Cytochrome P450 Epoxidegenase Gene Function in Hypoxic Pulmonary Vasoconstriction and Pulmonary Vascular Remodeling

Peter Pokreisz, Ingrid Fleming, Ladislau Kiss, Eduardo Barbosa-Sicard, Beate Fisslthaler, John R. Falck, Bruce D. Hammock, In-Hae Kim, Zsolt Szelid, Pieter Vermeersch, Hilde Gillijns, Marijke Pellens, Friedrich Grimminger, Anton-Jan van Zonneveld, Desire Collen, Rudi Busse, Stefan Janssens

Abstract—We assessed pulmonary cytochrome P450 (CYP) epoxygenase expression and activity during hypoxia and explored the effects of modulating epoxygenase activity on pulmonary hypertension. The acute hypoxic vasoconstrictor response was studied in Swiss Webster mice, who express CYP2C29 in their lungs. Animals were pretreated with vehicle, the epoxygenase inhibitor (N-methylsulfonyl-6-[2-propargyloxyphenyl] hexanamide) or an inhibitor of the soluble epoxide hydrolase. Whereas the epoxygenase inhibitor attenuated hypoxic pulmonary constriction (by 52%), the soluble epoxide hydrolase inhibitor enhanced the response (by 39%), indicating that CYP epoxygenase–derived epoxyeicosatrienoic acids elicit pulmonary vasoconstriction. Aerosol gene transfer of recombinant adenovirus containing the human CYP2C9 significantly elevated mean pulmonary artery pressure and total pulmonary resistance indices, both of which were sensitive to the inhibitor sulfaphenazole. The prolonged exposure of mice to hypoxia increased CYP2C29 expression, and transcript levels increased 5-fold after exposure to normobaric hypoxia (FIO₂ 0.07) for 2 hours. This was followed by a 2-fold increase in protein expression and by a significant increase in epoxyeicosatrienoic acid production after 24 hours. Chronic hypoxia (7 days) elicited pulmonary hypertension and pulmonary vascular remodeling, effects that were significantly attenuated in animals continually treated with N-methylsulfonyl-6-[2-propargyloxyphenyl] hexanamide (−46% and −55%, respectively). Our results indicate that endogenously generated epoxygenase products are associated with hypoxic pulmonary hypertension in mice and that selective epoxygenase inhibition significantly reduces acute hypoxic pulmonary vasoconstriction and chronic hypoxia-induced pulmonary vascular remodeling. These observations indicate potential novel targets for the treatment of pulmonary hypertension and highlight a pivotal role for CYP epoxygenases in pulmonary responses to hypoxia. (Hypertension. 2006;47:762-770.)

Key Words: endothelium-derived factors ■ arachidonic acids ■ endothelium-derived factors ■ lung ■ remodeling

Pulmonary hypertension (PH) is a progressive disease of the pulmonary vasculature characterized by sustained pulmonary vasoconstriction, increased vascular resistance with excessive smooth muscle growth, and in situ thrombosis.1 Among the variety of signal transduction pathways in pulmonary endothelial cells influencing vascular reactivity and architectural changes, cyclooxygenase and lipoxygenase products of arachidonic acid metabolism have been well characterized, and the results have been clinically applied, but much less was known about the cytochrome P450 (CYP)–mediated effects on pulmonary circulation.2,3 Epoxide and hydroxy derivatives of arachidonic acid and their respective metabolites have species-, sex-, and organ-specific autocrine and paracrine effects in the cardiovascular system and modulate vasomotor tone, ion channel activity, and cell proliferation.4,5 In the blood vessels of the kidney, brain, and heart, CYP-derived epoxyeicosatrienoic acids (EETs) hyperpolarize smooth muscle cells resulting in vascular relaxation,6,7 whereas in isolated pulmonary arteries vasoconstrictor effects have been observed.8 The majority of EETs generated by endothelial and smooth muscle cells are metabolized by the soluble epoxide hydro-
lase (sEH) to form the corresponding dihydroxy derivatives (DHETs).\(^9\)

CYP isoforms in lungs generate a series of regiospecific and stereospecific epoxides in the airways, as well as in pulmonary artery microsomes\(^4,10\) with comparable product profiles being evident among some species (eg, human CYP 2C9 and mouse CYP 2C29). The biological role of epoxygenases in the regulation of the pulmonary circulation remains unclear and has largely been inferred from in vitro experiments that studied the consequences of the exogenous administration of synthetic EETs or nonspecific enzyme inhibitors. However, experiments in isolated perfused lungs and in pulmonary arterial rings have generated conflicting results\(^8,11\) and are not truly representative of function in vivo, inasmuch as vasomotor tone is markedly different in isolated lungs\(^12\) and isolated pulmonary artery rings are conduit vessels, which do not participate in the regulation of pulmonary vascular resistance.

