Hypoxia-induced pulmonary hypertension: comparison of soluble epoxide hydrolase deletion vs. inhibition

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Aims	The C-terminal domain of the soluble epoxide hydrolase (sEH) metabolizes epoxyeicosatrienoic acids (EETs) to their less active diols, while the N-terminal domain demonstrates lipid phosphatase activity. As EETs are potent vasoconstrictors in the pulmonary circulation, we assessed the development of pulmonary hypertension induced by exposure to hypoxia (10% O_2) for 21 days in wild-type (WT) and $sEH^{-/-}$ mice and compared the effects with chronic (4 months) sEH inhibition.
Methods and results	In isolated lungs from WT mice, acute hypoxic vasoconstriction (HPV) was potentiated by sEH inhibition and attenuated by an EET antagonist. After prolonged hypoxia, the acute HPV and sensitivity to the EET antagonist were increased, but potentiation of vasoconstriction following sEH inhibition was not evident. Chronic hypoxia also stimulated the muscularization of pulmonary arteries and decreased sEH expression in WT mice. In normoxic $sEH^{-/-}$ mice, acute HPV and small artery muscularization were greater than that in WT lungs and enhanced muscularization was accompanied with decreased voluntary exercise capacity. Acute HPV in $sEH^{-/-}$ mice was insensitive to sEH inhibition but inhibited by the EET antagonist and chronic hypoxia induced an exaggerated pulmonary vascular remodelling. In WT mice, chronic sEH inhibition increased serum EET levels but failed to affect acute HPV, right ventricle weight, pulmonary artery muscularization, or voluntary running distance. In human donor lungs, the sEH was expressed in the wall of pulmonary arteries, however, sEH expression was absent in samples from patients with pulmonary hypertension.
Conclusion	These data suggest that a decrease in sEH expression is intimately linked to pathophysiology of hypoxia-induced pulmonary remodelling and hypertension. However, as sEH inhibitors do not promote the development of pulmonary hypertension it seems likely that the N-terminal lipid phosphatase may play a role in the development of this disease.
Keywords	Cytochrome P450 • Epoxyeicosatrienoic acid • Hypoxia • Pulmonary hypertension • Soluble epoxide hydrolase

1. Introduction

Hypoxic vasoconstriction is unique to the pulmonary circulation and redistributes pulmonary blood flow from areas of low oxygen partial pressure to areas of normal or relativity high oxygen availability, thus optimizing the matching of perfusion and ventilation and preventing arterial hypoxemia.[1] However, chronic hypoxia leads to a sustained increase in pulmonary artery pressure (PAP) as well as to structural changes in the walls of the pulmonary vasculature, i.e. increased muscularization of the pulmonary vasculature and right heart hypertrophy.[2,3]

Arachidonic acid is not only metabolized via cyclooxygenase, and lipoxygenases as well as cytochrome P450 (CYP) enzymes generate a wide range of bioactive eicosanoids. The CYP epoxygenases, for

example, metabolize arachidonic acid to epoxyeicosatrienoic acids (EETs), which have been attributed anti-inflammatory properties and linked with NO- and prostacyclin-independent vasodilatation in the systemic circulation but vasoconstriction in the pulmonary circulation.[4,5] Intracellular levels of the EETs are regulated by the activity of the CYP epoxygenases that generate them as well as by the soluble epoxide hydrolase (sEH) which converts the EETs (8,9-, 11,12-, and 14,15-EET) to their corresponding dihydroxyeicosatrienoic acids (DHETs). Genetic deletion of the sEH gene (*EPHX2*) as well as the pharmacological inhibition of the enzyme increase plasma EET levels and potentiate their effects.[6] It follows that increased sEH expression, would be expected to decrease EET levels and support a pro-hypertensive, pro-inflammatory phenotype. Indeed, in the spontaneously hypertensive rat, sEH expression is elevated and contributes to the development of hypertension.[7] sEH inhibition/deletion is however not always associated with beneficial effects as sEH deletion reduces survival after cardiac arrest,[8] and genetic variation in the sEH gene (EPHX2) has been linked to a higher incidence of stroke in rats and humans.[9,10] Such reports suggest that the downregulation or chronic inhibition of the sEH could exert detrimental rather than beneficial effects.

We recently reported that CYP epoxygenases are involved in the pulmonary vasoconstriction induced by acute exposure to hypoxia via a mechanism involving the intracellular translocation and activation of transient receptor potential (TRP) C6 channels.[5] As the acute pulmonary vasoconstrictor response to hypoxia was potentiated by sEH inhibition as well as in sEH$^{-/-}$ mice,[5] and sEH inhibition has beneficial effects on lung inflammation,[11] the aim of the present investigation was to analyse the role of the sEH in pulmonary hypertension and vascular remodelling induced in mice by exposure to hypoxia (10% O_2) for 21 days. Moreover, given that the sEH is a bifunctional enzyme in which the C-terminal domain possesses epoxide hydrolase activity while the N-terminal domain demonstrates lipid phosphatase activity,[12,13] we compared the effects of sEH-deletion with chronic (4 months) treatment with sEH inhibitors.

