0.0
F Normoxic
F Hypoxic
M Normoxic
M Hypoxic

B

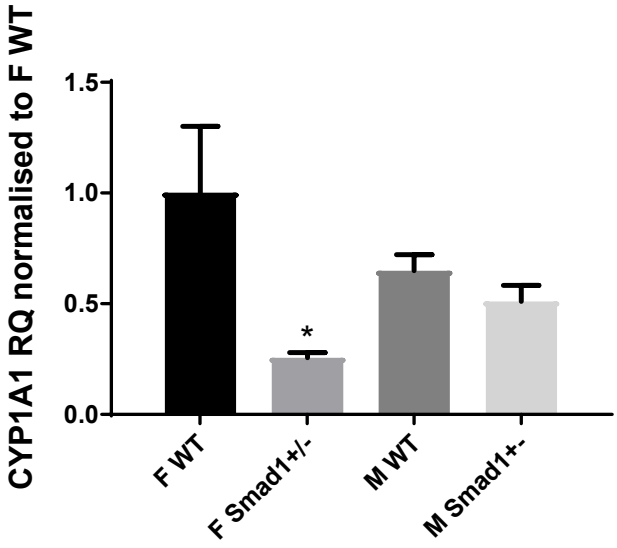

CYP1A1 RQ normalised to F WT
1.5
1.0
0.5
0.0
*
F WT
F Smad1+/-
M WT
M Smad1+-

Figure E2

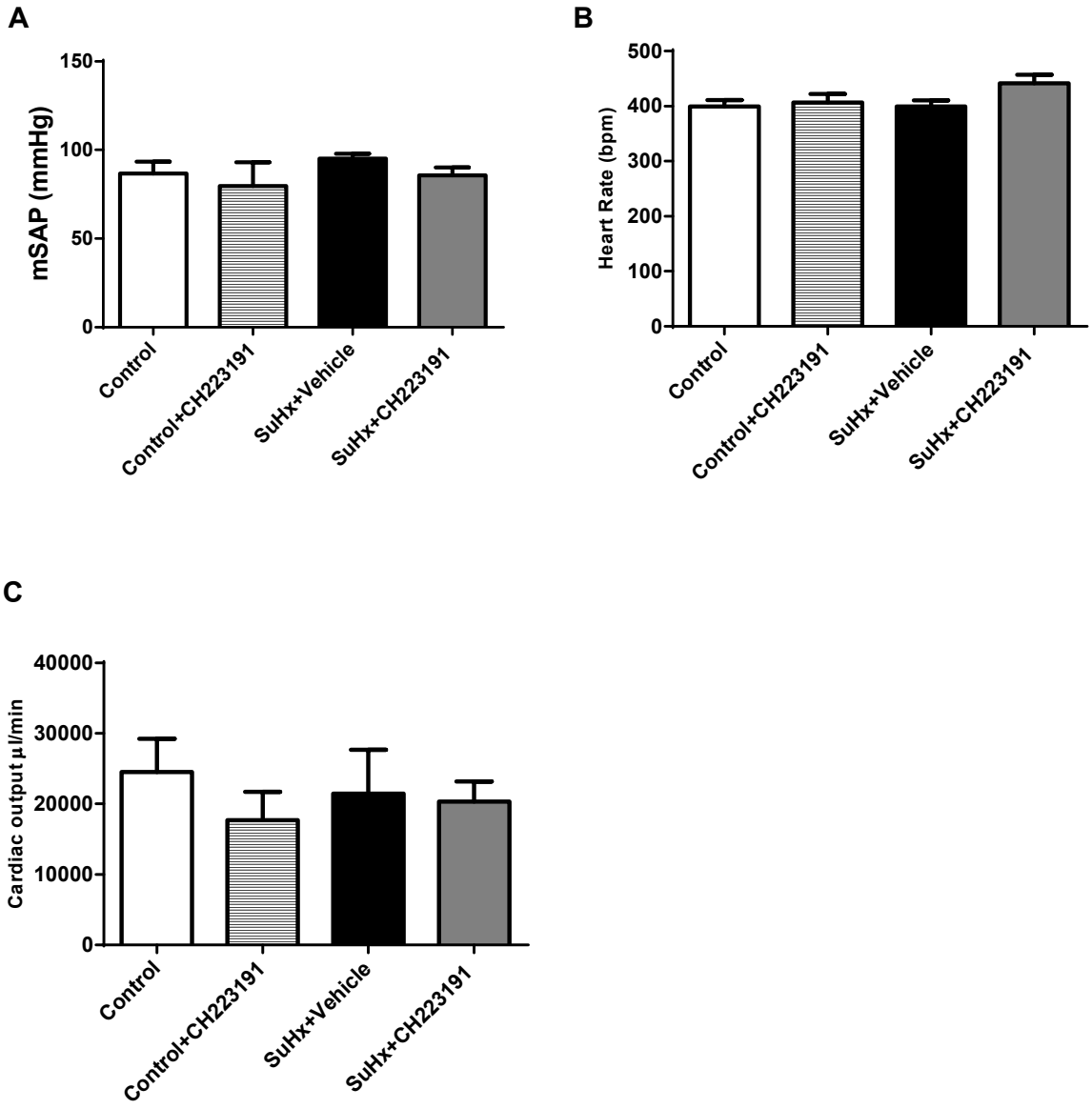

A
mSAP (mmHg)
150
100
50
0
Control
Control+CH223191
SuHx+Vehicle
SuHx+CH223191
B
Heart Rate (bpm)
500
400
300
200
100
0
Control
Control+CH223191
SuHx+Vehicle
SuHx+CH223191
C
Cardiac output µl/min
40000
30000
20000
10000
0
Control
Control+CH223191
SuHx+Vehicle
SuHx+CH223191

