Pulmonary Hypertension after Prolonged Hypoxic Exposure in Mice with a Congenital Deficiency of Cyp2j

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Abstract

Cytochrome P450 epoxygenase-derived epoxyeicosatrienoic acids contribute to the regulation of pulmonary vascular tone and hypoxic pulmonary vasoconstriction. We investigated whether the attenuated acute vasoconstrictor response to hypoxic exposure of *Cyp2j*^{-/-} mice would protect these mice against the pulmonary vascular remodeling and hypertension associated with prolonged exposure to hypoxia. *Cyp2j*^{-/-} and *Cyp2j*^{+/+} male and female mice continuously breathed an inspired oxygen fraction of 0.21 (normoxia) or 0.10 (hypoxia) in a normobaric chamber for 6 weeks. We assessed hemoglobin (Hb) concentrations, right ventricular (RV) systolic pressure (RVSP), and transthoracic echocardiographic parameters (pulmonary acceleration time [PAT] and RV wall thickness). Pulmonary *Cyp2c29*, *Cyp2c38*, and *sEH* mRNA levels were measured in *Cyp2j*^{-/-} and *Cyp2j*^{+/+} male mice. At baseline, *Cyp2j*^{-/-} and *Cyp2j*^{+/+} mice had similar Hb

levels and RVSP while breathing air. After 6 weeks of hypoxia, circulating Hb concentrations increased but did not differ between *Cyp2j*^{-/-} and *Cyp2j*^{+/+} mice. Chronic hypoxia increased RVSP in *Cyp2j*^{-/-} and *Cyp2j*^{+/+} mice of either gender. Exposure to chronic hypoxia decreased PAT and increased RV wall thickness in both genotypes and genders to a similar extent. Prolonged exposure to hypoxia produced similar levels of RV hypertrophy in both genotypes of either gender. Pulmonary *Cyp2c29*, *Cyp2c38*, and *sEH* mRNA levels did not differ between *Cyp2j*^{-/-} and *Cyp2j*^{+/+} male mice after breathing at normoxia or hypoxia for 6 weeks. These results suggest that murine *Cyp2j* deficiency does not attenuate the development of murine pulmonary vascular remodeling and hypertension associated with prolonged exposure to hypoxia in mice of both genders.

Keywords: hypoxia; cytochrome P450 epoxygenases; pulmonary vascular remodeling; right ventricular hypertrophy

Pulmonary hypertension (PH) due to increased vascular tone in lung vessels is important in many acute and chronic lung

diseases, such as pulmonary embolism and idiopathic pulmonary arterial hypertension. Although progress has been made with

therapeutic approaches to reduce vascular tone, all of the pathways influencing lung vascular tone have not been fully explored.

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Clinical Relevance

Products of cytochrome P450 epoxygenases have previously been implicated in the development of pulmonary hypertension. However, it is unknown which cytochrome P450 is the major contributor to the regulation of pulmonary hypertension and vascular remodeling. We report that a selective congenital *Cyp2j* deficiency does not attenuate the development of murine pulmonary hypertension after prolonged hypoxic exposure.

Recent experimental evidence suggests that arachidonic acid (AA) metabolites contribute significantly to regulating pulmonary vascular tone (1) and the development of PH (2, 3). AA is metabolized by cytochrome P450 (CYP) epoxygenases to a variety of bioactive eicosanoids (3–5). The CYP epoxygenases *Cyp2c* and *Cyp2j* catalyze the formation of epoxyeicosatrienoic acids (EETs), which have antiinflammatory properties and are associated with vasodilation of the systemic circulation (6, 7). In the pulmonary vasculature, EETs cause vasoconstriction (8) and are involved in the regulation of hypoxic pulmonary vasoconstriction (HPV) (6, 9) and the PH associated with prolonged exposure to hypoxia (2, 3). EETs are converted to their corresponding diols (dihydroxyeicosatrienoic acids [DHETs]) by soluble epoxide hydrolase (sEH) (10).

Several experimental studies have examined the role of the AA cascade in contributing to PH by studying the effects of chronic breathing at low ambient oxygen tensions to induce PH, pulmonary vascular remodeling, and right ventricular (RV) hypertrophy (1, 3). Ichinose and colleagues studied mice with a congenital deficiency of cyclic phospholipase A_2 (*cPLA*₂), an enzyme that lies upstream of CYP epoxygenases and releases AA from phospholipid membranes. The absence of *cPLA*₂ activity completely prevented acute HPV in mice. However, congenital *cPLA*₂ deficiency did not protect mice from developing chronic PH and vascular remodeling when *cPLA*₂^{−/−} mice breathed at an inspired oxygen fraction ($F_{I_{O_2}}$) of 0.10 for 3 weeks (1).

