Vascular Medicine

Activity of the Estrogen-Metabolizing Enzyme Cytochrome P450 1B1 Influences the Development of Pulmonary Arterial Hypertension

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

Methods and Results—Here, we investigated the putative pathogenic role of CYP1B1 in PAH. Quantitative reverse transcription–polymerase chain reaction, immunoblotting, and in situ analysis revealed that pulmonary CYP1B1 is increased in hypoxic PAH, hypoxic+SU5416 PAH, and human PAH and is highly expressed within the pulmonary vascular wall. PAH was assessed in mice via measurement of right ventricular hypertrophy, pulmonary vascular remodeling, and right ventricular systolic pressure. Hypoxic PAH was attenuated in CYP1B1−/− mice, and the potent CYP1B1 inhibitor 2,3′,4,5′-tetramethoxystilbene (TMS; 3 mg·kg−1·d−1 IP) significantly attenuated hypoxic PAH and hypoxic+SU5416 PAH in vivo. TMS also abolished estrogen-induced proliferation in human pulmonary artery smooth muscle cells and PAH–pulmonary artery smooth muscle cells. The estrogen metabolite 16α-hydroxyestrone provoked human pulmonary artery smooth muscle cell proliferation, and this mitogenic effect was greatly pronounced in PAH–pulmonary artery smooth muscle cells. ELISA analysis revealed that 16α-hydroxyestrone concentration was elevated in PAH, consistent with CYP1B1 overexpression and activity. Finally, administration of the CYP1B1 metabolite 16α-hydroxyestrone (1.5 mg·kg−1·d−1 IP) 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. (Circulation. 2012;126:1087-1098.)

Key Words: cardiovascular diseases ■ estrogens ■ hypertension, pulmonary ■ metabolism ■ models, animal

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lated from IPAH patients. 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. Intriguingly, CYP1B1 is also increased in the pulmonary arteries of these mice. Fourth, the estrogen precursor dehydroepiandrosterone is a potent suppressor of CYP1B1 expression, whereas dehydroepiandrosterone has previously been shown to prevent and reverse chronic hypoxic PAH. Cumulatively, these multiple lines of evidence converge to suggest the existence of a pathogenic link between CYP1B1 and the pathogenesis of PAH.

CYP1B1, a member of the cytochrome P450 family of enzymes, is expressed in the lung where it rapidly catalyzes the 4-hydroxylation of estrogen to form 4-hydroxyestrogen, which metabolizes estrogen via 16-hydroxylation, resulting in formation of the potent mitogen 16-hydroxyestrone. Aberrant CYP1B1 expression and overactivity are common across lung cancer, breast cancer, ovarian cancer, renal cell carcinoma, primary congenital glaucoma, and systemic hypertension.

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 overexpression and activity are 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 ad libitum. All animal procedures conform with the UK 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 (age, 10–12 weeks) were generated on a C57BL/6 background as previously described. Age-matched C57BL/6 mice were studied as control.

**Chronic Hypoxia**

The development of chronic hypoxic PAH in mice was achieved with hypobaric hypoxia as previously described.

**Chronic Hypoxia+SU5416**

To establish experimental PAH that exhibits a vascular pathology more consistent with human PAH, we used the chronic hypoxic+SU5416 PAH model. Mice were exposed to 21 days of normoxia or chronic hypoxia and simultaneously administered the vascular endothelial growth factor receptor inhibitor SU5416 (Sigma UK, 20 mg/kg SC) at 0, 7, and 14 days as previously described. SU5416 was suspended in 0.5% (vol/vol) carboxymethylcellulose sodium, 0.9% (wt/vol) NaCl, 0.4% (vol/vol) polysorbate, and 0.9% (vol/vol) benzyl alcohol in dH2O.

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

To assess the contribution of CYP1B1 in chronic hypoxic PAH and hypoxic+SU5416 PAH, 8- to 12-week-old C57BL/6 mice were administered the highly potent and selective CYP1B1 inhibitor 2,3',4,5'-tetramethoxystilbene (TMS; Tocris, UK; 3 mg · kg−1 · d−1) or vehicle (4% ethanol [vol/vol] dH2O) daily via intraperitoneal injection for the entire duration of experimental insult. Normoxic vehicle-dosed littermate mice were studied as control.