2. Methods

For an extended version of the methods section please see Supplementary material online.

2.1 Chemicals

The sEH inhibitors 1-adamantyl-3-cyclohexylurea (ACU), *trans*-4-[4-(3-adamantan-1-ylureido)cyclohexyloxy]-benzoic acid (sEHI-1471) and *cis*-4-[4-(3-adamantan-1-ylureido)cyclohexyloxy]-benzoic acid (sEHI-1675) were synthesized as described.[14,15] The EET antagonist 14,15-epoxyeicosa-5(*Z*)-enoic acid (14,15-EEZE) was kindly provided by John R. Falck (Dallas, USA). 14,15-EET was obtained from Cayman Chemicals (Massy, France) and the antibody against the sEH was kindly provided by Michael Arand (Zürich, Switzerland). All other substances were obtained from Sigma.

2.2 Animals

C57BL/6 mice (6 weeks old) were purchased from Charles River (Sulzfeld, Germany) and the sEH$^{-/-}$ mice[16] were obtained from Dr Frank Gonzalez (National Institutes of Health, Bethesda, Maryland)

and then cross-bred for eight generations onto the C57BL/6 background. Seven-week-old C57BL/6 mice received either solvent (ethanol: 0.3 vol%), sEHI-1675 (5 mg/L), or sEHI-1471 (8 mg/L) in the drinking water for 4 months. Mice were housed in conditions, handled and sacrificed using methods that conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23). Both the university animal care committee and the federal authority for animal research (Regierungspräsidium Darmstadt, Hessen, Germany) approved the study protocols (#F28/06 and F28/11).

Mice were housed in a ventilated chamber (BioSpherix, Lacona, USA) for up to 21 days and maintained under either normoxic conditions (21% O_2) or exposed to normobaric hypoxia (10% O_2). Thereafter, lungs were isolated and frozen either for western blotting and immunohistochemical analysis, or for morphometric analysis as described.[17] In some animals, the right ventricle was dissected from the left ventricle and septum, dried and weighed to obtain the right to left ventricle plus septum ratio.

2.3 Isolated buffer-perfused mouse lung

Changes in pulmonary perfusion pressure were assessed in the isolated buffer-perfused mouse lung, as described.[18]

2.4 Arachidonic acid metabolism and sEH activity

Mice were anesthetized with isoflurane, and blood was immediately collected from the right ventricle. The serum was extracted twice into ethyl acetate, evaporated under nitrogen, resuspended in methanol/water (vol. 1.1). The activity of the sEH was determined using cell lysates generated as described.[19] The eicosanoid profiles generated were determined with a Sciex API4000 mass spectrometer operating in multiple reaction monitoring (MRM) mode as described.[4]

2.5 Real-time quantitative reverse transcription PCR

Total RNA from mouse lungs or cultured rat pulmonary smooth muscle cells was isolated using Trizol. Equal amounts of total RNA were reverse transcribed with Superscript III (Invitrogen, Germany) using random hexamer primers. The cDNA was amplified by real-time PCR using SybrGreen in an MX4000 (Stratagene) with the appropriate primers. Mouse RNA signals were normalized to 18S using Assay-On-Demand (Applied Biosystems; Foster City, CA, USA) and for rat pulmonary smooth muscle cells, signals were normalized to elongation factor-2.

2.6 sEH promoter assay

HEK293 cells were cotransfected with sEH reporter plasmids, generated as described previously;[20] and a plasmid encoding β-galactosidase under the control of the cytomegalo virus promoter (pcDNA3.1myc-His/LacZ; Invitrogen, Paisely, UK). Relative promoter activity was calculated as the ratio of luciferase to galacosidase activity and normalized to total protein content.

2.7 Human samples

Human lung tissue was obtained from five donors and five patients with idiopathic PAH (IPAH) undergoing lung transplantation. The investigation conforms with the principles outlined in the Declaration of Helsinki' (see *Cardiovascular Research* 1997;**35:**2−4). The studies were approved by the Ethics Committee of the Justus-Liebig-University School of Medicine (AZ 31/93).

2.8 Statistical analysis

Data are expressed as mean ± SEM and statistical evaluation was performed using Student's *t*-test for unpaired data or one-way ANOVA followed by a Bonferroni *t*-test when appropriate. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1 Effect of chronic hypoxia on the acute hypoxic vasoconstriction and sensitivity to sEH inhibition