Because the expression and activity of several CYP proteins are sensitive to changes in oxygen tension,\(^13,14\) it is tempting to suggest that their expression and/or activity may be implicated in the development of hypoxic PH. The aims of the present investigation were: (1) to assess the role of CYP epoxygenase gene function in murine lungs in normoxia and after exposure to normobaric hypoxia; (2) to investigate the role CYP-derived products in acute hypoxic pulmonary vasoconstriction; (3) to monitor the hemodynamic consequences of lung-specific epoxygenase overexpression, and (4) to determine whether pharmacological modulation of endogenous CYP activity could influence chronic hypoxia-induced PH.

Methods

Experimental Animals and Chemicals

The Ethics Committee for Animal Experimentation, University of Leuven, approved all of the protocols on Swiss Webster pathogen-free mice (30 to 35 g, supplied by the university animal facility). For all of the hemodynamic measurements, mice were anesthetized using urethane (1.4 g/kg body weight), whereas a short-acting ketamine xylazine (100 mg and 12 mg/kg body weight, respectively) combined anesthetic was used for subcutaneous implantation of osmotic minipumps and aerosol gene transfer. Unless indicated, all of the chemicals were purchased from Sigma-Aldrich, Merck, or Acros. The epoxygenase inhibitor N-methylsulfonyl-6-(2-propargyloxyphenyl) hexanamide (MSPPOH) was described previously.\(^15\) A novel water soluble sEH inhibitor, 1-adamantan-1-yl-3-[5-(2-ethoxyethoxy)ethoxy] pentyll) urea (IK-950), was synthesized and tested (details can be found in an online supplement available at http://www.hypertensionaha.org).

Role of Endogenous Epoxygenase in Acute Hypoxic Pulmonary Vasoconstriction

To evaluate the effect of epoxygenase or epoxide hydrolase inhibition on acute hypoxic pulmonary vasoconstriction (HPV), mice received 30 mg/Kg body weight MSPPOH or 10 mg/kg body weight IK-950 in 50 μL vehicle or vehicle alone (45% 2-hydroxypropyl-β-cyclodextrin in physiological solution) via tail vein injection.\(^16,17\) After 1 hour, mice were anesthetized, ventilated with room air (250 μL tidal volume, 150 strokes/min), and a Millar catheter was introduced via the right jugular vein into the right ventricle. After baseline recordings, the ventilator was switched to 7% oxygen balanced with nitrogen, and right ventricular systolic pressure (RVSP) changes were recorded for additional 15 minutes.

Mouse Endogenous Pulmonary CYP2C Expression and Activity

Female mice were exposed to normobaric hypoxia (FIO\(_2\)=0.07) for 2, 6, 24, and 168 hours (n=5 for each time point). Littermates kept in room air served as controls (n=8). After euthanasia, the lungs were perfused with saline and dissected, snap frozen in liquid nitrogen, and stored at −80°C until additionally processed.

Transcript Levels of the Murine CYP2C29 Isoform

Real-time PCR was performed in duplicate (PE 7700, Applied Biosystems) using the specific probes for murine genes encoding hypoxanthon phospho-ribosyl transferase and CYP2C29 (FAM-5′-CTTATACAGACGCCATGTTCATGAGGTTCC-3′-TAMRA, forward primer 5′-CATGCAAGACAGGACCACA-3′, and reverse primer 5′-CTGCAATGGCGACGGTATG-3′). The results are expressed as relative copy numbers standardized to the housekeeping hypoxanthon phospho-ribosyl transferase gene.\(^18\)

Endogenous CYP2C Expression

Immunoblot analysis of extracted pulmonary proteins was performed using lungs harvested at each time point (see online data supplement), using polyclonal rabbit anti-CYP 2C (Acris, Hiddenshausen, Germany) and monoclonal anti-actin (MAB 1501, CHEMICON Europe, Ltd, Hampshire, United Kingdom).