**16α-Hydroxyestrone Study**

On the basis of the evidence that 16α-hydroxyestrone stimulated proliferation in hPASMCs, we assessed the effects of 16α-hydroxyestrone on the development of PAH in vivo. Female 10- to 12-week-old C57BL/6 mice were administered 16α-hydroxyestrone (Steroids, US; 1.5 mg · kg−1 · d−1) or vehicle (4% ethanol [vol/vol] dH2O) daily via intraperitoneal injection for 28 days before the assessment of PAH.

**Quantitative Reverse Transcription–Polymerase Chain Reaction**

CYP1B1 mRNA expression was assessed in the lungs of mice by quantitative reverse transcription–polymerase chain reaction as previously described. Briefly, lung tissue was lysed in radioimmuno-precipitation assay buffer via ultrasonic homogenization and 30 μg protein loaded for SDS-PAGE analysis. CYP1B1 and α-tubulin molecular weights were detected at 70 and 50 kDa, respectively. Densitometric analysis was performed with TotalLab TL100 software. Data are expressed as fold change compared with normoxic female mice.

**Immunoblotting**

Protein expression of CYP1B1 was assessed in the lung as previously described. Briefly, lung tissue was lysed in radioimmuno-precipitation assay buffer via ultrasonic homogenization and 30 μg protein loaded for SDS-PAGE analysis. CYP1B1 and α-tubulin molecular weights were detected at 70 and 50 kDa, respectively. Densitometric analysis was performed with TotalLab TL100 software. Data are expressed as 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 non-PAH) by immunohistochemistry. Detailed clinical characteristics of all PAH patients are described in the Table (patients 1–4, IPAH; patients 5–8, IPAH). All IPAH patients were confirmed as BMPR-II mutation positive, whereas all HPAH patients were BMPR-II mutation positive. Briefly, 5 μm sagittal sections were deparaffinized and rehydrated through a xylene-ethanol gradient. After epitope retrieval, endogenous peroxidase and biotin activity was blocked and lung tissue was incubated for 16 hours with anti-rabbit CYP1B1 antibody (Abcam, UK) or IgG control. After secondary incubation, CYP1B1 was visualized with the DAB substrate kit (Vector Laboratories, UK), which stained brown/dark brown.

**Quantitative Analysis of CYP1B1 Immunoreactivity**

CYP1B1 expression within the pulmonary vascular wall was quantitatively defined in murine and human lung via colorimetric analysis (version 6.1; Molecular Devices). The average pixel intensity of the annotated region was assigned a gray-scale range of 0 (black) to 255 (white), with intermediate intensities being assigned an appropriate numeric gray level. The vascular walls of pulmonary arteries with an external diameter <80 μm for mice and 200 μm for humans were annotated and analyzed quantitatively. To determine CYP1B1 stain intensity, the color 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.
Hemodynamic Measurements

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

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 is RV/LV+S.

Lung Histopathology

Five μm lung sagittal sections were stained with elastic Van Gieson and pulmonary arteries (<80 μm external diameter) microscopically assessed for degree of muscularization in a blinded fashion, as previously described.29 Remodeled arteries were confirmed by the presence of a double-elastic laminae. Briefly, percentage remodeling (percent of remodeled vessels) was defined for each animal by the number of remodeled 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 (Abcam, UK) using the same protocol as described previously. The presence and degree of pulmonary vascular occlusion formation were assessed in lungs from chronic hypoxic mice as previously described.30

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, 200–250 μm) 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 normalized against the 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

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

PASMC Proliferation

Because PASMC proliferation is a major pathological hallmark of vascular remodeling28 and CYP1B1 is highly expressed in smooth muscle cells that make up 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% confluence before 24 hour growth arrest in phenol red–free 0.2% FBS Dulbecco modified Eagle medium. 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 before 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 a low concentration (10 ng/mL) platelet-derived growth factor to maintain low basal cell turnover. Dulbecco modified Eagle medium including agonists/antagonists was replaced every 48 hours. For the last 24 hours, 0.2 μCi [3H] thymidine was added to each well, which is incorporated into replicating chromosomal DNA during mitosis. This is an extremely accurate method to assess PASMC proliferation, and we have previously shown that this method exhibits a high correlation with alternative proliferation assays in this cell type.14,33 [3H] thymidine incorporation was measured with a Wallac scintillation counter (PerkinElmer, UK), and data are expressed as fold change compared with control. All experiments were performed in triplicate (n=3).