Figure E3

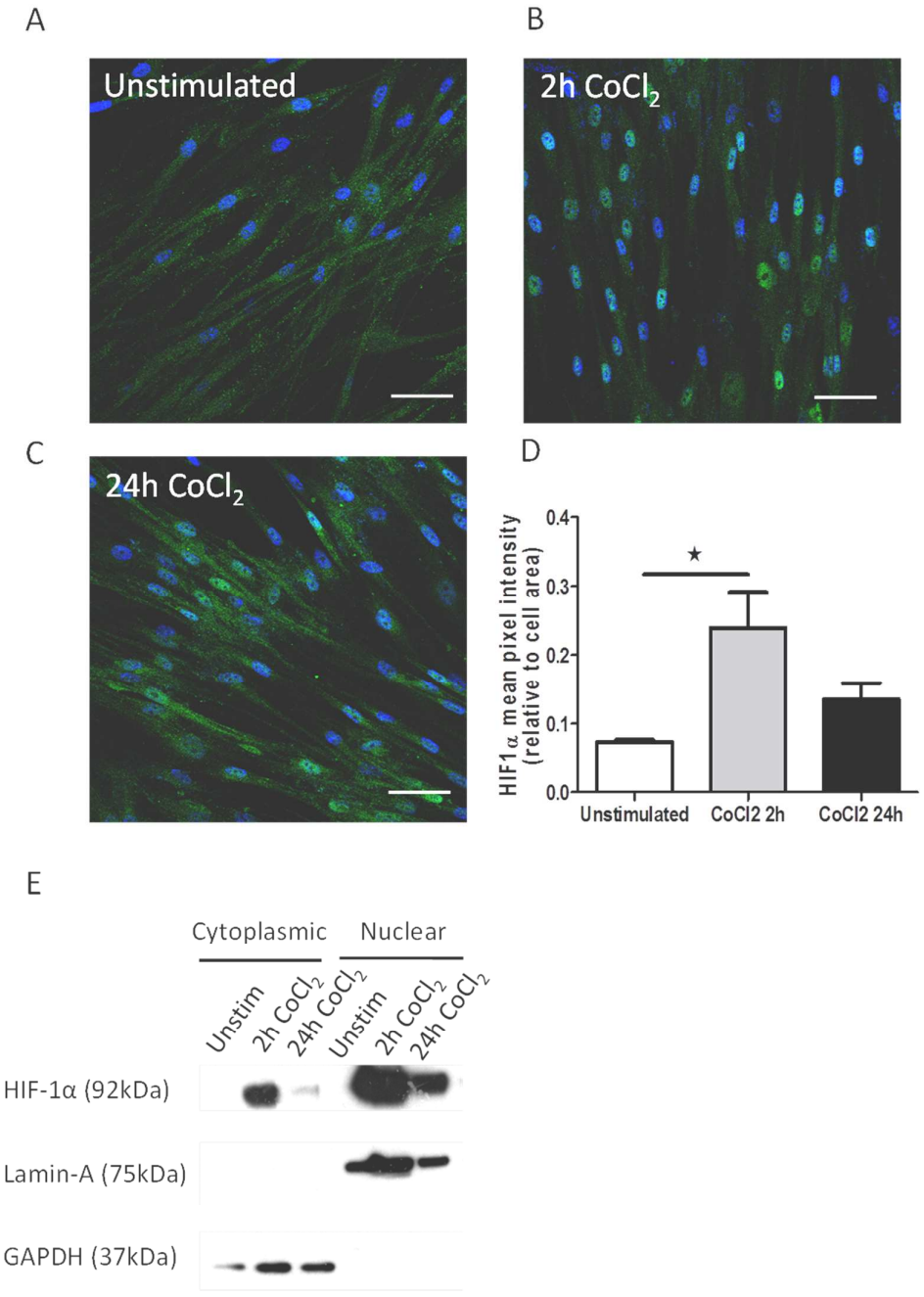

A
Unstimulated
B
2h CoCl2
C
24h CoCl2
D
HIF1α mean pixel intensity (relative to cell area)
0.4
0.3
0.2
0.1
0.0
★
Unstimulated
CoCl2 2h
CoCl2 24h
E
Cytoplasmic
Nuclear
Unstim
2h CoCl2
24h CoCl2
Unstim
2h CoCl2
24h CoCl2
HIF-1α (92kDa)
Lamin-A (75kDa)
GAPDH (37kDa)

