Activity of the Estrogen Metabolising Enzyme Cytochrome P450 1B1

Influences the Development of Pulmonary Arterial Hypertension

Running title: White et al.; CYP1B1 promotes the development of PAH

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Abstract:

**Background** - Pulmonary arterial hypertension (PAH) is a hyperproliferative vascular disorder predominantly observed in women. Estrogen is a potent mitogen in human pulmonary artery smooth muscle cells (hPASMCs) and contributes to PAH in vivo, however the mechanisms attributed to this causation remain obscure. Curiously, heightened expression of the estrogen-metabolising enzyme cytochrome P450 1B1 (CYP1B1) is reported in idiopathic PAH (IPAH) and murine models of PAH.

**Methods and Results** - Here, we investigated the putative pathogenic role of CYP1B1 in PAH. Quantitative RT-PCR, immunoblotting and in situ analysis revealed that pulmonary CYP1B1 is increased in hypoxic PAH, hypoxic + SU5416 PAH and human PAH and highly expressed within the pulmonary vascular wall. PAH was assessed in mice via measurement of right ventricular hypertrophy, pulmonary vascular remodelling and right ventricular systolic pressure. Hypoxic PAH was attenuated in CYP1B1-/- mice and the potent CYP1B1 inhibitor 2,3',4,5'-tetramethoxystilbene (TMS; 3mg/kg/day i.p.) significantly attenuated hypoxic PAH and hypoxic + SU5416 PAH in vivo. TMS also abolished estrogen-induced proliferation in hPASMCs and PAH-PASMCs. The estrogen metabolite 16α-hydroxyestrone provoked hPASMC proliferation and this mitogenic effect was greatly pronounced in PAH-PASMCs. ELISA analysis revealed that 16α-hydroxyestrone concentration was elevated in PAH, consistent with CYP1B1 over-expression and activity. Finally, administration of the CYP1B1 metabolite 16α-hydroxyestrone (1.5mg/kg/day i.p.) caused the development of PAH in mice.

**Conclusions** - Increased CYP1B1-mediated estrogen metabolism promotes the development of PAH, likely via the formation of mitogens including 16α-hydroxyestrone. Collectively, this study reveals a possible novel therapeutic target in clinical PAH.

**Key words:** animal model; cardiovascular disease; estrogen; hypertension, pulmonary; metabolism
Introduction

Idiopathic pulmonary arterial hypertension (IPAH) and heritable pulmonary arterial hypertension (HPAH) share a common vascular histopathology\(^1,2\), defined by pronounced vascular remodelling and complex vascular lesion formation arising from an accelerated rate of proliferation in all cell types which compose the pulmonary vascular wall (endothelial, smooth muscle and fibroblast)\(^3\)\(^-\)\(^6\).

The incidence of IPAH and HPAH is up to three-fold more common in women than men\(^7\)\(^-\)\(^9\). Sex hormones are likely to play a crucial role in these underlying gender differences. Specifically, multiple lines of evidence converge to implicate the estrogen pathway in PAH pathogenesis\(^10\)\(^-\)\(^12\). First, physiological concentrations (~1 nmol/L) of 17β estradiol (the predominant circulating estrogen in premenopausal women) are sufficient to increase expression of the estrogen metabolising enzyme cytochrome P450 1B1 (CYP1B1)\(^13\) and stimulate the proliferation of human pulmonary artery smooth muscle cells (hPASMCs)\(^14\)\(^,\)\(^15\). Second, the aberrant expression of CYP1B1 is reported in bone-morphogenetic protein type-II (BMPR-II) affected female HPAH patients\(^16\)\(^,\)\(^17\) and hPASMCs isolated from IPAH patients\(^13\). Third, female susceptibility is observed in mice expressing the human serotonin transporter gene construct (SERT; SERT+ mice), and this is dependent on the presence of circulating 17β estradiol\(^14\). Intriguingly, CYP1B1 is also increased in the pulmonary arteries of these mice\(^13\). Fourth, the estrogen precursor dehydroepiandrosterone is a potent suppressor of CYP1B1 expression\(^18\), whilst dehydroepiandrosterone has been previously shown to prevent and reverse chronic hypoxic PAH\(^19\). Cumulatively, these multiple lines of evidence converge to suggest the existence of a pathogenic link between CYP1B1 and the pathogenesis of PAH.

CYP1B1 is a member of the cytochrome P450 family of enzymes and is expressed in the
lung where it rapidly catalyses the 4-hydroxylation of estrogen to form 4-hydroxyestrogen\textsuperscript{20}. CYP1B1 also metabolises estrogen via 16-hydroxylation, resulting in formation of the potent mitogen 16-hydroxyestrogen\textsuperscript{21}. Aberrant CYP1B1 expression and over-activity is common across lung cancer\textsuperscript{22}, breast cancer\textsuperscript{23}, ovarian cancer\textsuperscript{24}, renal cell carcinoma\textsuperscript{25}, primary congenital glaucoma\textsuperscript{26} and systemic hypertension\textsuperscript{27}.