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 with specific alkaline-phosphatase–labeled conjugation, and quantification was determined by 405-nm spectrophotometry analysis (SpectraMax M2, Molecular Devices, US). All experiments were performed in triplicate (n=4 to 5).

Statistics

All data are expressed as mean±SEM. Wilcoxon rank sum, non-parametric ANOVA with the Conover and Iman rank transform method,34 logistic regression models fitted with generalized estimating equations methods, and linear regression models were used for statistical comparison as appropriate and are referred to in detail in the figure legends. Separate male and female analyses were carried out as required because 2×2×2 ANOVA on ranks has previously been shown to perform poorly. In addition, we were concerned primarily with the effects of either CYP1B1 knockout or TMS in these studies in both males and females. Pairwise comparisons were adjusted for multiple testing with the Bonferroni procedure in which P<0.05. Statistical analysis was performed with R version 2.14 (www.r-project.org) and Minitab version 16 (Minitab Inc, US).

Table. Clinical Characteristics of Patients With Human Pulmonary Artery Hypertension

<table>
<thead>
<tr>
<th>Patient</th>
<th>Group 1 PAH</th>
<th>Age, y</th>
<th>Sex</th>
<th>Mean PAP (RHC), mm Hg</th>
<th>Tissue Collection, Drug Therapy</th>
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<tbody>
<tr>
<td>1</td>
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<td>67</td>
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<tr>
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<td>30</td>
<td>Female</td>
<td>46</td>
<td>Transplant, iloprost and sildenafil</td>
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<tr>
<td>3</td>
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<td>56</td>
<td>Transplant, epoprostenol</td>
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<tr>
<td>4</td>
<td>HPAH</td>
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<td>Male</td>
<td>54</td>
<td>Transplant, iloprost and sildenafil</td>
</tr>
<tr>
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<td>IPAH</td>
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<td>Female</td>
<td>44</td>
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<td>NA</td>
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<tr>
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<td>43</td>
<td>Male</td>
<td>NA</td>
<td>Transplant, Eisenmenger syndrome</td>
</tr>
</tbody>
</table>

PAH indicates pulmonary arterial hypertension; PAP, pulmonary artery pressure; RHC, right heart catheterization; HPAH, heritable PAH; IPAH, idiopathic PAH; and APAH, associated PAH. Note that all HPAH patients were BMPR-II mutation carriers and all IPAH patients were not BMPR-II mutation carriers.
Results

Pulmonary CYP1B1 Expression Is Increased in Murine PAH and Human PAH

CYP1B1 mRNA and protein were upregulated in the lungs of chronic hypoxic mice compared with normoxic littermate controls (Figure 1A and 1B). CYP1B1 mRNA and protein expression was also upregulated in chronic hypoxic SU5416 PAH (Figure 1C and 1D). Immunohistochemistry analysis revealed that pulmonary vascular–specific CYP1B1 is upregulated within the pulmonary arteries in chronic hypoxic and chronic hypoxic SU5416 PAH (Figure 2A and 2B). In human PAH lung, pulmonary vascular CYP1B1 is similarly overexpressed in IPAH and HPAH compared with non-PAH lung, as confirmed by in situ analysis (Figure 2C). CYP1B1 appears to be expressed within all cell types that make up the vascular wall, including Von Willebrand factor–positive and smooth muscle actin–positive cells, indicative of endothelial and smooth muscle cells, respectively (Figure I in the online-only Data Supplement). In mice, chronic hypoxic exposure did not affect CYP1B1 expression in the right or left ventricle (Figure II in the online-only Data Supplement), inciting the existence of pulmonary-specific upregulation of CYP1B1 during PAH. Collectively, these results confirm CYP1B1 upregulation in at least 2 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 with wild-type 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 compared with wild-type controls, whereas pulmonary vascular reactivity was unaffected in normoxic or hypoxic female CYP1B1−/− mice (Figure 3C and 3D). Pulmonary vascular remodeling was also attenuated in male CYP1B1−/− mice, whereas no changes were reported in female CYP1B1−/− mice (Figure 3E and 3F).