Analysis of CYP-Derived Eicosanoids

The conversion of arachidonic acid to EETs and DHETs was assayed in lung microsome preparations and analyzed using liquid chromatography with tandem mass spectrometry detection (see online data supplement).

Adenovirus-Mediated Gene Transfer of CYP 2C9

Construction of Recombinant Adenoviruses

Adenoviral vectors containing a double expression cassette for green fluorescent protein (GFP) and the 1.9-kb human CYP 2C9 cDNA (AdCYP2C9) were produced using the AdEasy system.\(^19\) Adenoviral vectors carrying no transgene (AdR5) or expressing the bacterial β-galactosidase (AdLacZ) were used as controls.\(^20\)

Effect of AdCYP2C9-Derived Eicosanoids on Pulmonary Artery Smooth Muscle Cells In Vitro

Cultured murine pulmonary artery smooth muscle cells were challenged for 10 or 60 minutes with conditioned media from uninfected and AdCYP2C9- and AdR5-infected HeLa cells (see data supplement). The expression and phosphorylation status of extracellular signal-regulated protein kinases (ERK1/2, Cellular Signaling Technology) was evaluated using immunoblot analysis.

Gene Transfer in Murine Lungs

The exposed trachea of spontaneously breathing, anesthetized mice was punctured using a 26-gauge needle, and 2 separate boluses of adenoviral vectors (5×10\(^{10}\) optical particle units [OPUs]),\(^21\) in 50 μL of physiological solution containing 5% sucrose) were aerosolized in the right and left main bronchi using the PennCentury Microsprayer (DeLong Distributors). After aerosol treatment, the muscle and skin layers were closed, and animals were allowed to recover.

Detection of Transgene Expression and Immunohistological Analysis in Murine Lungs

Three days after AdCYP2C9 delivery, GFP expression was examined in excised lungs using the dissection microscope connected to a digital camera (Zeiss, LP505-nm filter) and laser scanning confocal microscopy (Axiovert 100 mol/L, LSM510 software, Zeiss; Ar laser, excitation 488 nm, emission filter LP505). Proteins from transfected and control lungs were extracted and immunoblotted using polyclonal rabbit anti-CYP 2C and monoclonal anti-actin (MAB 1501, Chemicon) as described. In AdLacZ-infected mice, β-galactosidase expression was examined using histochemical staining.\(^22\) Inflammation was evaluated on perfusion-fixed (4% formaldehyde) tissue sections by comparing the number of CD45 and Mac-3 positive cells.

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in control and in adenovirus-infected lungs (1:100, anti-CD45, No. 553076, and anti-Mac-3, No. 553322, BD Pharmingen).

**Hemodynamic Measurements After Gene Transfer**

Animals were aerosolized with AdRR5 and AdCYP2C9 and received daily intraperitoneal injections of sulfaphenazole (5 mg/kg), a CYP2C9-specific inhibitor,22,23 or equal volume of vehicle. Untreated animals served as control. After 3 days, right ventricular and pulmonary artery pressures were recorded in anesthetized, temperature-controlled (Hugo-Sachs) mice using a fluid-filled Hyman-catheter (NuMed) inserted via the right jugular vein together with a pediatric 0.01-in guide wire (Guidant) under fluoroscopic guidance (Siemens). Cardiac output was measured using a thermodilution method.24 Total pulmonary resistance index (TPRI) was calculated and normalized to body weight.

Systemic blood pressures were recorded in the left carotid artery using the 1.4F Millar catheter (Millar Instruments Inc). Electronic signals were processed using the PowerLab system and Chart 4.1 software (ADInstruments).

**Epoxygenases Inhibition in Chronic Hypoxic PH**

Osmotic mini-pumps (Alzet, 1 µL/h, 7 days; Durect Corp) containing MSPPOH (30 mg/kg per day) or vehicle alone (as above) were implanted subcutaneously. After recovery, mice were exposed to 7% hypoxia for 7 days. Animals kept in room air served as controls. After 7 days, RVSP and systemic pressure were recorded in the left carotid artery using the 1.4F Millar catheter (Millar Instruments Inc). Electronic signals were processed using the PowerLab system and Chart 4.1 software (ADInstruments).