Here, we investigated the possible attribution of pathogenic estrogen metabolism in the genesis and progression of PAH. In the present study, we demonstrate that CYP1B1 over-expression and activity is associated with the development of PAH, suggesting a pathogenic role for CYP1B1 in disease pathogenesis.

Methods

Ethical Information

Mice were housed in a 12 hour light-dark cycle with access to food and water \textit{ad libitum}. All animal procedures conform 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). Animal approval was also granted by the University Ethical Review Panel. Experimental procedures using human lung tissue and hPASMCs 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.

CYP1B1-/- Mice

CYP1B1-/- mice (10-12 weeks) were generated on a C57BL/6 background as previously described\textsuperscript{28}. Age-matched C57BL/6 mice were studied as control.
**Chronic Hypoxia**

The development of chronic hypoxic PAH in mice was achieved using hypobaric hypoxia, as previously described\(^29\).

**Chronic Hypoxia + SU5416**

To establish experimental PAH that exhibit a vascular pathology more consistent with human PAH, we utilized the chronic hypoxic + SU5416 PAH model. Mice were exposed to 21 days normoxia or chronic hypoxia and simultaneously administered the vascular endothelial growth factor receptor (VEGFR) inhibitor SU5416 (Sigma UK, 20mg/kg, s.c.) at 0, 7 and 14 days, as previously described\(^30\). SU5416 was suspended in 0.5% [w/v] carboxymethylcellulose sodium, 0.9% [w/v] NaCl, 0.4% [v/v] polysorbate and 0.9% [v/v] benzyl alcohol in dH\(_2\)O.

**2,3',4,5'-tetramethoxystilbene Study**

To assess the contribution of CYP1B1 in chronic hypoxic PAH and hypoxic + SU5416 PAH, 8-12 week old C57BL/6 mice were administered the highly potent and selective CYP1B1 inhibitor 2,3',4,5'-tetramethoxystilbene\(^31\) (TMS, Tocris, UK; 3mg/kg/day) or vehicle (4% ethanol (v/v) dH\(_2\)O) each day via intra-peritoneal injection for the entire duration of experimental insult. Normoxic vehicle-dosed littermate mice were studied as control.

**16α-Hydroxyestrone Study**

Based on the evidence that 16α-hydroxyestrone stimulated proliferation in hPASMCs, we assessed the effects of 16α-hydroxyestrone on the development of PAH *in vivo*. 10-12 week old female C57BL/6 littermate mice were administered 16α-hydroxyestrone (Steraloids, USA; 1.5mg/kg/day) or vehicle (4% ethanol (v/v) dH\(_2\)O) daily via intra-peritoneal injection for 28 days prior to the assessment of PAH.

**Quantitative RT-PCR**
CYP1B1 mRNA expression was assessed in the lungs of mice by quantitative RT-PCR, as previously described\textsuperscript{13,30}. ΔΔCT values were determined using Opticon2 software and values normalised against actin. Data are expressed as fold-change versus normoxic female mice.

**Immunoblotting**

Protein expression of CYP1B1 was assessed in the lung as previously described\textsuperscript{32}. Briefly, lung tissue was lysed in RIPA buffer via ultrasonic homogenization and 30μg protein loaded for SDS-PAGE analysis. CYP1B1 and α tubulin molecular weights were detected at 70kDa and 50kDa, respectively. Densitometrical analysis was performed using TotalLab TL100 software. Data are expressed relative to α tubulin density.

**Immunolocalization of CYP1B1 in Lung**

Pulmonary vascular CYP1B1 expression was investigated in murine lung (n=4) and human lung (n=4 IPAH; n=4 HPAH; n=4 non-PAH) by immunohistochemistry. Detailed clinical characteristics of all PAH patients are described in Table 1 (HPAH patients 1-4, IPAH patients 5-8). All IPAH patients were confirmed as BMPR-II mutation negative whilst all HPAH patients were BMPR-II mutation positive. Briefly, 5μm sagittal sections were deparaffinized and re-hydrated through a xylene-ethanol gradient. Following epitope retrieval, endogenous peroxidase and biotin activity was blocked and lung tissue incubated for 16 hours with anti-rabbit CYP1B1 antibody (Abcam, UK) or IgG control. Following secondary incubation, CYP1B1 was visualized using the DAB substrate kit (Vector Laboratories, UK), which stained as brown/dark-brown in colour.

**Quantitative analysis of CYP1B1 immunoreactivity**

CYP1B1 expression within the pulmonary vascular wall was quantitatively defined in murine and human lung via colorometric analysis (version 6.1, Molecular devices, USA). The average
pixel intensity of the annotated region was assigned a greyscale range of 0 (black) to 255 (white), with intermediate intensities being assigned an appropriate numerical grey level. The vascular wall of pulmonary arteries with an external diameter less than 80μm for mice and 200μm for humans were annotated and analyzed quantitatively. To determine CYP1B1 stain intensity the colour threshold was set to detect pixel intensity between 0 and 156. The percentage threshold area detected was then expressed as the percentage of CYP1B1 immunoreactivity within the vascular wall. For both murine and human lung, the mean value of CYP1B1 staining intensity was derived from the average value calculated from 15 resistance pulmonary arteries from each lung.