CYP epoxygenases are abundantly expressed in the lung (11), and *CYP2J* in particular is widely expressed in human

pulmonary endothelium (12). To investigate the contribution of CYP epoxygenases to chronic hypoxic PH, Pokreisz and colleagues (3) studied wild-type mice that were exposed to chronic hypoxia ($F_{I_{O_2}}$, 0.07) for 7 days with and without simultaneous treatment with N-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide (MS-PPOH), an inhibitor of CYP epoxygenases. Chronic hypoxic PH and vascular remodeling were attenuated in mice treated with MS-PPOH. However, MS-PPOH produces nonselective inhibition of CYP epoxygenases and thus cannot isolate the role of a particular CYP enzyme and determine its contribution to the development of chronic PH.

The aim of the present study was to selectively explore the role of *Cyp2j* in chronic hypoxic PH. We studied male and female mice with a targeted deletion of the entire murine *Cyp2j* locus syntenic to human *CYP2J2*. Because *Cyp2j*^{−/−} mice have impaired acute HPV (13), we hypothesized that *Cyp2j* deficiency might protect mice from developing chronic hypoxic PH and RV hypertrophy. Therefore, *Cyp2j*^{−/−} mice and their wild-type littermates (*Cyp2j*^{+/+}) of both genders were exposed to low ambient oxygen tensions (10%) for 6 weeks. Echocardiographic parameters (pulmonary acceleration time [PAT] and RV wall thickness) and RVSP were measured in closed-chest lightly anesthetized mice to obtain hemodynamic values with minimally invasive techniques. In addition, we measured circulating hemoglobin (Hb) concentrations and assessed RV hypertrophy by determining Fulton's ratio at autopsy. Pulmonary vascular remodeling was studied by quantification of intraacinar pulmonary vessels containing α -smooth muscle actin (α -SMA)-positive cells in their walls. To assess whether members of the *Cyp2c* subfamily and sEH could compensate for the lifelong *Cyp2j* deficiency, we measured pulmonary *Cyp2c29*, *Cyp2c38*, and *sEH* mRNA levels in *Cyp2j*^{−/−} and *Cyp2j*^{+/+} male mice.

We report that congenital *Cyp2j* deficiency did not attenuate the degree of PH, RV hypertrophy, or pulmonary vascular remodeling in mice of either gender breathing at low oxygen tensions for 6 weeks.

Materials and Methods

All animal experiments were approved by the Subcommittee on Research Animal Care

of the Massachusetts General Hospital, Boston, MA. We studied 59 10- to 20-week-old mice [B6(Cg)-*Tyr*^{−2J/J}] of both genders weighing 19 to 30 g (see Figure E1 in the online supplement), including *Cyp2j*^{−/−} mice on the B6(Cg)-*Tyr*^{−2J/J} background and their wild-type (*Cyp2j*^{+/+}) littermates.

Exposure to a Low-Oxygen Environment

Mice breathed at an $F_{I_{O_2}}$ of 0.10 (hypoxia) or 0.21 (normoxia) for 6 weeks as previously described (14). Additional information is available in the online supplement.

Hb Concentration

Hb concentrations were evaluated in mice before and after 6 weeks of normoxia or hypoxia as described previously (15). Additional information is available in the online supplement.

Transthoracic Echocardiography

Transthoracic echocardiography was obtained in lightly sedated mice (isoflurane 1%) breathing spontaneously using a 30-MHz transducer (Vevo 770; Visualsonics, Toronto, Ontario, Canada). We measured RV wall thickness and PAT to noninvasively estimate pulmonary arterial pressure before (baseline) and after exposure of mice to a hypoxic environment for 3 and 6 weeks (16). During baseline echocardiographic measurements before hypoxic exposure, mice breathed at $F_{I_{O_2}}$ 0.21. To maintain the hypoxic environment, during echocardiographic assessment of PAT and RV wall thickness after hypoxic exposure, the mice breathed at $F_{I_{O_2}}$ 0.1. Additional information is available in the online supplement.

Hemodynamic Measurements in Anesthetized Closed-Chest Mice

RV systolic pressure (RVSP) was measured as previously described (17). Additional information is available in the online supplement.