**Statistical Analyses**

All of the values are given as the mean±SEM. ANOVA or ANOVA for repeated measurements was performed and Student–Newman–Keuls or Bonferroni tests were subsequently used to identify differences between respective treatment groups. Data were analyzed using the PRISM software (Graphpad Inc), and significance was defined as $P<0.05$.

**Results**

**Role of Endogenous CYP Epoxygenases in Hypoxic Pulmonary Vasoconstriction**

Hypoxia induced a rapid (within 1 minute) increase in RVSP that was maintained over the experimental period (up to 15 minutes). Pretreatment with MSPPOH (30 mg/kg) significantly inhibited hypoxia-induced pulmonary vasoconstriction and reduced RVSP, whereas the sEH inhibitor IK-950 (10 mg/kg) potentiated the vasoconstrictor response (Figure 1). In sEH inhibitor-treated mice, the acute hypoxia-induced increase in RVSP was more rapid than in animals treated with vehicle or MSPPOH (31 ± 1.2 versus 27 ± 0.4 and 26 ± 0.6 mm Hg, respectively; $P<0.01$). In contrast, mean systemic blood pressure was unaffected (76 ± 6 mm Hg in vehicle-treated mice versus 78 ± 8 mm Hg in MSPPOH- and 79 ± 0.3 in IK-950-treated mice, $n=5$ in each group).

**Adenovirus-Mediated CYP2C9 Transfer**

To study the consequences of increased CYP2C activity on pulmonary hemodynamics, we generated a CYP2C9-expressing adenovirus. Conditioned medium from AdCYP2C9-infected but not from AdRR5-infected or uninfected HeLa cells contained abundant amounts of 11,12- and 14,15-EETs (see online data supplement) and was able to elicit the phosphorylation/activation of ERK 1 and 2, whereas the basal ERK expression remained unchanged (Figure 2).

Successful CYP2C9 gene transfer was confirmed by immunoblot analysis (Figure 3A). Aerosol administration of AdLacZ (5×10¹⁰ OPU) resulted in homogeneous β-galactosidase expression in both lungs. β-Galactosidase (as evidenced by the blue coloration of the nuclei) was expressed in epithelial cells of distal airways, terminal bronchioli, and alveolar lining cells, as well as in perivascular cells (Figure 3B), but was not detected in...
control lungs (Figure 3C). GFP expression was detectable in all of the freshly isolated lung lobes and extended from the large airways to the pleural surfaces but was absent from the heart of infected mice (Figure 3D). Confocal laser scanning microscopy with 3D reconstruction imaging confirmed equal distribution of the transgene throughout all lobes of the lung (Figure 3E). To assess whether inflammatory cell infiltration could contribute to the observed changes in vasomotor tone, we compared the expression of CD45, a common leukocyte antigen (Figure 3F and 3G) and Mac3, a macrophage specific antigen (Figure 3H and 3I) in both AdCYP2C9-infected (Figure 3G and 3I) and uninfected control lungs (Figure 3F and 3H). There were no significant differences in inflammatory cell infiltration in adenovirus-infected lungs.

**Hemodynamic Measurements After CYP2C9 Overexpression**

Three days after aerosol gene transfer RVSP and mean pulmonary artery pressure (mPAP) were significantly higher in AdCYP2C9-infected animals than in AdRR5-infected or
control animals (Figure 4A and 4B). The increase in RVSP and mPAP in AdCYP2C9-infected mice was prevented by the CYP2C9-specific inhibitor sulfaphenazole, which on its own did not influence EET production by endogenous murine epoxygenases (data not shown). The cardiac index was similar in AdCYP2C9-infected animals in the presence or absence of sulfaphenazole (5 mg/kg) and in AdRR5-infected and control mice. The TPRI was significantly higher in AdCYP2C9-infected than in AdRR5-infected or control animals, and the increase was prevented by the CYP2C9 inhibitor (Figure 4C). Vehicle alone had no effect on mPAP or TPRI, and mean systemic blood pressure was comparable in AdCYP2C9-infected and control animals (78 mm Hg versus 76 mm Hg), indicating that overexpression of CYP2C9 selectively affected the pulmonary vasculature.