**Haemodynamic Measurements**

Heart rate, right ventricular systolic pressure (RVSP) and systemic arterial pressure were measured and analysed as previously described\(^29\), \(^30\).

**Right Ventricular Hypertrophy**

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

**Lung Histopathology**

5μm sagittal sections of lung were elastica Van Gieson stained and microscopically assessed for the muscularization of pulmonary arteries (<80μm external diameter) in a blinded fashion, as previously described\(^29\). Remodelled arteries were confirmed by the presence of a double elastic laminae. Briefly, percentage remodelling (% remodelled vessels) was defined for each animal by the number of remodelled vessels divided by the total number (>80 per lung) of vessels observed in the lung. To visualize the degree of smooth muscle thickening, lungs were stained with α-
smooth muscle actin (α-SMA; Abcam, UK) using the same protocol as described previously. The presence and degree of pulmonary vascular occlusion formation was assessed in lungs from chronic hypoxic + SU5416 treated mice as previously described.29

Pulmonary Vascular Reactivity

The effect of CYP1B1 gene ablation versus TMS treatment in vivo on pulmonary vascular reactivity was studied. Intralobar pulmonary arteries (internal diameter 200μm-250μm) were dissected from the superior lobe of the left lung and studied for serotonin-induced pulmonary vascular contraction using small vessel wire myography, as previously described.29 Data were normalised against the maximal contractile response to 50mmol/L KCl. The 50% maximal contractile response (EC50) and maximal contractile response (Emax) were calculated for each group and respective values were compared across appropriate groups.

hPASMCs and PAH-PASMCs

Human pulmonary artery smooth muscle cells (hPASMCs) were provided by Professor N.W Morrell, University of Cambridge, UK. Briefly, hPASMCs were explanted from the distal pulmonary arteries of macroscopically normal lung tissue at transplant, with the patient having no reported presence of PAH. PAH-PASMCs were explanted from the distal pulmonary arteries of patients diagnosed with PAH (Patient 9, Table 1) immediately following pneumonectomy. Cultured hPASMCs were confirmed by both staining for α-SMA (>97% α-SMA+ cells) and morphological characterization.

PASMC Proliferation

Because PASMC proliferation is a major pathologic hallmark of vascular remodelling3 and CYP1B1 is highly expressed in smooth muscles cells which compose the pulmonary vascular wall, we were interested in assessing the role of CYP1B1 in smooth muscle cell proliferation.
hPASMCs (passage 3-6) were seeded in 24-well plates at a density of 20,000 per/well and grown to 60% confluency prior to 24 hour growth arrest in phenol-red free 0.2% foetal bovine serum (FBS) DMEM. Subsequently, hPASMCs were incubated with 17β estradiol or the required estrogen metabolite for 72 hours. For antagonist studies, the highly selective CYP1B1 inhibitor TMS was incubated with hPASMCs for at least 30 minutes prior to the addition of 17β estradiol. Because quiescent hPASMCs do not readily proliferate in response to 17β estradiol, all experiments were performed in the presence of low concentration (10ng/ml) platelet derived growth factor (PDGF) to maintain low basal cell turnover. DMEM including agonists/antagonists were replaced every 48 hours. For the last 24 hours, 0.2μCi [3H] thymidine was added to each well, which is incorporated into replicating chromosomal DNA during mitosis. This is an extremely accurate method to assess hPASMC proliferation, and we have previously shown this to exhibit a high correlation with alternative proliferation assays in this cell type.14,33 [3H] thymidine incorporation was measured using a Wallac scintillation counter (PerkinElmer, UK), and data are expressed as fold-change compared to control. All experiments n=3 in triplicate.

16α-hydroxyestrone ELISA Assay

16α-hydroxyestrone concentration in urine was quantified by ELISA analysis (ESTRAMET 2/16, Demeditec Diagnostics, Germany). Briefly, 16α-hydroxyestrone was assayed using specific alkaline-phosphatase labelled conjugation and quantification was determined by 405nm spectrophotometry analysis (SpectraMax M2, Molecular Devices, USA). All experiments n=4-5 and performed in triplicate.

Statistics

All data is expressed as mean ± SEM. Wilcoxon rank sum, nonparametric ANOVA using the
Conover and Iman Rank Transform method\(^{34}\), logistic regression models fitted using generalized estimating equations (GEE) methods and linear regression models were used for statistical comparison as appropriate, and referred to in detail in the Figure legends. Separate male and female analyses were carried out as required as 2x2x2 ANOVA on ranks has been previously shown to perform poorly. In addition, we were primarily concerned with the effects of either CYP1B1 knockout or TMS in these studies in both males and females. Pairwise comparisons were adjusted for multiple testing using the Bonferroni procedure where P<0.05. Statistical analysis was performed using R, v2.14 (www.r-project.org) and Minitab v16 (Minitab Inc, USA).