Quantitative Morphometry

After mice were killed (0.2 mg/kg pentobarbital, intraperitoneally), the airway and pulmonary vessels were perfused with 3% paraformaldehyde/0.1% glutaraldehyde in PBS at constant inflation pressures of 23 and 100 cm H₂O, respectively. Lung tissue was stored in 3% paraformaldehyde/0.1% glutaraldehyde for 24 hours and then

dehydrated and embedded in paraffin wax. Lung sections measuring 2 μm in thickness were then stained with an antibody against $\alpha\text{-SMA}$ (A5228; Sigma, St. Louis, MO) to evaluate pulmonary vascular remodeling. Additional information is available in the online supplement.

RV Hypertrophy

At the end of invasive hemodynamic studies, the mice were killed (0.2 mg/g pentobarbital, intraperitoneally), the heart was excised from the thoracic cavity, the atria removed, and the RV free wall was separated from the left ventricle, including the septum. Tissues were weighed (XE 100E; Denver Instruments, Arvada, CO). The ratio of the weight of the RV to the sum of the weights of the left ventricle and septum ($W_{\text{LV+S}}$) was calculated ($W_{\text{RV}}/W_{\text{LV+S}}$; Fulton's ratio).

RNA Preparation and Real-Time Quantitative PCR

The measurements of mRNA levels of *Cyp2c38*, *Cyp2c29*, *Ephx2*, and *Gapdh* were performed as previously described by using real-time quantitative PCR (13). *Ephx2* encodes sEH mRNA. The sequences of primers for *Cyp2c38* and *Cyp2c29* were listed previously (13). The forward primer for *Ephx2* is 5'-ACCACTCATGGATGAAAGCTACA-3', and the reverse primer is 5'-TCAGGTAGATTGGCTCCACAG-3'.

Statistical Analysis

All data are expressed as mean $\pm$ SEM. Statistical analysis was performed using Prism 5 software (GraphPad Software, Inc.). Additional information is available in the online supplement.

Results

All data presented in the main text of this manuscript were obtained from *Cyp2j^{+/+}* and *Cyp2j^{-/-}* male mice. To exclude the possibility that activity of EETs is gender dependent, we also studied whether the attenuated acute vasoconstrictor response to hypoxic exposure of *Cyp2j^{-/-}* female mice would protect female mice against the PH associated with prolonged exposure to hypoxia. In female as in male mice, we observed similar changes in Hb levels, hemodynamic parameters, transthoracic echocardiographic parameters (PAT and RV wall thickness), and Fulton's ratios in both genotypes after 6 weeks of hypoxic

breathing. Data obtained from female mice of both genotypes with hypoxic breathing are provided in the online supplement (Figures E2–E4; Table E1).

Hb Concentration

To evaluate whether *Cyp2j* deficiency altered the level of Hb during hypoxic exposure (18, 19), we measured circulating Hb concentrations in *Cyp2j^{+/+}* and *Cyp2j^{-/-}* mice before and after 6 weeks of exposure to hypoxia or normoxia. Hb concentration in whole blood, sampled from the tail vein, did not differ between *Cyp2j^{+/+}* and *Cyp2j^{-/-}* mice under normoxic conditions. After breathing at low oxygen tensions for 6 weeks, the Hb concentration was increased similarly in *Cyp2j^{+/+}* and *Cyp2j^{-/-}* mice ($P = 0.88$) (Figure 1).

Transthoracic Echocardiography

To obtain serial noninvasive measurements of RV wall thickness and to assess PAT, transthoracic echocardiography was performed in sedated *Cyp2j^{+/+}* and *Cyp2j^{-/-}* mice at baseline (before) and after hypoxic exposure for 3 and 6 weeks. PAT, which provides an estimate of pulmonary arterial pressure (16), and RV wall thickness (d_{RVfw}) did not differ between sedated *Cyp2j^{+/+}* and *Cyp2j^{-/-}* mice breathing air spontaneously (Figure 2). After hypoxic breathing for 3 or 6 weeks, PAT decreased equally in both genotypes (Figure 2A). The RV wall thickness increased progressively in *Cyp2j^{+/+}* and *Cyp2j^{-/-}* mice during chronic hypoxic exposure. No difference in RV wall thickness was observed between *Cyp2j^{+/+}* and *Cyp2j^{-/-}* mice after hypoxic breathing for 3 or 6 weeks (Figure 2B).