Figure E4

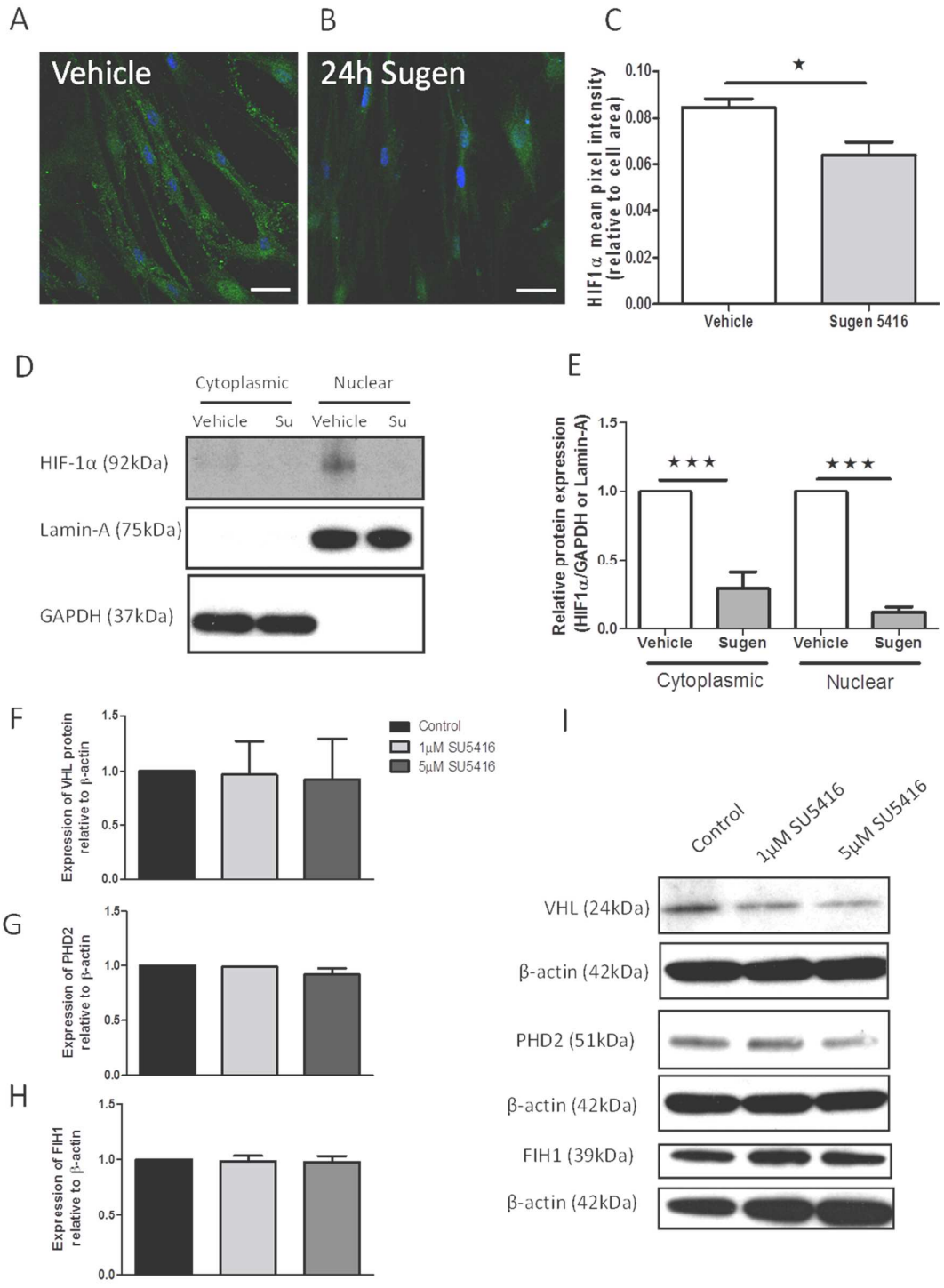

A
B
C
Vehicle
24h Sugen
HIF1α mean pixel intensity (relative to cell area)
0.10
0.08
0.06
0.04
0.02
0.00
★
Vehicle
Sugen 5416
D
Cytoplasmic
Nuclear
Vehicle
Su
Vehicle
Su
HIF-1α (92kDa)
Lamin-A (75kDa)
GAPDH (37kDa)
E
Relative protein expression (HIF1α/GAPDH or Lamin-A)
1.5
1.0
0.5
0.0
★★★
★★★
Vehicle
Sugen
Vehicle
Sugen
Cytoplasmic
Nuclear
F
Expression of VHL protein relative to β-actin
Control
1μM SU5416
5μM SU5416
G
Expression of PHD2 relative to β-actin
H
Expression of FIH1 relative to β-actin