CYP1B1 Inhibition in Chronic Hypoxic PAH

In chronic hypoxia, TMS attenuated RVH (Figure 4A) and RVSP (Figure 4B) in male and female mice compared with vehicle-dosed littermate controls. Pulmonary vascular contraction was not significantly attenuated in normoxic or chronic hypoxic TMS-dosed female and male mice (Figure 4C and 4D). Pulmonary vascular remodeling was also significantly attenuated by TMS in hypoxic mice (Figure 4E and 4F). In normoxia, TMS had no effect on RVH, RVSP, or pulmonary vascular remodeling.

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 that exhibits vascular pathology more consistent with

Figure 1. CYP1B1 is upregulated in murine pulmonary artery hypertension. 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). Two-way rank transform nonparametric ANOVA with Bonferroni pairwise comparisons.
human PAH. In chronic hypoxia, TMS attenuated RVH (Figure 5A), RVSP (Figure 5B), and pulmonary vascular remodeling (Figure 5C and 5D). TMS had a significant effect on occlusive lesion formation, with the number of fully occluded vessels being significantly reduced in TMS-dosed mice (Figure 5E and 5F). This effect was most significant in the female mice. In normoxia, TMS had no effect in RVH, RVSP, or pulmonary vascular remodeling. The combined experimental insult of chronic hypoxia and SU5416 had no effect in systemic arterial pressure.

**Contribution of CYP1B1 in 17β Estradiol–Induced Proliferation**

We and others have previously shown that physiological concentrations of 17β estradiol stimulate proliferation in hPASMCs; however, the mechanisms coordinating 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 overexpressed in IPAH-PASMCs. 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-hydroxyestrogen was the only metabolite that stimulated proliferation, and this was observed in a concentration-dependent manner. Intriguingly, 16α-hydroxyestrone evoked a hyperproliferative response in PAH-PASMCs compared with non-PAH (control) hPASMCs (Figure 6D).

**16α-Hydroxyestrone Promotes PAH in Mice**

Consistent with increased CYP1B1 overexpression and activity in PAH, 16α-hydroxyestrone urinary concentration was significantly increased in chronic hypoxic mice (Figure 7A). From these findings, we hypothesized that the CYP1B1-derived mitogen 16α-hydroxyestrone directly promotes the development of PAH in vivo. To test this directly, mice were administered 16α-hydroxyestrone for 28 consecutive days. This resulted in an increase of RVH (Figure 7B) and RVSP (Figure 7C) compared with 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 remodeling 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 uncover a novel role for CYP1B1 in the genesis and progression of PAH. We show...
that pulmonary CYP1B1 is increased in 2 independent models of experimental PAH and human PAH (IPAH and HPAH). As confirmed by in situ analysis, CYP1B1 is upregulated in the remodeled vasculature during experimental and human PAH and expressed within all cell types that compose the vascular wall, including 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 CYP1B1null mice and mice given the potent CYP1B1 inhibitor TMS. We observed that male CYP1B1null mice exhibited a significant attenuation in the development of PAH in response to chronic hypoxia.