**Results**

**Pulmonary CYP1B1 expression is increased in murine PAH and human PAH**

CYP1B1 mRNA and protein was upregulated in the lungs of chronic hypoxic mice compared to normoxic littermate controls (**figure 1a-b**). CYP1B1 mRNA and protein expression was also upregulated in chronic hypoxic + SU5416 PAH (**figure 1c-d**). Immunohistochemistry analysis revealed that pulmonary vascular-specific CYP1B1 is upregulated within the pulmonary arteries in chronic hypoxic and chronic hypoxic + SU5416 PAH (**figure 2a-b**). In human PAH lung, pulmonary vascular CYP1B1 is similarly over-expressed in IPAH and HPAH compared to non-PAH lung, as confirmed by in situ analysis (**figure 2c**). CYP1B1 appears expressed within all cell types which compose the vascular wall including Von Willebrand factor-positive and \(\alpha\)-SMA-positive cells, indicative of endothelial and smooth muscle cells respectively (**supplemental figure S1**). In mice, chronic hypoxic exposure did not affect CYP1B1 expression in the right or left ventricle (**supplemental figure S2**), inciting the existence of pulmonary-specific upregulation of CYP1B1 during PAH. Collectively, these results confirm
CYP1B1 upregulation in at least two independent models of murine PAH (chronic hypoxic PAH and chronic hypoxic + SU5416 PAH) and human PAH (IPAH and HPAH).

**Chronic hypoxic PAH in CYP1B1-/- mice**

In response to chronic hypoxia, male CYP1B1-/- mice showed significant attenuation of RVH and RVSP compared to wildtype mice (figure 3). Similarly, female CYP1B1-/- mice displayed an attenuation of RVH (figure 3a), however this was reported in the absence of significant attenuation in RVSP (figure 3b). No changes in systemic arterial pressure were observed (data not shown). Maximal pulmonary vascular contraction was attenuated in chronic hypoxic male CYP1B1-/- mice versus wildtype controls, whilst pulmonary vascular reactivity was unaffected in normoxic or hypoxic female CYP1B1-/- mice (figure 3c-d). Pulmonary vascular remodelling was also attenuated in male CYP1B1-/- mice, whilst no changes were reported in female CYP1B1-/- mice (figure 3e-f).

**CYP1B1 inhibition in chronic hypoxic PAH**

In chronic hypoxia, TMS attenuated RVH (figure 4a) and RVSP (figure 4b) in male and female mice versus vehicle-dosed littermate controls. Pulmonary vascular contraction was not significantly attenuated in normoxic or chronic hypoxic TMS-dosed female and male mice (figure 4c-d). Pulmonary vascular remodelling was also significantly attenuated by TMS in hypoxic mice (figure 4e-f). In normoxia, TMS had no effect on RVH, RVSP or pulmonary vascular remodelling.

**Effect of CYP1B1 inhibition in chronic hypoxic + SU5416 PAH**

The therapeutic viability of CYP1B1 was further tested in chronic hypoxic + SU5416 PAH, an experimental model of PAH which exhibits vascular pathology more consistent with human PAH. In chronic hypoxia, TMS attenuated RVH (figure 5a), RVSP (figure 5b) and pulmonary vascular remodelling.
vascular remodelling (figure 5c-d). TMS had a significant effect on occlusive lesion formation with the number of fully occluded vessels being significantly reduced in TMS-dosed mice (Figures 5e, f). This effect was most significant in the female mice. In normoxia, TMS had no effect in RVH, RVSP or pulmonary vascular remodelling. The combined experimental insult of chronic hypoxia and SU5416 had no effect in systemic arterial pressure.30.

**Contribution of CYP1B1 in 17β estradiol-induced proliferation**

We and others have previously shown that physiological concentrations of 17β estradiol stimulate proliferation in hPASMCs14,15, however the mechanisms co-ordinating this have yet to be fully delineated. Here, we defined the role of CYP1B1 in 17β estradiol-induced hPASMC proliferation. The potent CYP1B1 inhibitor TMS abolished 17β estradiol hPASMC proliferation in a concentration-dependent manner (figure 6a). This inhibitory effect was ~100-fold more potent in hPASMCs isolated from PAH patients (figure 6b). Consistent with this, CYP1B1 is over-expressed in lPAH-PASMCs.13 To further investigate the CYP1B1 metabolites mediating proliferation, the proliferative effects of all CYP1B1-derived estrogen metabolites (2-, 4- and 16-hydroxyestrogens; figure 6c) were examined. From all metabolites, 16-hydroxyestrone was the only metabolite which stimulated proliferation, and this was observed in a concentration-dependent manner. Intriguingly, 16α-hydroxyestrone evoked a hyperproliferative response in PAH-PASMCs versus non-PAH (control) hPASMCs (figure 6d).