I
Control
1μM SU5416
5μM SU5416
VHL (24kDa)
β-actin (42kDa)
PHD2 (51kDa)
β-actin (42kDa)
FIH1 (39kDa)
β-actin (42kDa)

Table 1 Age (where known) and disease status of patients (all female) from whom cells were derived.

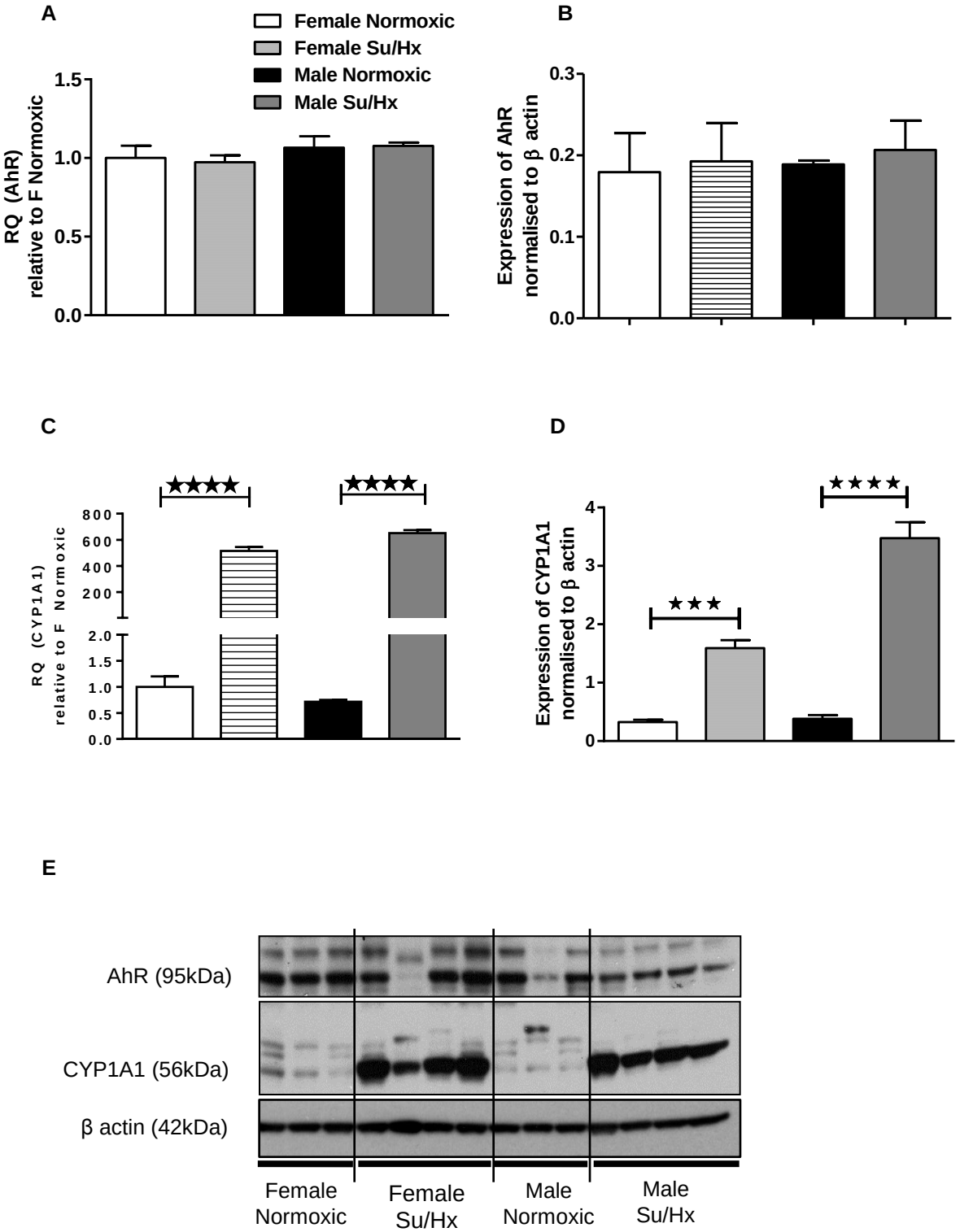
Cell type	*Cell line*	*Age*	*Disease status*
Female hPASMC	**35MP**	41	BMPRII mutant (N903S)
	37MP	24	IPAH
	38MP		IPAH
	73MP	30	BMPRII mutant (R899X)
	113MP	45	IPAH
	115MP	53	Associated PAH (Congenital Heart Disease)
Female BOEC	**PAH9**	62	IPAH
	PAH15	24	IPAH
	B4	37	BMPRII mutant (W9X)