Acute hypoxic challenge (1% O_2, 10 min) of lungs from wild-type mice housed under normoxic conditions resulted in an increase in PAP (*Figure 1A*). As reported previously,[5] inhibition of the sEH with ACU (3 μmol/L) enhanced the acute hypoxic vasoconstriction. The EET antagonist, 14,15-EEZE (10 μmol/L), attenuated the increase in PAP in the absence of the sEH inhibitor and completely prevented the increased constriction induced by ACU (*Figure 1A*).
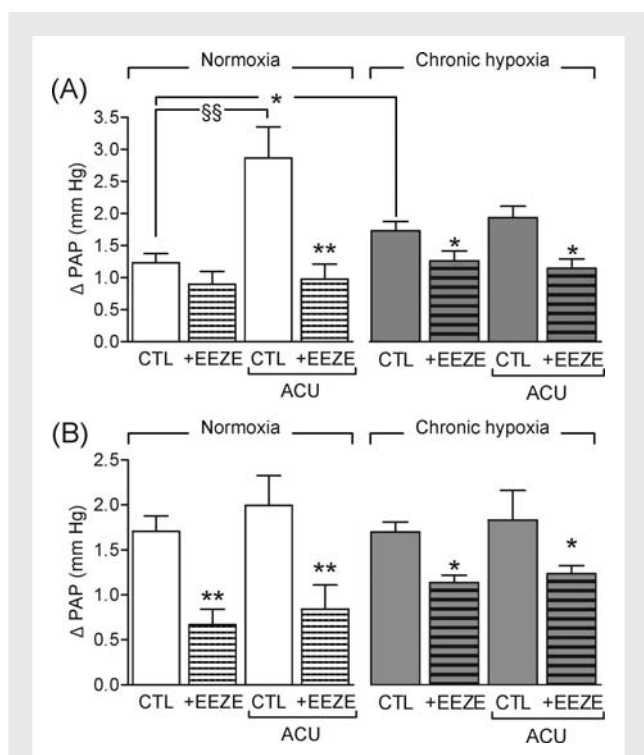
Figure I Effect of chronic hypoxia, EET antagonism, and sEH inhibition on the acute hypoxia-induced vasoconstriction in isolated perfused mouse lungs. (*A*) Wild-type and (*B*) sEH$^{-/-}$ mice were kept under normoxic conditions or exposed to hypoxia (10% O_2) for 21 days. Thereafter, hypoxic pulmonary vasoconstriction (1% O_2, 10 min) was assessed in the absence (CTL) and presence of 14,15-EEZE (EEZE, 10 μmol/L), ACU (3 μmol/L), or a combination of both. The bar graphs summarize data obtained in 5–8 independent experiments; *$P < 0.05$, *$P < 0.01$ vs. the appropriate CTL and §§$P < 0.01$ vs. ACU by one-way ANOVA.

An exaggerated response to acute hypoxia was observed in lungs isolated from animals exposed to 10% O_2 for 21 days but pharmacological inhibition of the sEH was without further effect (*Figure 1A*). In the latter tissues however, 14,15-EEZE significantly decreased PAP indicating that EET production contributed to the pulmonary vasoconstriction (*Figure 1A*). The increase in PAP elicited by acute hypoxia was greater in lungs from sEH$^{-/-}$ mice than from wild-type animals (ΔPAP 1.7 ± 0.17 vs. 1.2 ± 0.14 mmHg in sEH$^{-/-}$ and wild-type lungs, respectively, $n = 8$; $P < 0.05$) and, as expected, ACU did not further potentiate this response (*Figure 1B*). Prolonged exposure to hypoxia however failed to alter acute hypoxic pulmonary vasoconstriction in lungs from sEH$^{-/-}$ mice even though the EET antagonist was still able to significantly decrease the acute hypoxic pulmonary vasoconstrictor response (*Figure 1B*).

3.2 Effect of chronic hypoxia on right heart hypertrophy, pulmonary artery muscularization, and voluntary exercise capacity

The ratio of the right/left ventricle and septum did not differ between wild-type and sEH$^{-/-}$ mice maintained in normoxic conditions. Chronic hypoxia induced right heart hypertrophy in both strains, with more marked effects being evident in sEH$^{-/-}$ mice (*Figure 2A*).

In the isolated buffer-perfused mouse lung, baseline PAP, which reflects pulmonary vascular resistance, did not differ significantly between lungs from wild-type and sEH$^{-/-}$ mice maintained under normoxic conditions. Prolonged exposure of the animals to hypoxia, however, significantly enhanced baseline PAP in lungs from both strains (data not shown).

In animals maintained under normoxic conditions, the number of non-muscularized small (20–70 μm) vessels was significantly lower in sEH$^{-/-}$ than in wild-type mice and the number of partially muscularized vessels significantly increased (*Figure 2B*). No differences in the extent of muscularization of the 70–150 μm and >150–1000 μm vessels was observed under normoxic conditions.

Following chronic hypoxia there was a decrease in the number of non-muscularized 50–70 μm pulmonary vessels and a corresponding increase in the number of fully muscularized vessels in both mouse strains. The most marked effects were however observed in vessels between 70 and 150 μm and the deletion of the sEH was associated with a significant increase in the number of fully muscularized vessels (*Figure 2C*) and corresponding decrease in the number of non-muscularized vessels.