Hemodynamic Measurements in Anesthetized Closed-Chest *Cyp2j^{+/+}* and *Cyp2j^{-/-}* Mice

To further ascertain whether *Cyp2j* deficiency could prevent mice from developing PH associated with prolonged hypoxic exposure, invasive hemodynamic measurements were performed in *Cyp2j^{+/+}* and *Cyp2j^{-/-}* mice, comparing exposure with hypoxia or normoxia for 6 weeks. HR, SAP, and RVSP were measured in anesthetized closed-chest mice during mechanical ventilation at FiO_2 1. Hemodynamic parameters, including HR, SAP, and RVSP, did not differ between *Cyp2j^{+/+}* and *Cyp2j^{-/-}* mice breathing air for 6 weeks. Prolonged hypoxic exposure

increased RVSP similarly in *Cyp2j^{+/+}* and *Cyp2j^{-/-}* mice as compared with mice breathing air (Table 1). The HR, SAP, and RVSP levels did not differ between *Cyp2j^{+/+}* and *Cyp2j^{-/-}* mice after prolonged exposure to hypoxia.

Pulmonary Vascular Morphology

To evaluate whether murine *Cyp2j* deficiency alters pulmonary vascular remodeling (the number of intraacinar vessels with $\alpha\text{-SMA}$ -positive cells) after chronic hypoxic exposure, the number of pulmonary vessels with ≥ 15 and < 50 μm external diameter was assessed in *Cyp2j^{+/+}* and *Cyp2j^{-/-}* mice after breathing at hypoxia or normoxia for 6 weeks. Figure 3 shows representative histological images of lungs obtained from *Cyp2j^{+/+}* and *Cyp2j^{-/-}* mice. A similar number of pulmonary vessels was analyzed for both genotypes (Figure 4A). Mice of both genotypes breathing air had a similar percentage of intraacinar vessels with cells expressing $\alpha\text{-SMA}$ (Figure 4B). However, the number of pulmonary vessels with perivascular and endothelial cells expressing $\alpha\text{-SMA}$ was greater in *Cyp2j^{-/-}* than in *Cyp2j^{+/+}* mice after hypoxia ($P = 0.03$) (Figure 4B). In the vessels we analyzed, the $\alpha\text{-SMA}$ -positive cells were on or adjacent to the luminal surface (i.e., a mixed population of endothelial or perivascular cells).

RV Hypertrophy

To explore whether *Cyp2j* deficiency attenuates the development of RV hypertrophy associated with prolonged

Figure 1. Hb concentration in *Cyp2j^{+/+}* and *Cyp2j^{-/-}* male mice after breathing at hypoxia or normoxia for 6 weeks (*Cyp2j^{+/+}* normoxia [$n = 8$]; *Cyp2j^{+/+}* hypoxia [$n = 8$]; *Cyp2j^{-/-}* normoxia [$n = 7$]; *Cyp2j^{-/-}* hypoxia [$n = 11$]). * $P = 0.0001$ (differs versus value of the same parameter in normoxic group). All data are mean $\pm$ SEM (see the online supplement for Hb data in female mice). Hb, hemoglobin.

Figure 2. (A) Pulmonary acceleration time (PAT) and (B) right ventricular wall thickness (d_{RVW}) in *Cyp2j*^{+/+} and *Cyp2j*^{-/-} male mice measured by pulsed-wave Doppler. * $P = 0.02$ (differs versus baseline value); # $P = 0.0001$ (differs versus value at 3 weeks of breathing at low oxygen tensions). All data are mean $\pm$ SEM (see the online supplement for PAT and d_{RVW} data in female mice).

exposure to hypoxia, calculations of Fulton's ratio (W_{RV}/W_{LV+S}) were performed at autopsy in *Cyp2j*^{+/+} and *Cyp2j*^{-/-} mice. The ratio of W_{RV}/W_{LV+S} did not differ between *Cyp2j*^{+/+} and *Cyp2j*^{-/-} mice under normoxic conditions (Figure 5). After breathing at low oxygen tensions for 6 weeks, W_{RV}/W_{LV+S} increased equally in *Cyp2j*^{+/+} and *Cyp2j*^{-/-} mice (0.39 ± 0.014 and 0.36 ± 0.011 , respectively; $P = 0.22$).