Figure 3. Chronic hypoxic pulmonary arterial hypertension (PAH) in CYP1B1null mice. A, Right ventricular hypertrophy analysis in normoxic and chronic hypoxic wild-type (WT) and CYP1B1null female and male mice (n=8–12). Normoxia vs hypoxia P<0.0005; WT vs CYP1B1null female P=0.003, male P=0.0005. *P<0.05, **P<0.01, ***P<0.001 (pairwise comparison). RV/LV+S indicates right ventricular free wall and left ventricle plus septum. B, Right ventricular systolic pressure (RVSP) measurements in normoxic and chronic hypoxic WT and CYP1B1null female and male mice (n=7–11). Normoxia vs hypoxia, P<0.0005; WT vs CYP1B1null female, P=0.279; male, P=0.007. *P<0.05, **P<0.01 (pairwise comparison). C, Pulmonary vascular reactivity in normoxic WT and CYP1B1null female and male mice (n=5–7). No significant differences. Emax, no significant differences. D, Pulmonary vascular reactivity in chronic hypoxic WT and CYP1B1null female and male mice (n=5–7), pEC50, no significant differences. E, Pulmonary vascular remodeling in normoxic and chronic hypoxic WT and CYP1B1null female and male mice (n=5). No significant differences. Emax, no significant differences; hypoxia: WT vs CYP1B1null, P=0.0005; male vs female, P=0.015. ***P<0.001 (pairwise comparison). E, Representative α-smooth muscle actin–stained pulmonary arteries in normoxic and chronic hypoxic WT and CYP1B1null female and male mice. Scale bar=20 μm. Two-way rank transform nonparametric ANOVA (on female and male data for A, B, and E) with Bonferroni pairwise comparisons.
hypoxic insult, as reported through a significant attenuation in RVH, RVSP, pulmonary vascular contraction, and pulmonary vascular remodeling. In contrast, female CYP1B1−/− mice showed significant attenuation in RVH, which was reported in the absence of any changes in 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 remodeling.

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 wild-type mice. TMS-dosed mice exhibited significant attenuation in PAH response to chronic hypoxia.

Figure 4. The CYP1B1 inhibitor 2,3',4,5'-tetramethoxystilbene (TMS) attenuates the development of chronic hypoxic pulmonary arterial hypertension (PAH). A, Right ventricular hypertrophy analysis in normoxic and chronic hypoxic vehicle- and TMS-treated female and male mice (n=7–10). Normoxia vs hypoxia, P<0.005; vehicle vs TMS: female, P=0.183; male, P=0.036. *P<0.05, ***P<0.001 (pairwise comparison). RV/LV+S indicates right ventricular free wall and left ventricle plus septum. B, Right ventricular systolic pressure (RVSP) measurements in normoxic and chronic hypoxic vehicle- and TMS-dosed female and male mice (n=6–10). Normoxia vs hypoxia P<0.0005; 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). pEC50, 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). pEC50, no significant differences. Emax, no significant differences. E, Pulmonary vascular remodeling in normoxic and chronic hypoxic vehicle- and TMS-dosed female and male mice (n=5–7). Normoxia vs hypoxia, P<0.0005; vehicle vs TMS: female, P<0.04; male, P<0.0005. *P<0.05, **P<0.01, ***P<0.001 (pairwise comparison). F, Representative α-smooth muscle actin–stained pulmonary arteries in normoxic and chronic hypoxic vehicle- and TMS-dosed female and male mice. Scale bar=20 μm. Two-way rank transform nonparametric ANOVA (on female and male data for A, B, and E), with Bonferroni pairwise comparisons.
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 that 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 previously been shown to exhibit severe vascular remodeling 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 remodeling. The total number of occluded pulmonary vascular lesions was also significantly reduced in TMS-dosed mice compared with vehicle-dosed littermate controls.