Given that pulmonary hypertension is characterized by a decreased exercise performance,[21] we determined whether or not the enhanced muscularization of pulmonary resistance arteries detected in sEH$^{-/-}$ animals affected their exercise capacity. Mice were housed in standard cages and provided with a running wheel, while wild-type mice ran an average of 7.9 ± 0.6 km per day, sEH$^{-/-}$ mice ran significantly less i.e. approximately 5.6 ± 0.5 km ($n = 7$ per group, $P = 0.024$).
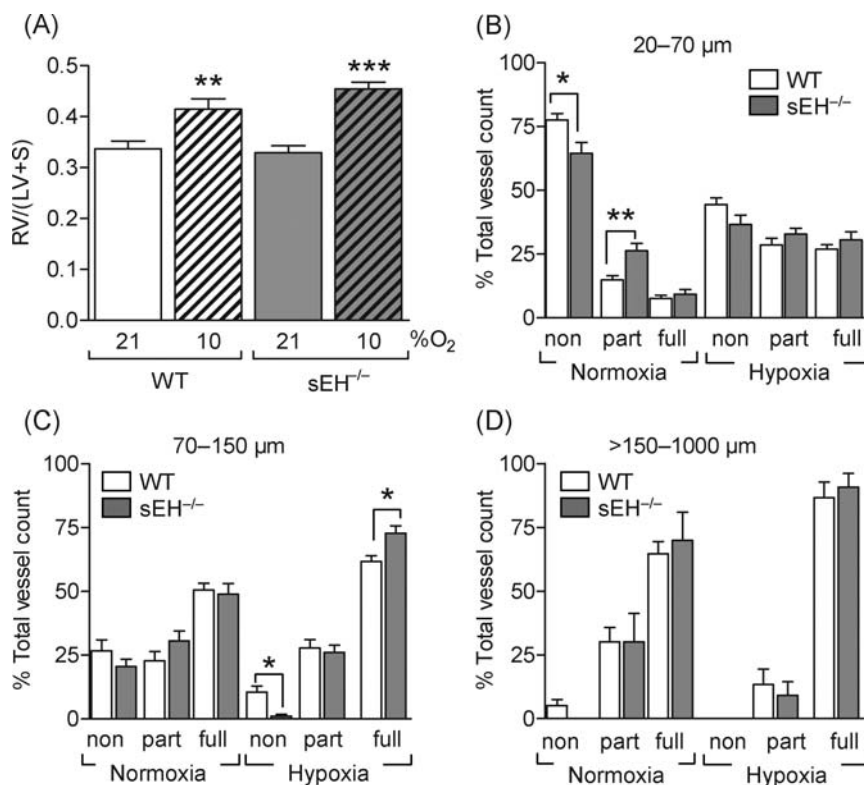
Figure 2 Effect of chronic hypoxia on right heart hypertrophy and pulmonary artery muscularization in wild-type (WT) and sEH$^{-/-}$ mice kept in normoxic conditions (21% O_2) or exposed to chronic hypoxia (10% O_2) for 21 days. (A) Ratio of the right ventricular wall (RV) and the left-ventricular wall and septum (LV+S) weight. (B–D). The degree of muscularization (non-muscularized, non; partially muscularized, part; fully muscularized, full) in vessels classed as (B) small (20–70 μm diameter), (C) medium (>70–150 μm diameter), and (D) large (>150–1000 μm diameter) in lungs from wild-type (WT) and sEH$^{-/-}$ mice either kept in normoxic conditions or exposed to chronic hypoxia (10% O_2) for 21 days. The bar graphs summarize data obtained using at least five animals in each group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. the corresponding normoxic control by one-way ANOVA (A) and Student's t-test (B–D).

3.3 Effect of hypoxia on the expression of the sEH

As the muscularization index was enhanced in normoxic sEH$^{-/-}$ vs. wild-type mice and hypoxia abolished pulmonary sensitivity to sEH inhibition in control animals, we hypothesized that the phenotype observed in lungs from wild-type mice could partially be accounted for by a decrease in the expression of the sEH.

In lungs from wild-type animals, the sEH was expressed in pulmonary artery smooth muscle cells (*Figure 3A*). Exposure of these animals to hypoxia for 21 days decreased the expression of the enzyme as detected by histochemistry (*Figure 3A*), western blotting (*Figure 3B*) and RT–qPCR (*Figure 3C*). The decrease in sEH expression was paralleled by a decrease in enzyme activity assessed as the conversion of 14,15-EET to 14,15-DHET (*Figure 3D*).

To determine whether or not hypoxia could directly affect the expression of the sEH, we assessed its effect on the activity of the sEH promoter using a luciferase gene based reporter assay. As reported previously,[20] significant luciferase activity was detected in HEK293 cells transfected with the 4 kb sEH promoter. Hypoxia rapidly (within 4 h) decreased promoter activity, an

effect that was maintained over 24 h (*Figure 4A*). Similar results were obtained using a 1.5 kb promoter construct. Moreover, in isolated rat pulmonary artery smooth muscle cells, which we have previously reported to express the sEH,[5] hypoxia significantly decreased sEH mRNA expression (*Figure 4B*).

3.4 sEH expression and primary pulmonary hypertension in humans

In human lungs from healthy donors, the sEH was expressed in vascular smooth muscle cells, i.e. α-smooth muscle actin positive cells (*Figure 5*). Although expression of the sEH was detectable in all of the samples obtained from healthy donors, it was not possible to detect sEH protein in samples (0/5) obtained from patients with idiopathic pulmonary artery hypertension undergoing lung transplantation (*Figure 5*).