Table 1. Invasive Hemodynamic Measurements in *Cyp2j*^{+/+} and *Cyp2j*^{-/-} Male Mice after Breathing at Normoxia or Hypoxia for 6 wk

Genotype	n	FiO ₂	HR (bpm)	SAP (mm Hg)	RVSP (mm Hg)
<i>Cyp2j</i> ^{+/+}	8	0.21	539 $\pm$ 20*	91 $\pm$ 3	25 $\pm$ 1
<i>Cyp2j</i> ^{-/-}	6	0.21	540 $\pm$ 16	91 $\pm$ 3	22 $\pm$ 2
<i>Cyp2j</i> ^{+/+}	8	0.1	526 $\pm$ 16	87 $\pm$ 3	39 $\pm$ 3 [†]
<i>Cyp2j</i> ^{-/-}	8	0.1	508 $\pm$ 19	90 $\pm$ 5	33 $\pm$ 1 [†]

Definition of abbreviations: FiO₂, fraction of inspired oxygen; HR, heart rate; RVSP, right ventricular systolic pressure; SAP, mean systemic arterial pressure.

*Data are mean $\pm$ SEM (see the online supplement for hemodynamic parameters data in female mice).

[†] $P = 0.0001$ differs versus the same value at normoxia.

Effects of Chronic Hypoxia on Pulmonary *Cyp2c29*, *Cyp2c38*, and *sEH* mRNA Levels in *Cyp2j*^{+/+} and *Cyp2j*^{-/-} Mice

In addition to members of *Cyp2j* subfamily, members of the *Cyp2c* family of cytochrome P450 enzymes, including *Cyp2c29* and *Cyp2c38*, metabolize AA to EETs (20, 21). To explore whether members of the *Cyp2c* family of cytochrome P450 epoxygenases compensate for the lifelong *Cyp2j* deficiency during 6 weeks of hypoxic breathing, we measured pulmonary *Cyp2c29* and *Cyp2c38* mRNA levels in *Cyp2j*^{+/+} and *Cyp2j*^{-/-} male mice breathing at FiO₂ 0.1 or 0.21 for 6 weeks. Pulmonary *Cyp2c29* and *Cyp2c38* mRNA levels did not differ between *Cyp2j*^{+/+} and *Cyp2j*^{-/-} male mice breathing at FiO₂ 0.21 or 0.1 for 6 weeks (Figures 6A and 6B). Breathing at FiO₂ 0.1 for 6 weeks decreased pulmonary *Cyp2c38* mRNA levels in *Cyp2j*^{+/+} and *Cyp2j*^{-/-} male mice to a similar extent and did not change pulmonary *Cyp2c29* mRNA levels in both genotypes (Figure 6B).

Because sEH converts EETs to their corresponding diols (DHETs), reduced sEH activity could counteract lifelong *Cyp2j* deficiency by reducing the metabolism of EETs, thereby potentiating pulmonary vasoconstriction mediated via EETs during the 6 weeks of hypoxic breathing. Thus, we measured pulmonary *sEH* mRNA levels in *Cyp2j*^{+/+} and *Cyp2j*^{-/-} male mice breathing at FiO₂ 0.1 or 0.21 for 6 weeks. Pulmonary *sEH* mRNA levels did not differ between *Cyp2j*^{+/+} and *Cyp2j*^{-/-} male mice breathing at FiO₂ 0.21 or 0.1 (Figure 6C).

We also measured pulmonary *Cyp2c29*, *Cyp2c38*, and *sEH* mRNA levels relative to GAPDH. Because the *Gapdh* promoter region contains hypoxia-responsive elements

(22), exposure of mice we studied to a hypoxic environment might alter the expression of *Gapdh*. Therefore, we measured pulmonary *Gapdh* expression (relative to actin) in the lungs of *Cyp2j*^{-/-} and *Cyp2j*^{+/+} male mice exposed to hypoxia and normoxia for 6 weeks. We did not detect any difference in *Gapdh* expression (relative to actin) between *Cyp2j*^{-/-} and *Cyp2j*^{+/+} mice exposed to hypoxia or normoxia for 6 weeks (Figure E5).

Discussion

We investigated the effects of congenital *Cyp2j* deficiency on the development of PH and vascular remodeling associated with prolonged hypoxic exposure in mice of both genders. The major finding of the present study is that, despite the absence of HPV upon acute exposure of *Cyp2j*^{-/-} mice to hypoxia, a congenital deficiency of *Cyp2j* in mice did not prevent the development of PH and RV hypertrophy associated with prolonged (6 wk) hypoxic exposure in mice of both genders. As a secondary finding, we report that *Cyp2j* deficiency increases the number of pulmonary vessels with cells expressing α -SMA during prolonged hypoxic exposure.