**16α-hydroxyestrone promotes PAH in mice**

Consistent with increased CYP1B1 over-expression and activity in PAH, 16α-hydroxyestrone urinary concentration was significantly increased in chronic hypoxic mice (figure 7a). Based on these findings, we hypothesised that the CYP1B1-derived mitogen 16α-hydroxyestrone directly promotes the development of PAH in vivo. To test this directly, mice were administered 16α-
hydroxyestrone consecutive for 28 days. This resulted in an increase of RVH (figure 7b) and RVSP (figure 7c) versus vehicle-dosed littermate mice. No changes in pulmonary vascular contraction (figure 7d) were observed. In support of the PAH-promoting effects of this mitogen, pulmonary vascular remodelling was also increased in 16α-hydroxyestrone dosed mice (figure 7e). No changes in systemic arterial pressure were reported in these mice (data not shown).

Discussion

The collective data presented here uncovers a novel role for CYP1B1 in the genesis and progression of PAH. We show that pulmonary CYP1B1 is increased in two independent models of experimental PAH and human PAH (IPAH and HPAH). As confirmed by in situ analysis, CYP1B1 is upregulated in the remodelled vasculature during experimental and human PAH and expressed within all cell types which compose the vascular wall, including the endothelial cells and smooth muscle cells. Given this observation, we hypothesized that CYP1B1 upregulation contributes to the genesis and progression of PAH. To test this directly, we assessed the effects of CYP1B1 loss-of-function in the development of PAH in vivo, through the investigation of CYP1B1-/- mice and mice given the potent CYP1B1 inhibitor TMS. We observed that male CYP1B1-/- mice exhibited a significant attenuation in the development of PAH in response to chronic hypoxic insult, as reported through a significant attenuation in RVH, RVSP, pulmonary vascular contraction and pulmonary vascular remodelling. In contrast, female CYP1B1-/- mice showed significant attenuation in RVH which was reported in the absence of any changes to vascular pathology or pulmonary hemodynamics. Although the reasons for these disparate findings in females are unclear, given the importance of CYP1B1 function in cardiomyocyte survival and proliferation we suspect that the embryonic knockout of CYP1B1 likely contributes
to changes in ventricular remodelling.

In order to support our findings in CYP1B1/-/- mice, we tested the putative beneficial effects of the potent and selective CYP1B1 inhibitor TMS in the development of chronic hypoxic PAH in wildtype mice. TMS-dosed mice exhibited significant attenuation in PAH response to chronic hypoxia, as reported through a beneficial reduction in RVH, RVSP and pulmonary vascular remodelling.

It is well established that the chronic hypoxic murine PAH model fails to recapitulate the severe plexogenic arteriopathy often present in human PAH. To address this limitation, we further tested the potential beneficial effects of TMS in an experimental model of PAH which exhibits a vascular pathology more consistent to human PAH. Severe experimental PAH was evoked in mice through the simultaneous insult of chronic hypoxia and administration of the vascular endothelial growth factor receptor antagonist SU5416. This model has been previously shown to exhibit severe vascular remodelling and the existence of occlusive pulmonary lesions. Here, we observed that TMS significantly attenuated the development of severe PAH in these mice, as reported through a reduction in RVH, RVSP and pulmonary vascular remodelling. The total number of occluded pulmonary vascular lesions was also significantly reduced in TMS-dosed mice versus vehicle-dosed littermate controls.

*In vitro*, we demonstrate that the inhibition of CYP1B1 with TMS abolishes the proliferative effects of 17β estradiol in hPASMCs. This data provides intriguing evidence that proliferation is dependent on the formation of mitogenic 17β estradiol metabolites via CYP1B1 metabolism. Intriguingly, TMS potency was 100-fold more potent at inhibiting proliferation in PAH-PASMCs, indicative of CYP1B1 over-expression and activity during PAH. To precisely define those mitogen(s) which stimulate hPASMC proliferation, we compared the proliferative
effects of all CYP1B1 metabolites including 2-, 4- and 16-hydroxyestrogens. Intriguingly, from all metabolites assessed 16α-hydroxyestrone was the only to stimulate proliferation and this was observed in a concentration-dependent manner. In line with this, the bioavailability of 16α-hydroxyestrone has been shown to be increased clinically in female BMPR-II mutation carriers affected by PAH compared to unaffected mutation carriers.35 Intriguingly, the proliferative effects of this mitogen were significantly more pronounced in PAH-PASMCs versus hPASMCs. 16α-hydroxyestrone has been previously shown to cause proliferation via the upregulation of cyclin D1 expression, which is an important cell cycle regulator in PASMCs.37 This observation may be relevant to 16α-hydroxyestrone pathogenesis in PAH, given that cyclin D1 expression is also increased in the lung during PAH in vivo. In the present study, we show that 16α-hydroxyestrone concentration is significantly increased in murine PAH in vivo. To test this metabolite directly, we studied the effects of 16α-hydroxyestrone in vivo. Consistent with its mitogenic effects in vitro, the administration of 16α-hydroxyestrone induced the development of PAH in mice, as reported by increased RVH, RVSP and pulmonary vascular remodelling.