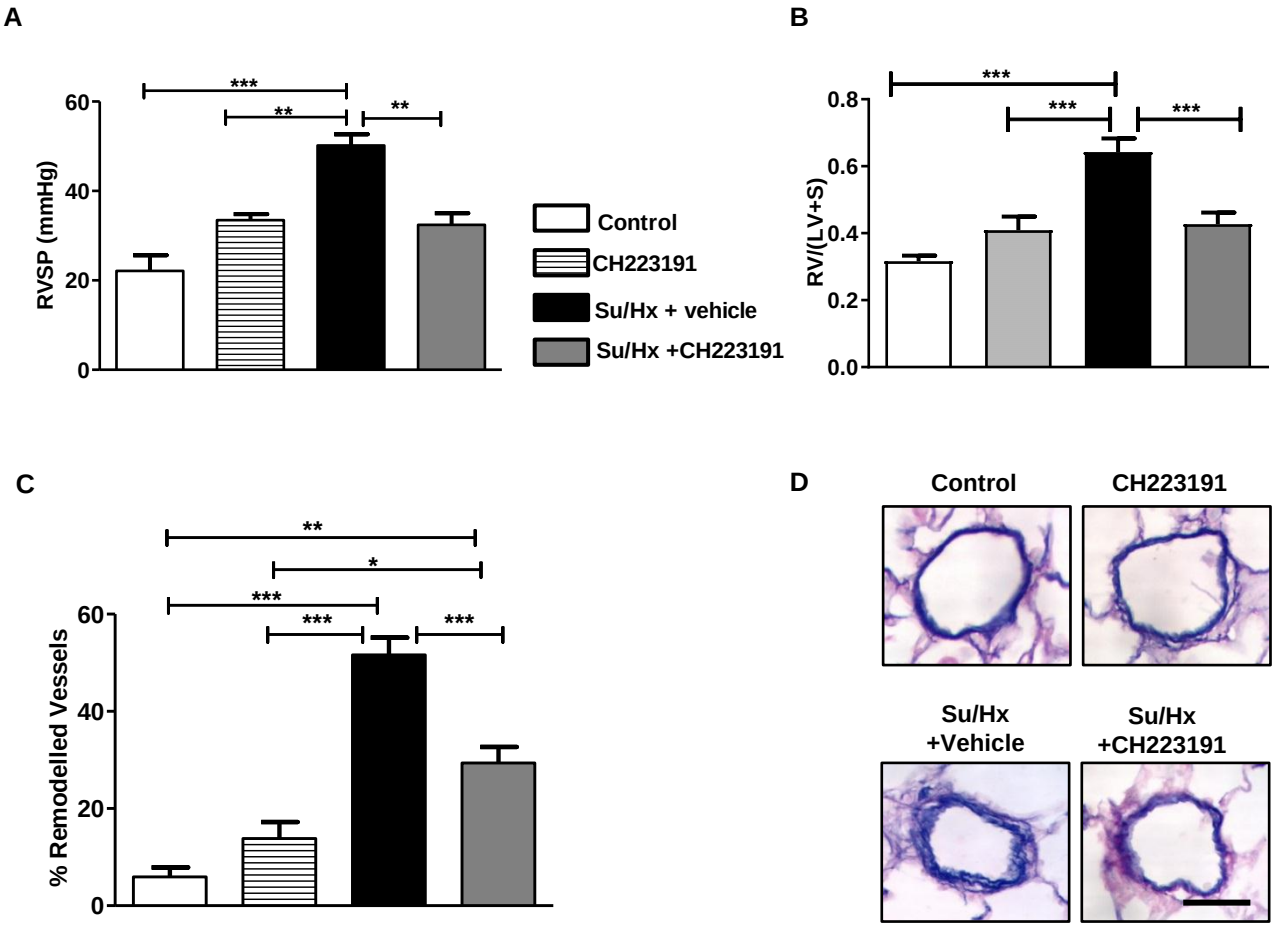
References

1. Ormiston ML, Toshner MR, Kiskin FN, Huang CJZ, Groves E, Morrell NW, Rana AA. Generation and Culture of Blood Outgrowth Endothelial Cells from Human Peripheral Blood. *J Vis Exp* 2015.