3.5 Effect of prolonged sEH inhibition on vascular remodelling and voluntary exercise

To determine whether the long-term treatment of wild-type mice with sEH inhibitors could reproduce the effects of sEH deletion,
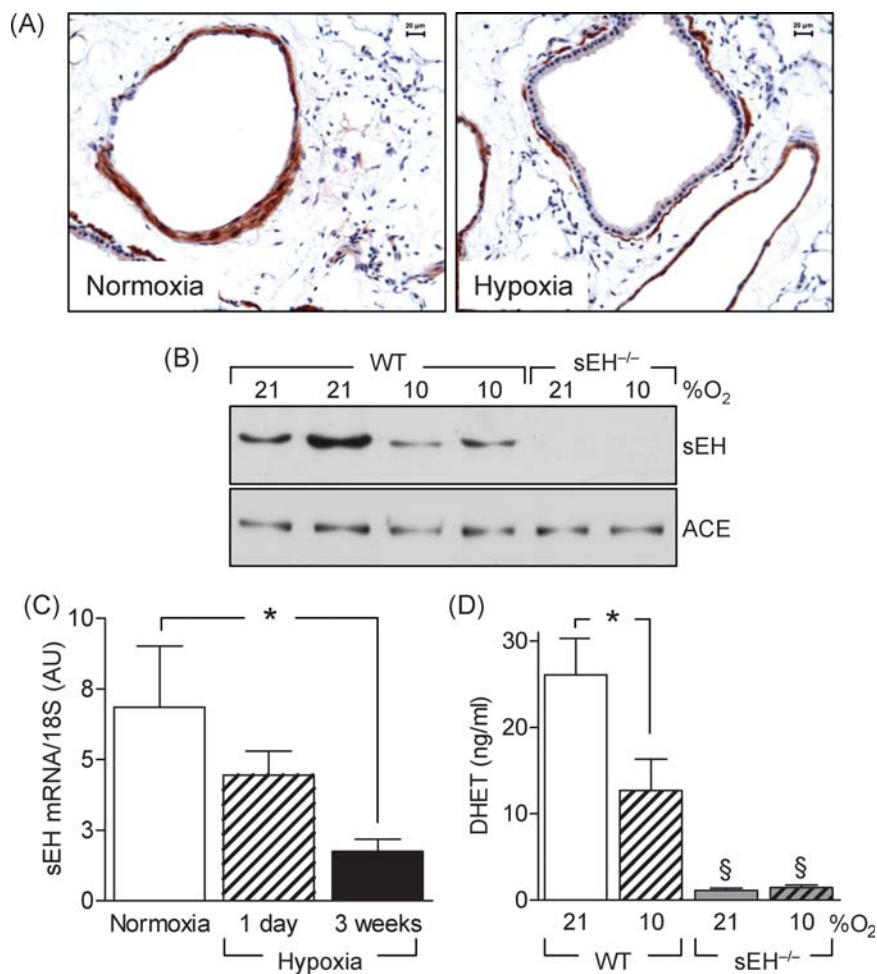
Figure 3 Effect of chronic hypoxia on the expression/activity of the sEH in murine lungs. (*A*) Immunohistochemical analysis showing the expression of the sEH in lungs from wild-type mice kept under normoxic conditions or after exposure to 21 days hypoxia (10% O₂). Representative western blots (*B*) and RT−qPCR analysis (*C*) showing the effects of hypoxia on pulmonary sEH expression. For western blotting the angiotensin converting enzyme (ACE) was used to demonstrate the equal loading of the lanes and samples from sEH$^{-/-}$ lungs were used as a negative control. (*D*) Effect of chronic hypoxia on the generation of 14,15-DHET from 14,15-EET by lung homogenates from wild-type (WT) mice or sEH$^{-/-}$ mice. The graphs summarize data obtained in 4−16 animals; *$P < 0.05$ vs. normoxia and §$P < 0.05$ vs. wild-type hypoxia by one-way ANOVA.

mice were treated with solvent, sEHI-1471, or sEHI-1675 for 4 months.

Prolonged sEH inhibition resulted in significantly elevated serum levels of 5,6-, 8,9-, 11,12-, and 14,15-EETs/DHETs (see Supplementary material online, *Figure S1*) but failed to affect the ratio of the right/left ventricle and septum (*Figure 6A*). Moreover, although the sEH$^{-/-}$ animals displayed a clearly enhanced muscularization compared with wild-type mice, chronic sEH inhibition was unable to reproduce this phenotype (*Figure 6B−D*). Correspondingly, there was no significant effect of sEH inhibitor treatment on distances run per day by 6-month-old wild-type animals given either solvent; 5.4 ± 0.6 km, sEHI-1471; 5.8 ± 0.8 km, or sEHI-1675; 5.1 ± 1.1 km (*n* = 7 per group) for 4 months.