Collectively, these data suggest that the formation of 16α-hydroxyestrone via the CYP1B1 metabolism of 17β estradiol is an important contributor to the genesis and progression of PAH. However, we suspect that these changes in CYP1B1 expression and activity advertently regulate the formation of multiple metabolites beyond 16α-hydroxyestrone, and these collective net changes in metabolic profile also contribute to pulmonary vascular cell survival and proliferation.

In humans, CYP1B1 is a known modifier gene in bone morphogenetic protein receptor type II (BMPR-II) affected PAH, and has been previously cited as a promising therapeutic target in disease.16,17 Beyond PAH, the therapeutic value of CYP1B1 inhibition in the treatment and
management of cancer is currently under Phase II clinical trial investigation\textsuperscript{39}. Here, our data suggests that the pathogenic metabolism of estrogen via CYP1B1 is influential in PAH progression, making this a promising therapeutic target in PAH.

Although multiple epidemiological studies have implied a causative relationship between estrogen exposure and the development of PAH in humans\textsuperscript{10-12}, little evidence exists to support this. Indeed, paradoxical findings from studies in rodent models have suggested that estrogen\textsuperscript{40-43} and its bioactive metabolites\textsuperscript{44-46} may actually prove beneficial against the development and progression of PAH. Also, estrogen inhibits pulmonary artery vasoconstriction and promotes pulmonary artery vasodilatation in rats\textsuperscript{47,48}. These inconsistencies between human PAH and experimental PAH have diffused our current understanding of pathologic estrogen signalling in PAH. Novel emerging animal models have gone some way in explaining these anomalies. \textit{In vivo}, we have previously shown that pulmonary CYP1B1 is significantly upregulated in mice expressing the human serotonin transporter gene (SERT+ mice)\textsuperscript{13}. In SERT+ PAH, only female mice exhibit spontaneous PAH implying a causative role for female hormones. Indeed, the presence of circulating $17\beta$-estradiol is a requisite for PAH in these mice\textsuperscript{14}. In line with this, $17\beta$-estradiol growth effects in hPASMCs is dependent on serotonin signalling\textsuperscript{14}. Hence, we propose that in the presence of pre-existing pulmonary hypertensive insult, estrogen can be causative in the development of PAH.

These findings may be pertinent to the widely reported gender differences observed in several forms of human PAH. We speculate that heightened circulating levels of estrogen coupled with CYP1B1 upregulation may render pre-menopausal women more susceptible to the increased formation of metabolites such as $16\alpha$-hydroxyestrone, subsequently leading to the increased risk of PAH development.
In conclusion, we provide unique evidence to support a role for CYP1B1 in the genesis and progression of PAH. Aberrant CYP1B1 expression promotes pathologic estrogen metabolism within the pulmonary vasculature, resulting in the formation of smooth muscle mitogens including 16α-hydroxyestrone. Further studies to establish the viability of CYP1B1 as a therapeutic target in human PAH are merited.

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Conflict of Interest Disclosures: None.

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Table 1. Clinical characteristics of patients with human PAH

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<th>Patient ID</th>
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<th>Age</th>
<th>Gender</th>
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Clinical characteristics of human PAH patients. HPAH, heritable PAH; IPAH, idiopathic PAH; APAH, associated PAH; n/a, not available; PAP, pulmonary artery pressure; RHC, right heart catheterization. Note that all HPAH patients were BMPR-II mutation carriers whilst all IPAH patients were not BMPR-II mutation carriers.

Figure Legends:

Figure 1. CYP1B1 is upregulated in murine PAH. (a) CYP1B1 RNA in normoxic and chronic hypoxic female and male murine lung (n=5); Male vs female P<0.0005, normoxic vs hypoxic P<0.0005, *** P< 0.001 (pairwise comparison). (b) CYP1B1 protein expression and representative Western blot analysis in normoxic and chronic hypoxic female and male murine lung (n=5); Male vs female P<0.0005, normoxic vs hypoxic P=0.001, *P<0.05 (pairwise comparison). (c) CYP1B1 RNA in normoxic and chronic hypoxic + SU5416 female and male murine lung (n=7); Male vs female P=0.136, normoxic vs hypoxic P<0.0005, ** P< 0.001 (pairwise comparison). (d) CYP1B1 protein expression and representative Western blot analysis in normoxic and chronic hypoxic + SU5416 female and male murine lung (n=7); Male vs female P=0.073, normoxic vs hypoxic P<0.0005, * P< 0.05, **P<0.01 (pairwise comparison). 2-way Rank Transform nonparametric ANOVA, with Bonferroni pairwise comparisons.
Figure 2. CYP1B1 immunolocalization in murine and human pulmonary arteries during PAH. (a) CYP1B1 immunolocalization and quantification in normoxic and chronic hypoxic murine pulmonary arteries (n=4); Male vs female P=0.025, hypoxic vs normoxic ***P<0.0005, two factor GEE. (b) CYP1B1 immunolocalization and quantification in normoxic and chronic hypoxic + SU5416 murine pulmonary arteries (n=5); Male vs female P=0.036, hypoxic vs normoxic ***P<0.0005, two factor GEE. (c) CYP1B1 immunolocalization in human pulmonary arteries. Examples shown are from a male, age 56 with IPAH and a female, age 26 with HPAH (with BMPR2 mutation). Quantification was from n=4 subjects, ≥ 60 vessels of <80μm external diameter in total, in human non-PAH lung, IPAH lung and HPAH lung (see Table 1 for clinical characteristics of the human patients); Group P<0.0005, non-PAH vs IPAH ***P<0.0005, non-PAH vs HPAH ***P<0.0005, one factor GEE. CYP1B1 stains as brown/dark brown. Scale bar 20μm in murine and 100μm in human.