Chronic exposure to hypoxia in many species is a major cause of PH and increased pulmonary vascular resistance associated with pulmonary vascular remodeling (23). Recent studies emphasize the role of CYP epoxygenases and their metabolites in the regulation of pulmonary vascular tone during acute and chronic hypoxic exposure (2, 3). Michaelis and colleagues observed increased CYP2C protein levels in endothelial cells obtained from human umbilical veins after 8 hours of exposure to 1% O₂ (7). Earley and colleagues demonstrated that CYP2C9 protein and EET levels were increased in the mesenteric vessels of rats after 2 days of hypoxic breathing (24). CYP enzymes are widely expressed in the lung, and CYP2J2 expression is particularly prominent in the human pulmonary endothelium (12). Pokreisz and colleagues reported a transient increase of pulmonary CYP2C protein levels in mice after breathing 7% O₂ for 1 week. In their study, doubling of CYP2C protein levels in whole lungs was measured after 24 hours, returning to baseline levels at 1 week of hypoxia (3).

Figure 3. Representative images of pulmonary vasculature of *Cyp2j*^{+/+} and *Cyp2j*^{-/-} male mice after 6 weeks of breathing at normoxia or hypoxia. * α -smooth muscle actin (SMA)-negative alveolar vessels; $\uparrow$ α -SMA-positive alveolar vessels. Scale bars, 25 μ m.

The availability of novel gene-targeting techniques allowed us to determine the role of murine *Cyp2j* in the regulation of chronic pulmonary vascular responses to hypoxic exposure in mice. In a previous study, we demonstrated that *Cyp2j*-deficient mice exhibit impaired HPV (13). Based on these data, in the present study we hypothesized that *Cyp2j* deficiency would prevent the development of chronic PH and vascular remodeling during prolonged exposure to hypoxia.

Because hypoxic breathing induces RV hypertrophy, we measured murine RV wall

thickness and PAT using transthoracic echocardiography, which provides an *in vivo* estimate of pulmonary arterial pressure (16). After breathing at low oxygen tensions for 3 and 6 weeks, the PAT decreased equally in both genotypes, indicating a similar increase of pulmonary arterial pressure in hypoxic *Cyp2j*^{+/+} and *Cyp2j*^{-/-} mice. We also performed invasive hemodynamic measurements in anesthetized and ventilated mice breathing hypoxic or normoxic gas mixtures for 6 weeks. PH, as measured by RVSP, was increased in hypoxic *Cyp2j*^{+/+} and *Cyp2j*^{-/-}

mice to a similar extent. After breathing at low oxygen tensions for 3 and 6 weeks, the right ventricle became equally hypertrophied in both genotypes, as assessed by echocardiographic wall thickness measurements. At autopsy after 6 weeks of hypoxic exposure, the degree of right heart hypertrophy (Fulton's ratio) did not differ between *Cyp2j*^{+/+} and *Cyp2j*^{-/-} mice. Taken together, these results demonstrate that congenital *Cyp2j* deficiency did not protect mice against PH and RV hypertrophy during 6 weeks of hypoxic breathing.

The products of AA metabolism by a variety of CYP epoxygenases (EETs) have been proposed to influence pulmonary vascular remodeling based on the observation that in mice a nonselective inhibitor of all CYP epoxygenases (MS-PPOH) attenuated pulmonary artery muscularization induced by exposure to hypoxia for 7 days (3). In contrast, Zheng and colleagues demonstrated that gene delivery of human *CYP2J2* (and presumably greater EET levels) ameliorated monocrotalin-induced pulmonary vascular remodeling in rats (25). Revermann and colleagues reported that inhibition of EET metabolism attenuated monocrotaline (MCT)-induced pulmonary vascular remodeling in rats (26). These results demonstrate that CYP epoxygenases and their metabolites (EETs) may play different roles in various models of pulmonary arterial hypertension.

To date, the most commonly used rodent models for studying PH include exposure of rodents to either a hypoxic environment or MCT (27). The response to MCT is variable among strains and animals due to differences in the hepatic metabolism of MCT by cytochrome P450 enzymes. MCT causes direct endothelial damage (28), which could impair EET signaling through endothelial cells (29). In the present study, we studied a murine model of hypoxia-induced PH because the murine hypoxia model reliably induces PH.