2. Suzuki K, Bose P, Leong-Quong RY, Fujita DJ, Riabowol K. REAP: A two minute cell fractionation method. *BMC Research Notes* 2010; 3: 294.

Figure 1


A
Female Normoxic
Female Su/Hx
Male Normoxic
Male Su/Hx
RQ (AhR) relative to F Normoxic
1.5
1.0
0.5
0.0
B
Expression of AhR normalised to β actin
0.3
0.2
0.1
0.0
C
RQ (CYP1A1) relative to F Normoxic
800
600
400
200
2.0
1.5
1.0
0.5
0.0
D
Expression of CYP1A1 normalised to β actin
4
3
2
1
0
E
AhR (95kDa)
CYP1A1 (56kDa)
β actin (42kDa)
Female Normoxic
Female Su/Hx
Male Normoxic
Male Su/Hx


A
RVSP (mmHg)
60
40
20
0
***
**
**
Control
CH223191
Su/Hx + vehicle
Su/Hx +CH223191
B
RV/(LV+S)
0.8
0.6
0.4
0.2
0.0
***
***
***
C
% Remodelled Vessels
60
40
20
0
**
*
***
***
***
D
Control
CH223191
Su/Hx
+Vehicle
Su/Hx
+CH223191

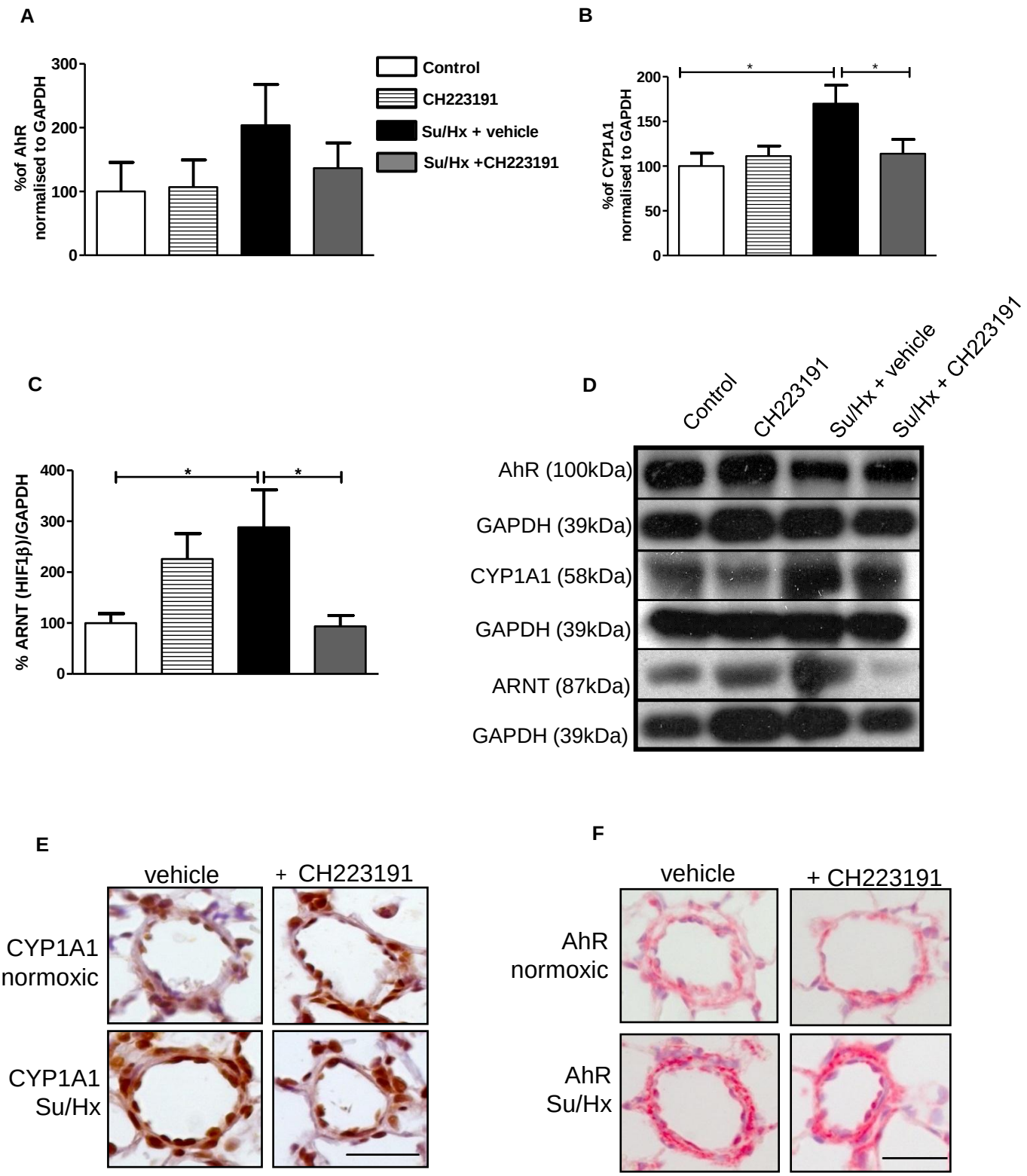
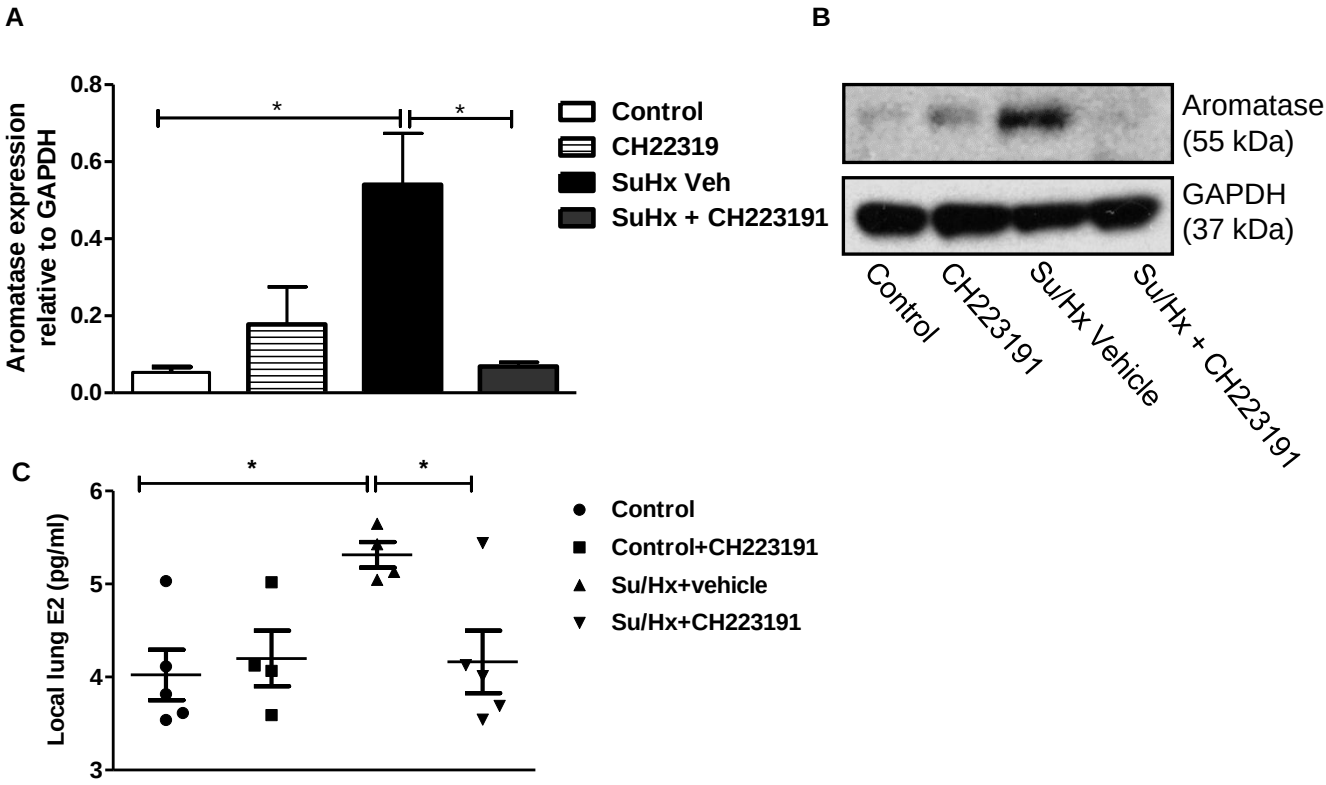