4. Discussion

The results of the present investigation indicate that deletion of the sEH mimics, to a certain extent, the pathophysiological changes induced in the lung by chronic (21 days) hypoxia. Certainly, the degree of muscularization of pulmonary arteries in sEH$^{-/-}$ mice maintained under normoxic conditions differed from that of wild-type mice and the acute hypoxia-induced pulmonary vasoconstriction was significantly elevated. Moreover, in wild-type animals, chronic hypoxia decreased pulmonary sensitivity to sEH inhibition at the same time as decreasing sEH mRNA and protein expression as well as activity. While it was not possible to demonstrate a causative link between sEH expression levels and the development of pulmonary hypertension in humans, we were not able to detect
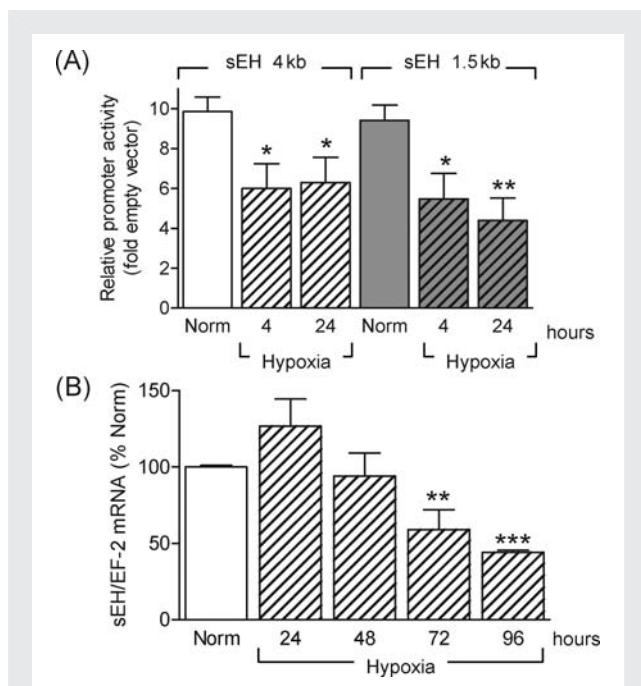
Figure 4 Effect of hypoxia on sEH expression *in vitro*. (A) HEK 293 cells were transfected with either a 4 or 1.5 kb form of the luciferase-coupled sEH promoter construct and activity was assessed after 24 h culture under normoxic (Norm) conditions or 4 or 24 h following exposure to hypoxia (1% O_2). (B) Effect of hypoxia (1% O_2) for up to 96 h on the expression of sEH mRNA (as determined by RT−qPCR) in rat pulmonary artery smooth muscle cells. The graphs summarize data obtained in 4−9 independent experiments; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. normoxia by one-way ANOVA.
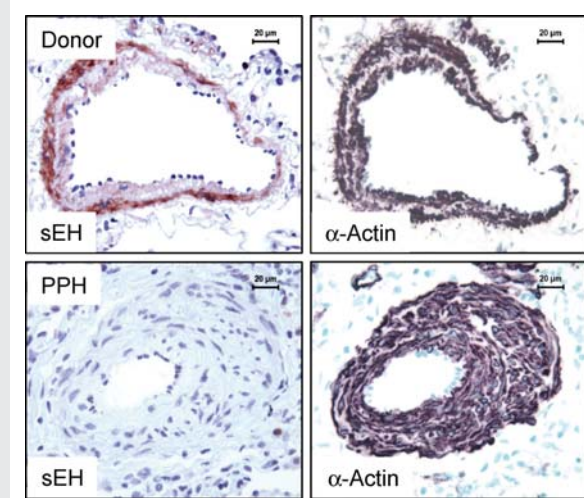

Figure 5 sEH expression in primary pulmonary hypertension. Immunohistochemical analysis showing the expression of the sEH and α-actin in a sample from a healthy human lung donor and a patient with primary pulmonary hypertension (PPH).

sEH protein expression in lungs from patients with pulmonary hypertension even though the enzyme was highly expressed in vascular smooth muscle cells in lungs from healthy donors. However, despite the fact that the genetic deletion of the sEH elicited effects similar to its hypoxia-induced downregulation, the chronic inhibition of epoxide hydrolase activity with two chemically distinct sEH inhibitors had no obvious effect on pulmonary vascular remodelling or exercise capacity. These data indicate that although the C-terminal epoxide hydrolase is involved in the acute regulation of pulmonary resistance, the N-terminal phosphatase domain may play the more important role in the long-term regulation of pulmonary morphology and the development of pulmonary hypertension.