**Effect of Hypoxia on the Expression and Activity of Endogenous CYP2C29**

CYP2C29 mRNA was detectable in the lungs of mice maintained under normoxic conditions and increased by ~5-fold after exposure of the animals to hypoxia for 2 hours. Transcript levels remained elevated in the lungs of animals subjected to hypoxia for 6 hours but returned to baseline levels after 24 hours of hypoxia and tended to rise again after 1 week (Figure 5A). The CYP2C protein levels also transiently increased after exposure to hypoxia. The 2-fold peak was detected after 24 hours and returned to baseline after 7 days of hypoxia (Figure 5B and 5C).

To determine whether the hypoxia-induced increase in CYP 2C expression was associated with an increased enzymatic activity, mice were exposed to 7% hypoxia and euthanized after 2, 6, and 24 hours and 7 days. Pulmonary microsomes from these mice rapidly converted arachidonic acid, and liquid chromatography with tandem mass spectrometry detection analysis of metabolite profile revealed significantly elevated levels of 8,9-, 11,12-, and 14,15-EETs/DHETs after 24 hours. In absolute values, more 8,9-EET/DHET was generated, whereas the ability to generate 11,12- and 14,15-EET/DHET was also detected in microsomes prepared from animals exposed to hypoxia for 1 week (Figure 6). Thus, although protein levels tended to decline with time of exposure to hypoxia, the ability to generate EETs/DHETs was maintained in the lungs of animals chronically exposed to hypoxia.

**Endogenous Epoxygenases in Chronic Hypoxic PH**

Mice exposed to chronic hypoxia (FIO₂=0.07; 7 days) developed PH, as evidenced by a significantly elevated RVSP, right ventricular hypertrophy, and remodeling of pulmonary resistance vessels. Simultaneous administration of the epoxygenase inhibitor reduced the increase in RVSP and attenuated vascular remodeling. In control and vehicle-treated animals, the RVSP increased from 24.7 ±0.9 mm Hg (n =6) at baseline to 34.9 ±0.5 mm Hg (n =8) and 35.0 ±0.7 mm Hg (n =9), respectively. In contrast, MSPPOH significantly reduced the pressure rise (29.4 ±0.9 mm Hg, n =9; P<0.01 versus vehicle and control; Figure 7A). Mean arterial pressures and heart rates were comparable in all of the groups (Table).

The changes in pulmonary hemodynamics were accompanied by significant architectural alterations in resistance vessels characterized by distal smooth muscle cell extension. We observed a significant reduction in the number of partially muscularized vessels in MSPPOH-treated mice compared with control or vehicle-treated mice (Figure 7B and 7C). Fully muscularized intraacinar vessels became clearly distinguishable after chronic hypoxia, especially in control and in vehicle-treated mice (1.4 ±0.4% and 2.4 ±0.8%, respectively, n =8 for each). This phenomenon was much less marked in MSPPOH-treated animals (0.9 ±0.5%, n=8; P<0.05). The reduction of PH after MSPPOH treatment resulted in a reduced afterload to the right heart and a concomitant reduction in right ventricular weight (P=0.07).

**Discussion**

CYP-derived eicosanoids are important regulators of systemic vascular homeostasis, but their role in the regulation of the pulmonary vascular tone and structure is unclear (reviewed in Reference 4). The results of the present investiga-

![Figure 4. Baseline RVSP (A), mPAP (B), and TPRI (C) in AdCYP2C9-infected, in AdRR5-infected, and in uninfected mice. Sulfaphenazole (5 mg/kg, daily IP injections) prevented the rise in RVSP, mPAP, and TPRI, *P<0.05 vs all.](http://hyper.ahajournals.org/doi/abs/10.1161/01.HYP.0000218315.56393.73?journalCode=hyper)
tion indicate that CYP2C-derived EETs increase pulmonary vascular resistance in response to an acute hypoxic challenge, as well as after a gene transfer–induced increase in CYP2C expression during normoxia. Epoxidegenerase inhibition using MSPPOH attenuated, whereas sEH inhibition with IK-950 enhanced the acute hypoxia-induced pulmonary vasoconstriction. Moreover, prolonged MSPPOH delivery for 7 days attenuated the chronic hypoxia-induced pressure rise and pulmonary vascular remodeling, suggesting that CYP2C enzymes represent a therapeutic target for the treatment of PH.