Figure 3. Chronic hypoxic PAH in CYP1B1-/- mice. (a) RVH analysis in normoxic and chronic hypoxic WT and CYP1B1-/- female and male mice (n=8-12). Normoxia vs hypoxia P<0.0005; WT vs CYP1B1 -/-: female P= 0.003, male P<0.0005. *P<0.05, **P<0.01, *** P< 0.001 (pairwise comparison). (b) RVSP measurements in normoxic and chronic hypoxic WT and CYP1B1-/- female and male mice (n=7-11). Normoxia vs hypoxia P<0.0005; WT vs CYP1B1 -/-: female P=0.279, male P<0.007. *P<0.05, *** P< 0.001 (pairwise comparison). (c) Pulmonary vascular reactivity in normoxic WT and CYP1B1-/- female and male mice (n=5-7). pEC50: no significant differences. Emax: no significant differences (d) Pulmonary vascular reactivity in chronic hypoxic WT and CYP1B1-/- female and male mice (n=5-7). pEC50: no significant differences. Emax: normoxia: no significant differences. Hypoxia: WT vs CYP1B1 P<0.0005;
male vs female P<0.015. ***P<0.001 (pairwise comparison). (e) Pulmonary vascular remodelling in normoxic and chronic hypoxic WT and CYP1B1-/- female and male mice (n=5). Normoxia vs hypoxia P<0.0005; WT vs CYP1B1 -/-: female P=0.156, male P=0.193. *P<0.05, *** P< 0.001 (pairwise comparison). (f) Representative α-SMA-stained pulmonary arteries in normoxic and chronic hypoxic WT and CYP1B1-/- female and male mice. Scale bar 20µm. 2-way Rank Transform nonparametric ANOVA (on female and male data for a, b and e), with Bonferroni pairwise comparisons.

**Figure 4.** The CYP1B1 inhibitor TMS attenuates the development of chronic hypoxic PAH. (a) RVH analysis in normoxic and chronic hypoxic vehicle and TMS-treated female and male mice (n=7-10). Normoxia vs hypoxia P<0005; vehicle vs TMS: female P=0.183, male P=0.036. *P<0.05, *** P< 0.001 (pairwise comparison). (b) RVSP measurements in normoxic and chronic hypoxic vehicle and TMS-dosed female and male mice (n=6-10). Normoxia vs hypoxia P<0005; vehicle vs TMS: female P=0.038, male P=0.354. *P<0.05, *** P< 0.001 (pairwise comparison).

(c) Pulmonary vascular reactivity in normoxic vehicle and TMS-dosed female and male mice (n=5-6). pEC$_{50}$: no significant differences. Emax: no significant differences. (d) Pulmonary vascular reactivity in chronic hypoxic vehicle and TMS-dosed female and male mice (n=4-6). pEC$_{50}$: no significant differences. Emax: no significant differences. (e) Pulmonary vascular remodelling in normoxic and chronic hypoxic vehicle and TMS-dosed female and male mice (n=5-7). Normoxia vs hypoxia P<0005; vehicle vs TMS: females P<0.04, males P<0.0005. *P<0.05. **P<0.01, *** P< 0.001 (pairwise comparison). (f) Representative α-SMA-stained pulmonary arteries in normoxic and chronic hypoxic vehicle and TMS-dosed female and male mice. Scale bar 20µm. 2-way Rank Transform nonparametric ANOVA (on female and male data
for a, b and e), with Bonferroni pairwise comparisons.

**Figure 5.** The CYP1B1 inhibitor TMS attenuates the development of hypoxic + SU5416 PAH. (a) RVH analysis in normoxic and chronic hypoxic + SU5416 vehicle and TMS-dosed female and male mice (n=7). Normoxia vs hypoxia + SU5416: female P=0.001, male<0.0005; vehicle vs TMS: females P=0.029, males P=0.162. *P<0.05, **P<0.01, *** P< 0.01 (pairwise comparison). (b) RVSP measurements in normoxic and chronic hypoxic + SU5416 vehicle and TMS-dosed female and male mice (n=6-7). Normoxia vs hypoxia + SU5416 P<0.0005; vehicle vs TMS: females P=0.009, males P=0.137. *P<0.05, **P<0.01, *** P< 0.001 (pairwise comparison). (c) Pulmonary vascular remodelling in normoxic and chronic hypoxic + SU5416 vehicle and TMS-dosed female and male mice (n=5-7). Normoxia vs hypoxia + SU5416 P<0.0005; vehicle vs TMS: P=0.262 (female), P=0.788 (male). **P<0.01, *** P< 0.001 (pairwise comparison). (d) Representative α-SMA-stained pulmonary arteries in normoxic and chronic hypoxic + SU5416 vehicle and TMS-dosed female and male mice. (e) Effect of TMS treatment on occlusive pulmonary lesion formation in chronic hypoxic + SU5416 PAH (n=6). Vehicle vs TMS: P< 0.0005, Male vs female P<0.001. *P<0.05 (pairwise comparison) (f) Representative image of an α-SMA-stained occlusive pulmonary lesion chronic hypoxic + SU5416 PAH. Scale bar 20μm. Rank Transform nonparametric ANOVA (on female and male data for a,b,c), with Bonferroni pairwise comparisons.