A role for CYP-derived EETs in pulmonary vascular remodelling has previously been postulated on the basis of the observation that a CYP epoxygenase inhibitor attenuated the pulmonary artery muscularization induced by exposure to hypoxia for 7 days.[22] The latter study also reported that hypoxia increases the expression of the murine epoxygenase CYP2C29. As an increase in CYP expression would be expected to result in increased EET levels it seemed logical to expect that prolonged exposure to hypoxia should also enhance vasoconstriction induced by the acute lowering of the O_2 tension. Indeed, in the mouse lung, chronic exposure to hypoxia induces just such a phenomenon, thus confirming previous reports.[17] That EETs underlie this effect was demonstrated by the finding that the potentiated response was not observed in lungs treated with the EET antagonist. Surprisingly, however, chronic exposure to hypoxia abrogated the sensitivity of the preparation to sEH inhibition so that no increase in the acute hypoxic vasoconstriction was observed following the application of the sEH inhibitor. Chronic exposure of sEH$^{-/-}$ mice to hypoxia on the other hand did not potentiate the acute hypoxic pulmonary vasoconstrictor response. Thus, from a functional point of view, responses of lungs from wild-type mice exposed to chronic hypoxia behaved similarly to lungs from normoxic sEH$^{-/-}$ mice.

As hypoxia increases CYP expression *in vitro*[4] and *in vivo*[22] and EETs stimulate both angiogenesis and vascular maturation,[23] we were interested in determining the role of the CYP/sEH pathway in the vascular remodelling induced by hypoxia. We observed that the genetic deletion of the sEH was associated with the remodelling of the pulmonary vasculature. Indeed, even under normoxic conditions, lungs from sEH$^{-/-}$ mice displayed a higher level of small artery muscularization than lungs from wild-type mice. That these results are indeed of functional relevance was demonstrated by the decreased exercise capacity in the sEH$^{-/-}$ animals. These findings suggest that hypertrophic mechanisms are activated in sEH$^{-/-}$ animals. EETs certainly have the potential to promote such a response as these epoxides are reported to enhance cell survival[24] as well as to elicit the phosphorylation of MAP kinases (e.g. p38 MAP kinase, ERK1/2) all of which can contribute to pulmonary smooth muscle cell hypertrophy.[25,26]

The molecular pathways by which chronic exposure to hypoxia causes vasoconstriction and pulmonary vascular remodelling remain to be completely elucidated. However, given that the sEH substrate, 11,12-EET, can elicit pulmonary vasoconstriction[5] as well as vascular maturation,[23] we hypothesized that the
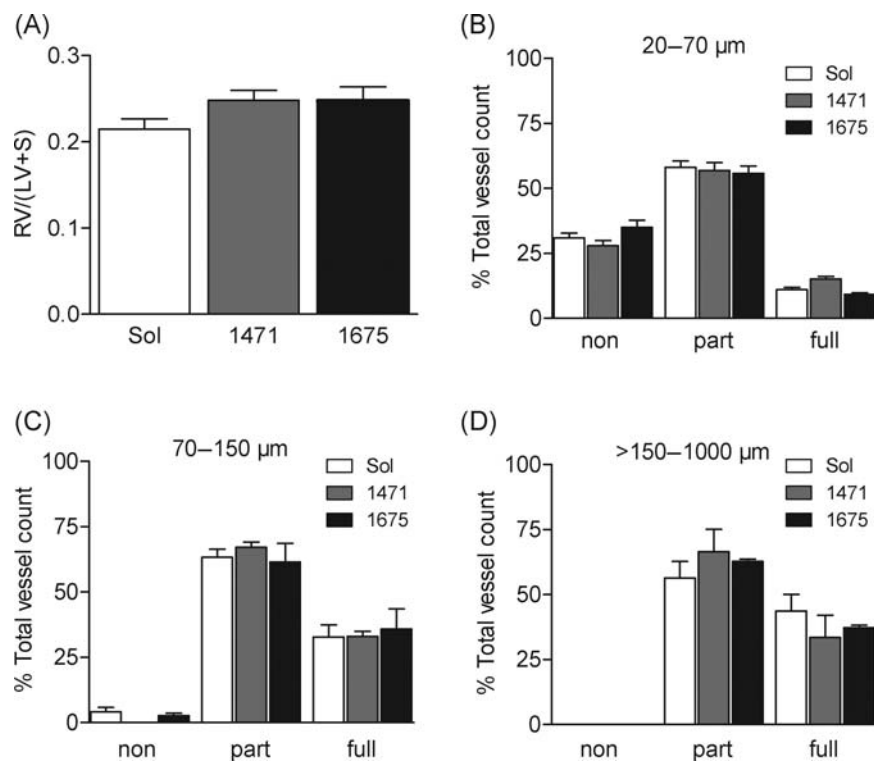
Figure 6 Effect of chronic sEH inhibition on cardiac and pulmonary remodelling. Wild-type animals were pre-treated with either solvent, sEHI-1675 or sEHI-1471 for 4 months. (*A*) Ratio of the right ventricular wall (RV) and the left-ventricular wall and septum (LV+S) weight. (*B–D*) The degree of pulmonary muscularization (non-muscularized, non; partially muscularized, part; fully muscularized, full) in vessels classed as (*B*) small (20–70 μm diameter), (*C*) medium (>70–150 μm diameter), and (*D*) large (>150–1000 μm diameter) was assessed. The bar graphs summarize data obtained with at least six animals in each group.