A
%of AhR normalised to GAPDH
300
200
100
0
Control
CH223191
Su/Hx + vehicle
Su/Hx +CH223191
B
%of CYP1A1 normalised to GAPDH
200
150
100
50
0
*
*
C
% ARNT (HIF1β)/GAPDH
400
300
200
100
0
*
*
D
Control
CH223191
Su/Hx + vehicle
Su/Hx + CH223191
AhR (100kDa)
GAPDH (39kDa)
CYP1A1 (58kDa)
GAPDH (39kDa)
ARNT (87kDa)
GAPDH (39kDa)
E
vehicle
+ CH223191
CYP1A1 normoxic
CYP1A1 Su/Hx
F
vehicle
+ CH223191
AhR normoxic
AhR Su/Hx

Figure 4


A
Aromatase expression relative to GAPDH
0.8
0.6
0.4
0.2
0.0
*
*
Control
CH22319
SuHx Veh
SuHx + CH223191
B
Aromatase (55 kDa)
GAPDH (37 kDa)
Control
CH223191
Su/Hx Vehicle
Su/Hx + CH223191
C
Local lung E2 (pg/ml)
6
5
4
3
*
*
Control
Control+CH223191
Su/Hx+vehicle
Su/Hx+CH223191

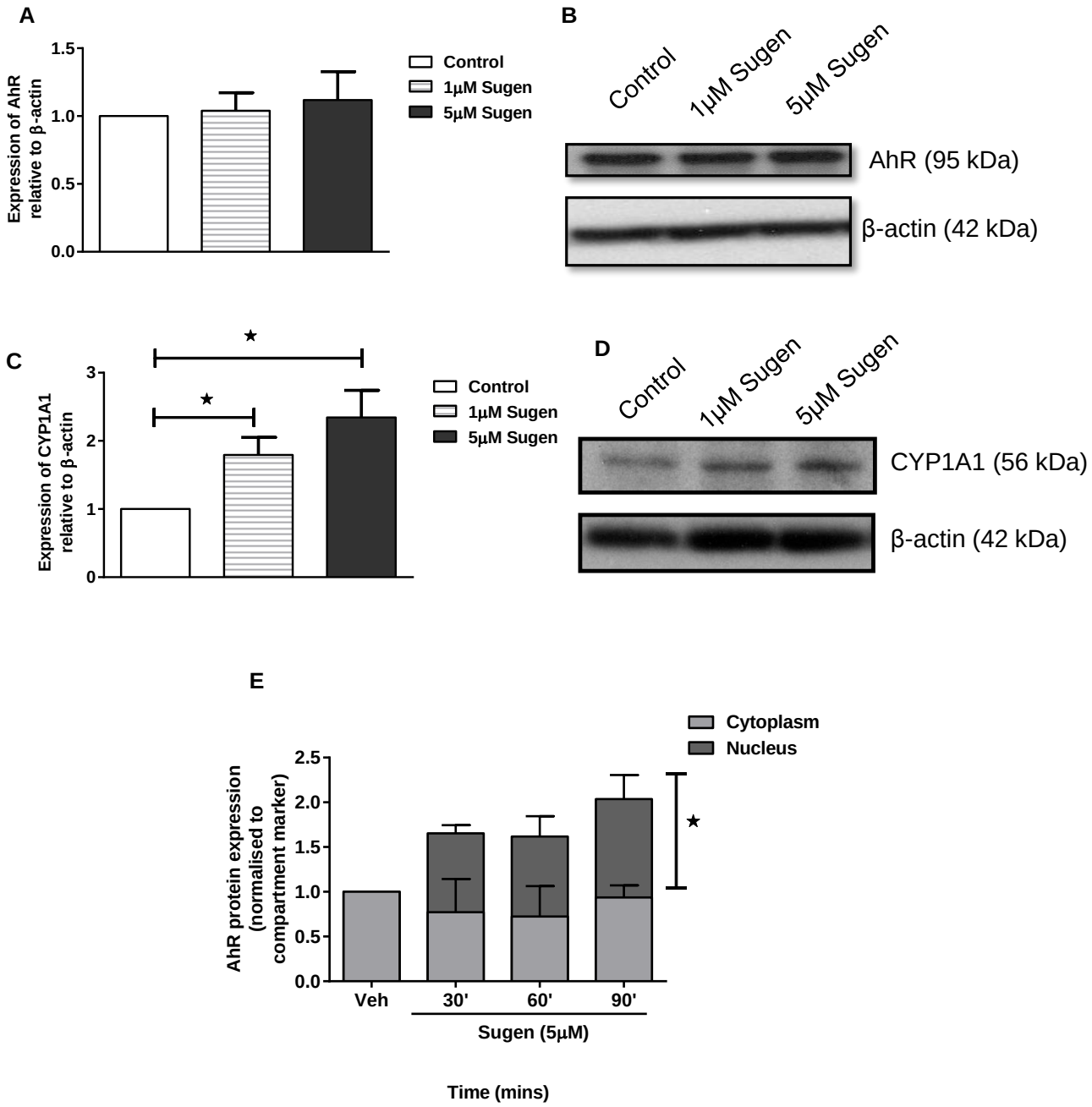

A
Expression of AhR relative to β-actin
1.5
1.0
0.5
0.0
Control
1μM Sugen
5μM Sugen
B
Control
1μM Sugen
5μM Sugen
AhR (95 kDa)
β-actin (42 kDa)
C
Expression of CYP1A1 relative to β-actin
3
2
1
0
★
★
Control
1μM Sugen
5μM Sugen
D
Control
1μM Sugen
5μM Sugen
CYP1A1 (56 kDa)
β-actin (42 kDa)
E
AhR protein expression (normalised to compartment marker)
2.5
2.0
1.5
1.0
0.5
0.0
Cytoplasm
Nucleus
★
Veh
30'
60'
90'
Sugen (5μM)
Time (mins)