In the present study, after 6 weeks of hypoxic exposure we observed a greater number of pulmonary vessels with perivascular and endothelial cells expressing α -SMA in *Cyp2j*^{-/-} as compared with *Cyp2j*^{+/+} male mice. This finding indicates that *Cyp2j* deficiency induced pulmonary vascular neomuscularization presumably by the distal migration of smooth muscle cells into alveolar cells (30). Alternatively, this

Figure 4. Quantitative morphometric analysis of pulmonary vessels containing α -SMA-positive cells in *Cyp2j*^{+/+} and *Cyp2j*^{-/-} male mice after 6 weeks of breathing at normoxia or hypoxia ($n = 5$ per group). (A) Number of intraacinar vessels ≥ 15 and < 50 μ m in external diameter. (B) Proportion of pulmonary vessels ≥ 15 and < 50 μ m in external diameter containing α -SMA-positive cells. * $P = 0.0001$ (differs versus value of the same parameter at normoxia). \$ $P = 0.003$ (differs versus value of the same parameter of *Cyp2j*^{+/+} mice after 6 wk of hypoxic breathing). All data are mean $\pm$ SEM.

Figure 5. Fulton's ratio (W_{RV}/W_{LV+S} ; i.e., the ratio of the weight of the right ventricle to the sum of the weights of the left ventricle and septum) in *Cyp2j*^{+/+} and *Cyp2j*^{-/-} male mice breathing air for 6 weeks (normoxia) and after 6 weeks of hypoxic breathing (*Cyp2j*^{+/+} normoxia [$n = 8$]; *Cyp2j*^{+/+} hypoxia [$n = 8$]; *Cyp2j*^{-/-} normoxia [$n = 7$]; *Cyp2j*^{-/-} hypoxia [$n = 11$]). * $P = 0.0001$ (differs versus value at normoxia). All data are mean $\pm$ SEM (see the online supplement for Fulton's ratio data in female mice).

neomuscularization may result from the development of precursor SMCs surrounding the pulmonary vessels (31, 32). The noted shift to α -SMA expression by endothelial cells we report likely occurs in the absence of the development of a filament network characteristic of contractile cells (31, 32). Prolonged exposure to decreased oxygen concentrations results in marked phenotypic changes in all of the cells that comprise the pulmonary artery (23). NF- κ B appears to be one of the central transcription factors responsible for these phenotypic changes (23) and is activated by hypoxia (33, 34). Node and colleagues demonstrated that CYP2J2-derived eicosanoids inhibit NF- κ B in human endothelial cells obtained from coronary arteries (35). It is conceivable that *Cyp2j* deficiency is associated with increased activity of NF- κ B during hypoxia, which might augment the phenotypic changes of

cells comprising the walls of the murine pulmonary vasculature.

Alternatively, it is possible that cytochrome P450 epoxygenases other than *Cyp2j* can contribute to the development of PH in *Cyp2j*^{-/-} mice. We have previously reported that *Cyp2c* genes are expressed in the lungs of *Cyp2j*^{-/-} mice of both genders (13). We reported that pulmonary *Cyp2c29* and *Cyp2c38* mRNA levels did not differ between *Cyp2j*^{+/+} and *Cyp2j*^{-/-} mice breathing at FiO_2 0.21 (13). Therefore, we hypothesized that *Cyp2c* family members could compensate for the absence of *Cyp2j* in the development of murine PH induced by chronic hypoxia. After 6 weeks of exposure to a hypoxic environment, pulmonary *Cyp2c29* and *Cyp2c38* mRNA levels did not differ between *Cyp2j*^{+/+} and *Cyp2j*^{-/-} male mice. Breathing at FiO_2 0.1 for 6 weeks decreased pulmonary *Cyp2c38* mRNA levels in *Cyp2j*^{+/+} and *Cyp2j*^{-/-} male mice to a similar extent and did not change pulmonary *Cyp2c29* mRNA levels in both genotypes. These findings indicate that these *Cyp2c* family members do not compensate for the life-long *Cyp2j* deficiency during 6 weeks of hypoxic breathing. Pokreisz and colleagues reported that pulmonary *Cyp2c29* mRNA increased ~ 5 -fold after exposure of wild-type mice to hypoxia (FiO_2 , 0.07) for 2 hours, remained elevated for 6 hours, and returned to baseline levels after 24 hours of hypoxic breathing (3). Pokreisz and colleagues also reported that pulmonary *Cyp2c* protein levels were increased after exposure of wild-type mice to hypoxia and returned to baseline after 7 days of hypoxic breathing (3).