Vascular tone is largely determined by a dynamic interaction between endothelium-dependent and -independent constricting and relaxing stimuli. Vasoactive compounds may exert different effects in the systemic than in the pulmonary circulation. The family of CYP enzymes (>3000 homologous sequences) is composed of epoxygenases and terminal hydroxylases, several of which are abundantly expressed in the pulmonary vasculature,4,26 but their biological role is incompletely understood. Therefore, we investigated CYP epoxygenases in an in vivo mouse model under normoxic and hypoxic conditions using gene transfer and selective pharmacological inhibitors.

Vasoconstriction in response to alveolar hypoxia directs blood flow from poorly to highly ventilated lung regions to facilitate gas exchange.27 Inhibition of endogenous epoxygenases by MSPPOH reduced the HPV response by half. However, some CYP enzymes (eg, CYP2C9) are capable of generating physiologically relevant amounts of superoxide anions (O$_2^-$),28 which can also act as vasoconstrictors and compromise the bioavailability of endothelium-derived NO.22,29 Because the epoxygenase inhibitors MSPPOH and sulfaphenazole attenuate the production of EETs and O$_2^-$, data obtained using these compounds alone do not provide sufficient evidence for a role for EETs in the regulation of pulmonary vascular tone. We, therefore, assessed the effects of a novel, water-soluble sEH inhibitor, which solely in-

Figure 5. Real-time PCR analysis of CYP2C29 expression in normoxic and hypoxic (FiO$_2$=0.07) murine lungs (A). Immunoblot (B) and densitometric analysis normalized to the actin signal (C) of CYP 2C protein expression in murine lungs during normoxia and after exposure to hypoxia for variable time points. *P<0.05 vs normoxia and 24-hour hypoxia.

A

B

C

Figure 5.
creases the accumulation of EETs and does not affect the production of \( \text{O}_2^\cdot \) by CYP2C enzymes (author’s unpublished observation). The sEH inhibitor IK-950 potentiated hypoxia-induced pulmonary vasoconstriction in mice indicating that EETs and not reactive oxygen species are responsible for the effects observed.

Our observations that EETs participate in pulmonary vasoconstriction are concordant with earlier results that the pressure rise in isolated human lungs after \( \text{Ca}^{2+} \) ionophore was preceded by a significant elevation of EET levels in the perfusion solution. They are also congruent with the lack of hypoxia-induced pulmonary vasoconstriction in mice deficient for cyto-

![Figure 6](image_url)

**Figure 6.** Liquid chromatography with tandem mass spectrometry detection analysis of total EETs and DHETs synthesized from arachidonic acid by pulmonary microsomes, which are freshly isolated from normoxic lungs and from lungs exposed to hypoxia for different duration (n=6 for each condition). *\( P<0.05 \); †\( P<0.01 \); ‡\( P<0.001 \) vs normoxia.

![Figure 7](image_url)

**Figure 7.** RVSP rise (A) and pulmonary artery muscularization (B and C) at baseline and after 1 week of chronic hypoxia (\( \text{FiO}_2 = 0.07 \)). Right ventricular pressure response and pulmonary vascular remodeling is demonstrated in nontreated control mice (CON), and in vehicle- (VEH) or in MSPPOH-treated (30 mg/kg per day) mice. *\( P<0.001 \) vs control and vehicle; †\( P<0.001 \) vs normoxia; ‡\( P<0.05 \) vs control and vehicle; §\( P<0.01 \) vs normoxia.
solic phospholipase A$_2$-α, which releases arachidonic acid from phospholipids and, therefore, constitutes the rate-limiting step in the biosynthesis of EETs.$^{31}$ Taken together, these observations highlight an important role for arachidonic acid–dependent signaling in pulmonary vasoconstriction.