development of pulmonary hypertension might be linked to a change in sEH expression. We found that the activity of the sEH promoter and thus the expression of sEH mRNA and protein were attenuated under hypoxic conditions. Indeed, hypoxia effectively decreased the expression of the sEH *in vivo* in animals exposed to hypoxia for as little as one day and decreased the activity of the sEH promoter in cells cultured under hypoxic conditions for only a few hours. Little is known about the mechanism(s) regulating the expression of the sEH although both the binding of SP-1[20] and c-Jun[27] to the sEH promoter have been reported to regulate activity. Irrespective of the mechanisms involved, the decrease in EET metabolism resulting from the hypoxia-induced downregulation of the sEH would be expected to enhance pulmonary EET levels, thus inducing pulmonary vasoconstriction. There are, however, alternative, less efficient ways of controlling cellular EET levels; for example β-oxidation or C2 elongation[6] that may partially compensate for the loss of the sEH and thus limit the pathology observed in sEH$^{-/-}$ animals. While the translation of such findings to the human situation is difficult we found that although the sEH was easily detectable in the medial wall of arteries from donor lungs, it was not detectable in samples from pulmonary hypertension patients. The loss of a vasoconstrictor metabolizing enzyme in human pulmonary hypertension is consistent with the findings in the animal experiments and

fits in the hypothesis that the sEH is implicated in the development of pulmonary hypertension and pulmonary vascular remodelling.

To determine whether the loss of epoxide hydrolase activity could account for the findings obtained, wild-type mice were treated with two chemically distinct sEH inhibitors for 4 months. The compounds used significantly increased EET/DHET ratios but neither sEHI-1471 nor sEHI-1675 reproduced either the pulmonary remodelling phenotype or the attenuated exercise capacity seen in the sEH$^{-/-}$ animals. Although studies using sEH inhibitors report generally anti-inflammatory/protective effects (for review see reference[28]) this is not always the case when sEH$^{-/-}$ mice are studied. Indeed, sEH deletion reduces survival after cardiac arrest,[8] and genetic variation in EPHX2 has been linked to a higher incidence of stroke in rats as well as in humans.[9,10] Moreover, although sEH$^{-/-}$ mice show morphological changes reminiscent of the early stages of pulmonary hypertension, sEH inhibition protects against the initial (but not late) stages of monocrotaline-induced pulmonary hypertension.[29] Such apparently contradictory findings between wild-type animals treated with sEH inhibitors and sEH$^{-/-}$ mice can most probably be attributed to the fact that the mammalian sEH protein has two active domains. Indeed, the functional protein is a homodimer composed of two 60 kDa monomers joined by a proline-rich bridge,[30] and each monomer consists of an N-terminal domain which displays

lipid phosphatase activity and a larger C-terminal which processes classical α/β-hydrolase activity.[12,13] sEH inhibitors act through inhibition of the hydrolase domain but do not affect the phosphatase activity.[12] Therefore, the loss of lipid-phosphate activity could account for the link between the expression/activity of the sEH and the development of pulmonary hypertension. Interestingly, most current sEH literature attributes the hydrolase domain to the cardiovascular effects seen in humans. However, the human sEH single nucleotide polymorphism most often associated with cardiovascular disease (R287Q) encodes a protein with significantly lower rather than elevated hydrolase activity.[31] Thus, solely incriminating the hydrolase domain for adverse cardio- and pulmonary-vascular effects seems premature and highlights the importance of further investigating the independent roles of the hydrolase and phosphatase domains.

Relatively little is known about the lipid phosphatase activity associated with the sEH, but it has been linked with cholesterol-related disorders, peroxisome proliferator-activated receptor activity, and the isoprenoid/ cholesterol biosynthesis pathway.[32] Indeed, in addition to demonstrating enhanced circulating EET levels,[16] male sEH$^{-/-}$ mice exhibit decreased plasma cholesterol and testosterone levels.[33] Moreover, it seems that isoprenoid pyro- and monophosphates are substrates for the N-terminal domain of the enzyme,[34,35] and these lipid phosphates which are metabolic precursors of cholesterol biosynthesis and are also utilized for isoprenylation of small G-proteins involved in multiple cell signalling pathways.[36] Unfortunately, no selective inhibitors of sEH lipid phosphatase exist that are appropriate for use in organ preparations such as the isolated-perfused mouse lung.

In summary, the results of the present investigation support a role for the sEH in the development of pulmonary hypertension and pulmonary vascular remodelling. However, as the same phenotype was not elicited by chronic sEH inhibition it seems that the effects observed following the downregulation of the sEH may be attributable to the loss of lipid phosphatase rather than epoxide hydrolase activity. This is an important point as, based on experimental data showing that sEH inhibitors attenuate hypertension in angiotensin II-treated mice[37] and protect stroke-prone spontaneously hypertensive rats from cerebral ischemia,[38] sEH inhibitors are currently being developed for the treatment of human hypertension and inflammation/atherosclerosis. Based on these results it appears that although these substances can affect the acute hypoxia-induced vasoconstriction in the lung, they are less likely to accelerate the development of pulmonary hypertension than previously feared.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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