In vitro, we demonstrate that the inhibition of CYP1B1 with TMS abolishes the proliferative effects of 17β estradiol in hPASMCs. These data provide 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 overexpression and activity during PAH. To precisely define those mitogen(s) that stimulate hPASMC proliferation, we compared the proliferative effects of all CYP1B1 metabolites, including 2-, 4-, and 16-hydroxyestrogens. Intriguingly, of all the metabolites assessed, 16α-hydroxyestrone was the only one that stimulated 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 with unaffected mutation carriers. Intriguingly, the proliferative effects of this mitogen were significantly more pronounced in PAH-PASMCs compared with hPASMCs. 16α-hydroxyestrone has previously been shown to cause proliferation via the upregulation of cyclin D1 expression, which is an important cell cycle regulator in PASMCs. 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 remodeling. 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 that 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 BMPR-II–affected PAH and has been previously cited as a promising therapeutic target in disease. Beyond PAH, the therapeutic value of CYP1B1 inhibition in the treatment and manage-
ment of cancer is currently under phase II clinical trial investigation. Here, our data suggest 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, little evidence exists to support this. Indeed, paradoxical findings from studies in rodent models have suggested that estrogen and its bioactive metabolites may actually prove beneficial against the development and progression of PAH. In addition, estrogen inhibits pulmonary artery vasoconstriction and promotes pulmonary artery vasodilatation in rats. These inconsistencies between human PAH and experimental PAH have diffused our current understanding of pathological estrogen signaling in PAH. Novel emerging animal models have helped to explain these anomalies. In vivo, we have previously shown that pulmonary CYP1B1 is significantly upregulated in SERT mice. In SERT PAH, only female mice exhibit spontaneous PAH, implying a causative role for female hormones. Indeed, the presence of circulating 17 estradiol is a requisite for PAH in these mice. In line with this, 17 estradiol growth effects in hPASMCs are dependent on serotonin signaling. Hence, we propose that in the presence of preexisting pulmonary hypertensive insult, estrogen can be causative in the development of PAH.

These findings may be pertinent to the widely reported sex differences observed in several forms of human PAH. We speculate that heightened circulating levels of estrogen coupled with CYP1B1 upregulation may render premenopausal women more susceptible to the increased formation of metabolites such as 16-hydroxyestrone, subsequently leading to the increased risk of PAH development.

Conclusions

We provide unique evidence to support a role for CYP1B1 in the genesis and progression of PAH. Aberrant CYP1B1 expression promotes pathological estrogen metabolism within the pulmonary vasculature, resulting in the formation of smooth muscle mitogens, including 16-hydroxyestrone. Further studies to establish the viability of CYP1B1 as a therapeutic target in human PAH are merited.

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Disclosures

None.

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CYP1B1 Promotes the Development of PAH


CLINICAL PERSPECTIVE

Pulmonary arterial hypertension (PAH) is a fatal condition with diverse origins that converge to promote pathological changes in the pulmonary vasculature. The nature of these origins is intriguing and stems from genetic and environmental factors to secondary risk factor–related disease. Estrogen is one such risk factor that has been causally related to PAH; however, the causation remains obscure. In this study, we have identified that the estrogen-metabolizing enzyme cytochrome P450 1B1 (CYP1B1) controls the formation of estrogen-derived mitogenic metabolites to drive vascular cell mitogenesis and PAH. Central to this, we report that CYP1B1 is robustly upregulated in 2 independent forms of clinical PAH, whereas the inhibition of this enzyme markedly inhibits the proliferative capacity of pulmonary artery smooth muscle cells isolated from PAH patients. In vivo, CYP1B1 is upregulated in 2 independent models of PAH, whereas the genetic ablation or pharmacological inhibition of CYP1B1 markedly attenuates the development of PAH, as reported in CYP1B1/−/− and 2,3',4,5'-tetramethoxystilbene–treated mice, respectively. Further investigation of estrogen metabolites reveals that 16α-hydroxyestrone is a key metabolite that robustly stimulates smooth muscle mitogenesis and promotes the development of PAH in mice in vivo. This study reinforces a pathogenic role of estrogen in human PAH and reveals for the first time the importance of estrogen metabolism in the genesis and progression of PAH.
Activity of the Estrogen-Metabolizing Enzyme Cytochrome P450 1B1 Influences the Development of Pulmonary Arterial Hypertension

Kevin White, Anne Katrine Johansen, Margaret Nilsen, Loredana Ciucian, Emma Wallace, Leigh Paton, Annabel Campbell, Ian Morecroft, Lynn Loughlin, John D. McClure, Matthew Thomas, Kirsty M. Mair and Margaret R. MacLean

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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.

CYPB1 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.  
   [Images of tissue sections labeled as a and b]
Figure S2.

(a) CYP1B1 fold change for normoxic and hypoxic conditions in females and males.

(b) CYP1B1 fold change for normoxic and hypoxic conditions in females and males.