**Figure 6.** 17β-estradiol induced proliferation in human PASMCs. (a) Effect of the CYP1B1 inhibitor TMS in 17β-estradiol proliferation in hPASMCs.*P<0.05 (Wilcoxon signed rank test); §§ P<0.01 Linear regression of log fold change vs log M TMS. (b) Effects of the CYP1B1
inhibitor TMS on 17β-estradiol proliferation in PAH-PASMCs. *P<0.05 (Wilcoxon signed rank test); §§§ P<0.001 Linear regression of log fold change vs log M TMS. (c) Proliferative effects of all CYP1B1 metabolites in hPASMCs. **P<0.01 (Wilcoxon signed rank test); §§ P<0.01 Linear regression of log fold change vs log M 16α-OHE-1 (d) Proliferative effects of the CYP1B1 metabolite 16α-hydroxyestrone (16α-OHE1) in hPASMCs and PAH-PASMCs. Linear regression of log fold change vs log M 16α-OHE-1 (§§§ P<0.001) and Non-PAH vs IPAH (# P<0.05) All experiments performed n=3 and repeated in triplicate.

**Figure 7.** 16α-hydroxyestrone (16α-OHE1) concentration is significantly increased in PAH and induces the development of PAH. (a) Urinary levels of 16α-OHE1 in normoxic and chronic hypoxic female mice (n=5). **P<0.01 (b) RVH analysis in vehicle and 16α-OHE1-dosed mice (n=7-9). Vehicle vs 16α-OHE1 **P<0.05 (c) RVSP measurements in vehicle and 16α-OHE1-dosed mice (n=7). Vehicle vs 16α-OHE1 **P<0.01 (d) Pulmonary vascular reactivity in vehicle and 16α-OHE1-dosed mice (n=6-8). pEC50: no significant differences. Emax: no significant differences (e) Pulmonary vascular remodelling in vehicle and 16-OHE1-treated mice. (n=5) Vehicle vs 16α-OHE1 *P<0.05. (f) Representative α-SMA-stained pulmonary arteries in vehicle and 16α-OHE1-treated mice. Scale bar 20µm. Wilcoxon Rank Sum.
Activity of the Estrogen Metabolising Enzyme Cytochrome P450 1B1 Influences the Development of Pulmonary Arterial Hypertension

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Supplemental Material
Methods

Localization of CYP1B1 in human pulmonary arteries

To determine CYP1B1 localization within pulmonary arteries, serial human lung sections were stained with CYP1B1, the smooth muscle marker αSMA and the endothelial marker von Willebrand Factor. Immunohistochemistry and quantitative methods are described in Methods.

CYP1B1 expression in non-pulmonary tissue

CYP1B1 mRNA was assessed in the right ventricle and left ventricle of mice by quantitative RT-PCR, as previously described\(^{13,30}\). ΔΔCT values were determined using Opticon2 software and values normalised against GAPDH. Data are expressed as fold-change versus normoxic female mice. CYP1B1 expression was analysed in 6 mice per group.

Results

CYP1B1 is highly expressed within the pulmonary vascular endothelium and smooth muscle

Immunohistochemistry analysis reveals that CYP1B1 is highly expressed in expressing vWF positive cells and α-SMA positive cells, indicative of the endothelium and smooth muscle respectively (figure S1).

CYP1B1 expression is unchanged in the heart during PAH

CYP1B1 expression was unchanged in both the right ventricle and left ventricle of mice following exposure to chronic hypoxia (figure S2). This observation was reported in both female and male mice.
**Figure S1.** CYP1B1 immunolocalisation in pulmonary vascular lesions from a 38 year old female diagnosed with HPAH (B) and a 26 year old male diagnosed with HPAH. Scale bar=100µM.

**Figure S2.** CYP1B1 is unchanged in the right ventricle and left ventricle during PAH in mice. (a) CYP1B1 mRNA expression in right ventricle of normoxic and chronic hypoxic mice (n=6). (b) CYP1B1 RNA expression in left ventricle of normoxic and chronic hypoxic mice (n=6).
Figure S1.

a. CYP1B1  α-SMA  vWF

b.
Figure S2.