The results of previous studies using chemically synthesized EETs or drug-induced CYP activation are not necessarily representative of the pathophysiological consequences of an increase in epoxygenase expression/activity in vivo.$^{4,7}$ To investigate the consequences of a sustained increase in pulmonary epoxygenase activity, we performed lung-specific gene transfer of human CYP2C9, which can be specifically inhibited by sulfaphenazole$^{23}$ and which has a very similar metabolic profile to the murine CYP2C29 isoform. We observed a selective increase in mPAP and resistance, which was inhibited by the specific CYP2C9 inhibitor, sulfaphenazole. These hemodynamic changes were associated with increased and sulfaphenazole-sensitive production of CYP2C9-derived EETs (see online data supplement).

The molecular mechanisms underlying CYP2C9-induced PH are currently unknown but could be related to paracrine constrictor effects of EETs or the stimulation of thromboxane-like metabolic products in neighboring smooth muscle cells.$^{9,32}$

Hypoxia induces the expression of human CYP 3A6 and the rat homologue to CYP2C9. On the other hand, in patients with chronic pulmonary disease, the expression and activity of several drug-metabolizing CYP enzymes decreases after acute moderate hypoxemia.$^{13,33}$ The promoters of the murine CYP2C29 (GenBank No. BC019908), as well as the human CYP2C9 (GenBank No. BC020754) genes, contain hypoxia-responsive elements. Our findings showing an increased CYP2C mRNA, and doubled protein expression confirmed their hypoxia-sensitive regulation. Moreover, microsomes isolated from the lungs of mice exposed to hypoxia have enhanced the ability to generate EETs. Murine pulmonary microsomes synthesize $\approx$100% more 8,9-, 11,12-, and 14,15-EETs/DHETs after exposure to hypoxia for 24 hours, and this significantly greater catalytic activity persists in microsomes prepared from animals exposed to hypoxia for 1 week. Thus, epoxygenases are regulated in an oxygen-sensitive manner with differential time-dependent changes at the transcriptional, translational, as well as enzyme activity level. These observations suggest that epoxygenases play an important role in the PH associated with hypoxia.

Chronic exposure to hypoxia is a major cause of PH and elevated pulmonary vascular resistance, which is associated with architectural remodeling, a hallmark of several chronic cardiopulmonary diseases. The results of the present investigation indicate that the hypoxia-induced increase in CYP epoxygenase expression and activity contributes the remodeling process inasmuch as an epoxygenase inhibitor attenuated the hypoxic response. Sustained inhibition of EET biosynthesis using MSPPOH reduced hypoxia-induced RVSP increase and the distal migration of smooth muscle cells to precapillary resistance vessels in the pulmonary circulation.

Whereas the effects of EETs on endothelial cells have been relatively well characterized,$^{34,35}$ little is known about their effects on smooth muscle cell migration, growth, and proliferation. However, whereas EETs stimulate endothelial cell proliferation and migration, they exert the opposite effects on the serum- or growth factor-induced migration of vascular smooth muscle cells explanted from systemic conduit arteries.$^{36}$ EETs elicit the phosphorylation of mitogen-activated protein (MAP) kinases, particularly of the p38 MAP kinase and ERK 1/2,$^5$ all of which contribute to pulmonary smooth muscle cell hypertrophy.$^{37}$ We observed a similar increase in the phosphorylation in these kinases in cultured mouse pulmonary artery smooth muscle cells exposed to conditioned medium containing CYP2C9-derived EETs, suggesting that activation of MAP kinases may, in part, mediate the alterations in the pulmonary smooth muscle cell phenotype associated with hypoxia-induced pulmonary remodeling.

Taken together, the results of the present investigation demonstrate that increased CYP epoxygenase activity in vivo participates in pulmonary vasoconstriction and initiates pulmonary vascular remodeling in mice. Selective inhibition of epoxygenase activity attenuates HPV and reduces chronic hypoxic PH and vascular remodeling.

**Perspectives**

The results of the present investigation demonstrate an important modulatory role for CYP epoxygenases in vivo in pulmonary vasoconstriction and pulmonary vascular remodeling in mice. Whether similar mechanisms are likely to participate in the pathophysiology of human PH remains to be determined. If confirmed, the attenuation of HPV and chronic hypoxia-induced pulmonary vascular remodeling by pharmacological inhibition of epoxygenase activity offers novel treatment options for PH.

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