Because sEH converts EETs to their corresponding diols (DHETs), a decrease of

sEH activity could potentially counteract a lifelong *Cyp2j* deficiency by reducing the metabolism of EETs, thereby potentiating murine pulmonary vasoconstriction mediated via EETs during 6 weeks of hypoxic breathing. Keseru and colleagues demonstrated that exposure of wild-type mice to hypoxia (FiO_2 0.1) for 3 weeks decreased murine pulmonary sEH expression (2). We report that pulmonary sEH mRNA levels did not differ between *Cyp2j*^{+/+} and *Cyp2j*^{-/-} male mice breathing at FiO_2 0.21 or 0.1 (Figure 6C). It is conceivable that in our study sEH expression was reduced at an earlier time, decreasing EET metabolism, and returning to baseline after 6 weeks of hypoxic breathing. Alternatively, it is possible that currently unappreciated compensatory metabolic mechanisms synthesized EETs in *Cyp2j*^{-/-} mice during breathing at FiO_2 0.1 for 6 weeks, thereby contributing to the development of PH in *Cyp2j*^{-/-} mice. Prolonged exposure to a hypoxic environment may increase basal pulmonary vascular tone by modifying pulmonary arterial myocyte function, augmenting synthesis of other vasoactive factors or enhancing responsiveness to these mediators (36), thereby contributing to the development of PH and vascular remodeling in *Cyp2j*^{-/-} mice.

Synthesis of EETs appears to be highly gender dependent in different vascular beds (37, 38). In the present work, we did not observe gender-specific differences in the development of PH associated with prolonged hypoxic exposure in *Cyp2j*^{-/-} mice. In our previous study, we reported a similar response to acute alveolar hypoxia in male and female *Cyp2j*^{-/-} mice (13). Thus, unlike other gender-specific

Figure 6. Pulmonary *Cyp2c29* (A), *Cyp2c38* (B), and soluble epoxide hydrolase (C) mRNA levels in *Cyp2j*^{+/+} and *Cyp2j*^{-/-} male mice ($n = 5$ per group) after breathing at hypoxia or normoxia for 6 weeks. * $P < 0.05$ (differs versus the same parameter in the normoxic group). All data are mean $\pm$ SEM. *Gapdh*, glyceraldehyde phosphate dehydrogenase.

pathways involved in the regulation of cardiovascular tone (e.g., the soluble guanylate cyclase-derived cGMP signaling pathway [39]), the cytochrome P450_{2j} epoxigenase signaling pathway appears not to be gender specific in the regulation of murine pulmonary vascular tone.

Prolonged exposure to hypoxia leads to increased erythropoiesis. Erythropoietin-dependent erythropoiesis is increased by certain AA metabolites, including PGE₂ (40, 41). Abraham and colleagues demonstrated that human bone marrow cells metabolize AA via CYP enzymes to compounds with powerful erythropoietic-potentiating activity (42). In the present study, prolonged hypoxic breathing increased Hb

levels equally in *Cyp2j*^{+/+} and *Cyp2j*^{-/-} mice; therefore, *Cyp2j* deficiency is unlikely to impair hypoxic erythropoiesis.

There are several limitations of our study: (1) we did not measure EETs and DHETs in mice of both genotypes breathing at normoxia or hypoxia; (2) transcriptional activity of *Cyp2c29*, *Cyp2c38*, and *sEH* mRNA might not necessarily represent the protein levels of *Cyp2c29*, *Cyp2c38*, and *sEH* in *Cyp2j*^{+/+} and *Cyp2j*^{+/+} mice; and (3) we did not perform cell culture studies exploring the contribution of *Cyp2j* to increasing the number of pulmonary vessels expressing α -SMA during prolonged hypoxic exposure.

In conclusion, we report that physiological development of PH, RV hypertrophy, and pulmonary vascular remodeling during prolonged hypoxic stress were not attenuated in mice with a targeted deletion of the entire murine *Cyp2j* locus syntenic to human *CYP2J2*. These findings indicate that, although *Cyp2j* appears involved in the regulation of acute HPV in mice (13), *Cyp2j* deficiency does not protect mice from the development of PH and vascular remodeling associated with prolonged exposure to hypoxia. ■

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

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