Figure 6

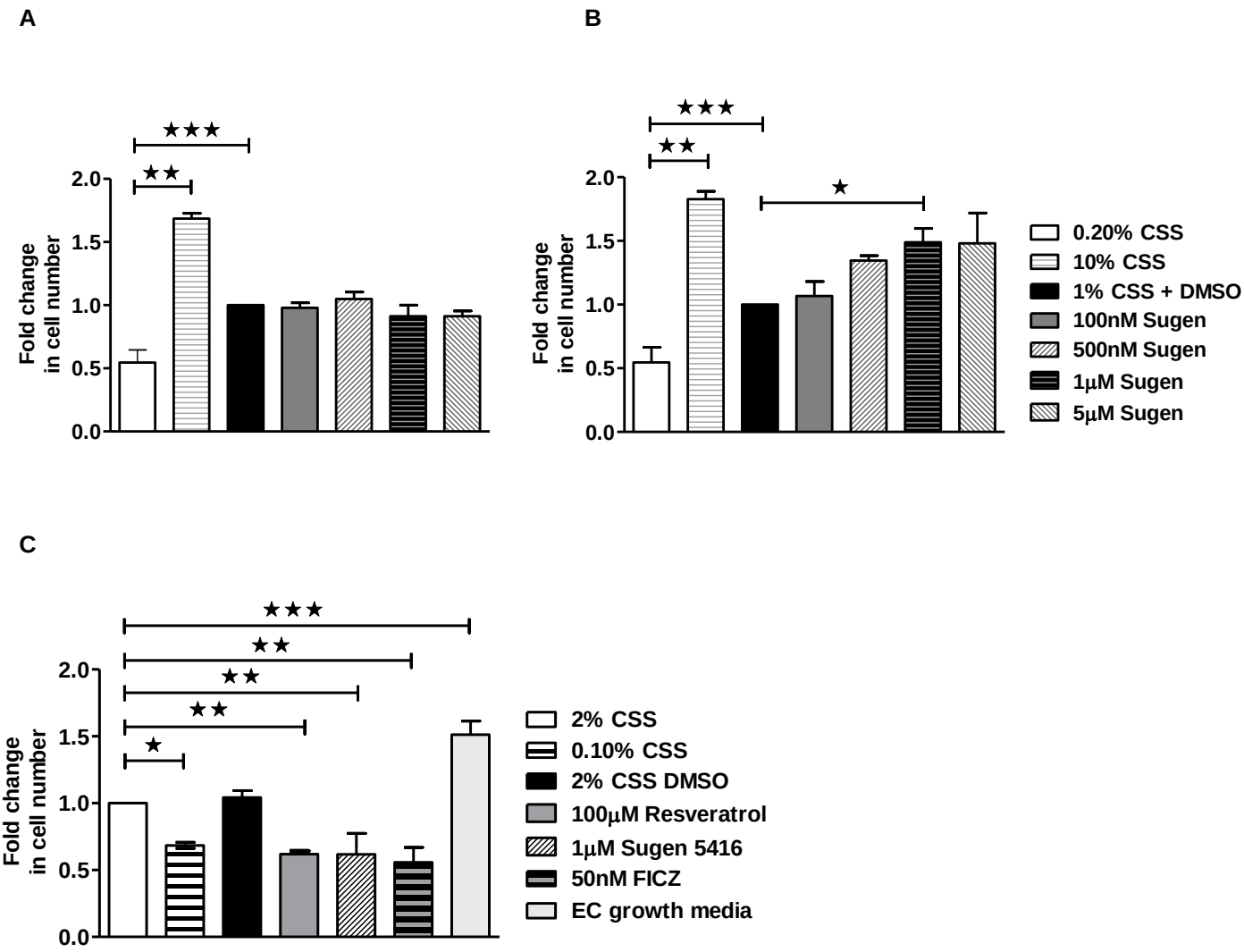
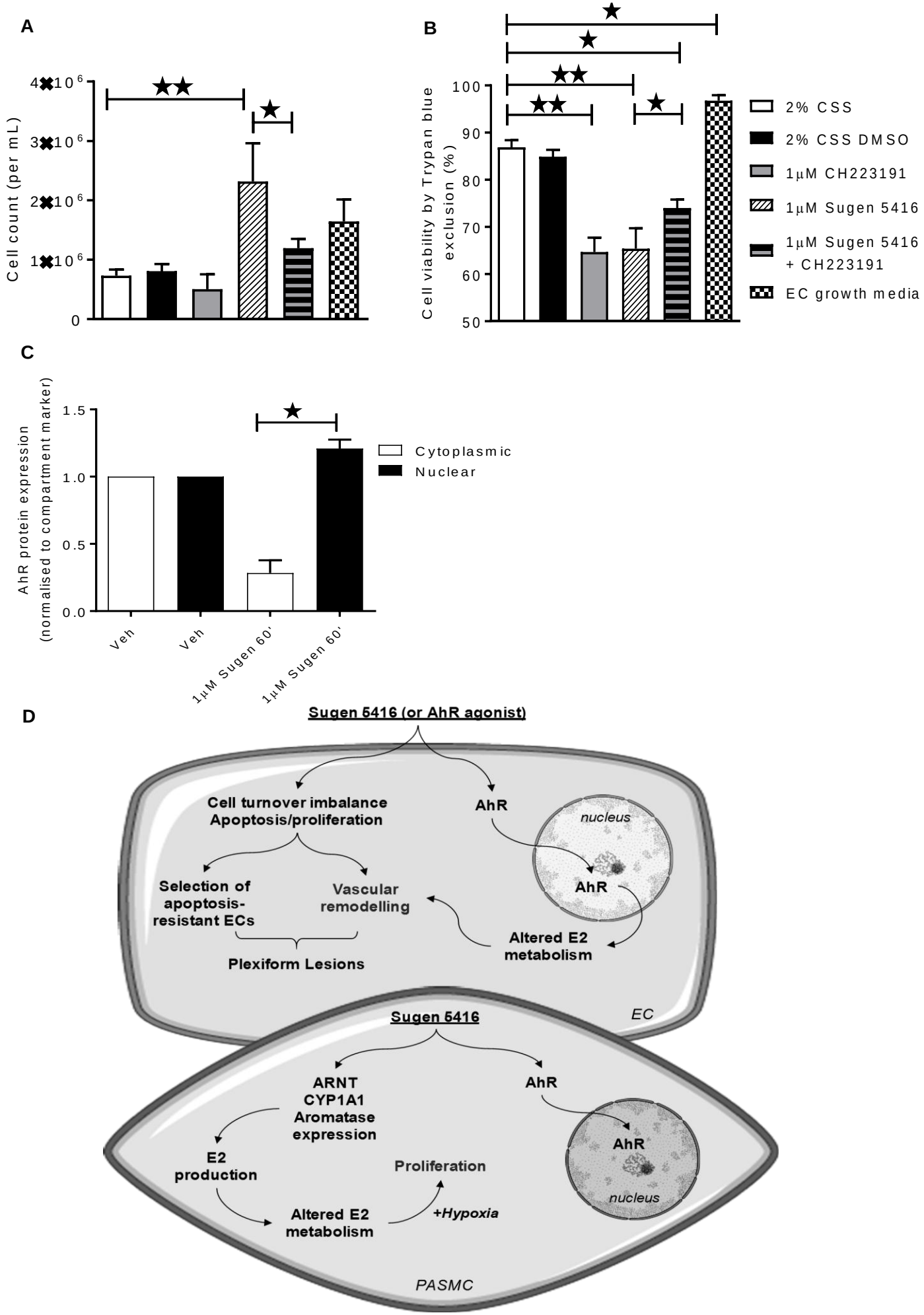

A
B
C
Fold change in cell number
2.0
1.5
1.0
0.5
0.0
★★★
★★
★
0.20% CSS
10% CSS
1% CSS + DMSO
100nM Sugen
500nM Sugen
1μM Sugen
5μM Sugen
2% CSS
0.10% CSS
2% CSS DMSO
100μM Resveratrol
1μM Sugen 5416
50nM FICZ
EC growth media

Figure 7


A
Cell count (per mL)
4✖10 6
3✖10 6
2✖10 6
1✖10 6
0
B
Cell viability by Trypan blue exclusion (%)
100
90
80
70
60
50
2% CSS
2% CSS DMSO
1μM CH223191
1μM Sugen 5416
1μM Sugen 5416 + CH223191
EC growth media
C
AhR protein expression (normalised to compartment marker)
1.5
1.0
0.5
0.0
Cytoplasmic
Nuclear
Veh
Veh
1μM Sugen 60'
1μM Sugen 60'
D
Sugen 5416 (or AhR agonist)
Cell turnover imbalance Apoptosis/proliferation
AhR
nucleus
AhR
Selection of apoptosis-resistant ECs
Vascular remodelling
Plexiform Lesions
Altered E2 metabolism
EC
Sugen 5416
ARNT CYP1A1 Aromatase expression
AhR
AhR
nucleus
E2 production
Proliferation
Altered E2 metabolism
+Hypoxia
PASMC