Epoxyeicosatrienoic acids and the soluble epoxide hydrolase are determinants of pulmonary artery pressure and the acute hypoxic pulmonary vasoconstrictor response

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ABSTRACT **Recent findings have indicated a role for cytochrome *P*-450 (CYP) epoxygenase-derived epoxyeicosatrienoic acids (EETs) in acute hypoxic pulmonary vasoconstriction (HPV). Given that the intracellular concentration of EETs is determined by the soluble epoxide hydrolase (sEH), we assessed the influence of the sEH and 11,12-EET on pulmonary artery pressure and HPV in the isolated mouse lung. In lungs from wild-type mice, HPV was significantly increased by sEH inhibition, an effect abolished by pretreatment with CYP epoxygenase inhibitors and the EET antagonist 14,15-EEZE. HPV and EET production were greater in lungs from sEH$^{-/-}$ mice than from wild-type mice and sEH inhibition had no further effect on HPV, while MSPPOH and 14,15-EEZE decreased the response. 11,12-EET increased pulmonary artery pressure in a concentration-dependent manner and enhanced HPV *via* a Rho-dependent mechanism. Both 11,12-EET and hypoxia elicited the membrane translocation of a transient receptor potential (TRP) C6-V5 fusion protein, the latter effect was sensitive to 14,15-EEZE. Moreover, while acute hypoxia and 11,12-EET increased pulmonary pressure in lungs from TRPC6$^{+/-}$ mice, lungs from TRPC6$^{-/-}$ mice did not respond to either stimuli. These data demonstrate that CYP-derived EETs are involved in HPV and that EET-induced pulmonary contraction under normoxic and hypoxic conditions involves a TRPC6-dependent pathway.—Keserü, B., Barbosa-Sicard, E., Popp, R., Fisslthaler, B., Dietrich, A., Gudermann, T., Hammock, B. D., Falck, J. R., Weissmann, N., Busse, R., Fleming, I. Epoxyeicosatrienoic acids and the soluble epoxide hydrolase are determinants of pulmonary artery pressure and the acute hypoxic pulmonary vasoconstrictor response. *FASEB J.* 22, 4306–4315 (2008)**

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ACUTE HYPOXIC PULMONARY vasoconstriction is an adaptive response of the pulmonary circulation that directs blood flow from poorly oxygenated to better ventilated areas, thereby maintaining pulmonary gas exchange (1). To date, the pulmonary oxygen sensor and the signaling cascade leading to hypoxic pulmonary vasoconstriction remain to be fully elucidated. However, current thinking tends to attribute the role of O_2 sensor to the mitochondrion and to postulate reactive oxygen species as intracellular mediators, which stimulate the activation of the ion channels that ultimately elicit the changes in intracellular Ca^{2+} and Rho kinase activity that lead to vasoconstriction [for reviews, see Moudgil *et al.* (2) and Weir and Olschewski (3)].

Arachidonic acid is metabolized *via* cyclooxygenase, lipoxygenase, and cytochrome *P*-450 (CYP) enzymes to generate a range of bioactive eicosanoids. CYP enzymes with an important role in cardiovascular function are the epoxygenases of the CYP2C and 2J gene families, which form four regioisomeric epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EET) and the ω-hydroxylases of the CYP4A family, which generate hydroxyeicosatetraenoic acids (19- and 20-HETE) (4). In the systemic circulation, EETs act as anti-inflammatory mediators and endothelium-derived hyperpolarizing factors mediating the nitric oxide and prostacyclin-independent but endothelium-dependent vasodilation observed in several vascular beds (5). 20-HETE, on the other hand, constricts renal, cerebral, coronary, and

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mesenteric arteries *via* effects on Ca^{2+}-activated K^+ channels and the Rho-kinase (4, 6). At the moment, the biological role of CYP-derived EETs in the pulmonary circulation is unclear because completely contradictory findings in different sized arteries isolated from canine and rabbit lungs have been published (7–10). However, it appears that CYP-derived EETs may elicit pulmonary vasoconstriction instead of vasodilatation, and it was recently reported that a CYP epoxygenase is implicated in acute hypoxia-induced pulmonary vasoconstriction, as well as in the pulmonary remodeling induced by chronic hypoxia (11).

Intracellular levels of the EETs are tightly regulated, and metabolism by the soluble epoxide hydrolase (sEH), which is the most important EET-metabolizing enzyme, occurs relatively quickly. The exception is the chemically unstable 5,6-EET, which is more rapidly metabolized by cyclooxygenase than by the sEH (12). Several of the EET metabolites generated, such as the sEH-derived dihydroxyeicosatrienoic acids (DHETs) are also biologically active, but generally less so than the parent epoxides. Moreover, the DHETs are not as readily incorporated into membrane lipids as the EETs and are thought to be the form in which the majority of endothelium-derived EETs leave the cell [for a review, see Spector and Norris (13)]. Inhibition of the sEH would therefore be expected to increase intracellular EET levels and prolong their vasodilator and anti-inflammatory actions. Therefore, the aim of the present investigation was to analyze in detail the role of CYP-derived EETs in hypoxic pulmonary vasoconstriction using a series of specific tools to inhibit CYP activity (CYP epoxygenase inhibitors), antagonize the actions of the EET (14,15-epoxyeicosa-5(Z)-enoic acid), or to prolong their half-life (sEH inhibitors). Moreover, the molecular mechanisms involved in mediating the hypoxia- and 11,12-EET-induced pulmonary vasoconstriction described were addressed using a combination of cultured pulmonary smooth muscle cells and genetically modified animals (sEH- and transient receptor potential (TRP) C6 channel-deficient mice).

MATERIALS AND METHODS

Chemicals

The sEH inhibitors 1-adamantyl-3-cyclohexylurea (ACU) and 1-adamantan-1-yl-3-{5-[2-(2-ethoxyethoxy)ethoxy]pentyl}urea (AEPU or IK-950), as well as the EET antagonist 14,15-epoxyeicosa-5(*Z*)-enoic acid (14,15-EEZE) and the CYP epoxygenase inhibitor MSPPOH, were synthesized as described (11, 14, 15). 11,12-EET, 14,15-EET, 11,12-DHET, and arachidonic acid were obtained from Cayman Chemicals (Massy, France), NADPH from Applichem (Darmstadt, Germany), U46619 from Alexis (Lörrach, Germany), and Y27632 dihydrochloride form Tocris (Ellisville, MO, USA). Fenbendazole and all other substances were purchased from Sigma (Deisenhofen, Germany).

Animals

$sEH^{-/-}$ mice were obtained from Dr. Frank Gonzalez (National Institutes of Health, Bethesda, MD, USA) and cross-bred for 8 generations onto the C57BL/6 background. $TRPC6^{-/-}$ and $TRPC6^{+/-}$ mice, generated as described (16), were bred by the animal facility at the University of Marburg. C57BL/6 mice (6–8 wk old) were purchased from Charles River (Sulzfeld, Germany). Mice were housed in conditions that conform to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH publication no. 85-23). Both the University Animal Care Committee and the Federal Authority for Animal Research at the Regierungspräsidium Darmstadt (Hessen, Germany) approved the study protocol (# F28/14).

Isolated buffer-perfused mouse lung

Changes in pulmonary perfusion pressure were assessed in the isolated buffer-perfused mouse lung, as described (17). Briefly, catheters were inserted into the pulmonary artery and left atrium, and buffer perfusion *via* the pulmonary artery was initiated at a flow of 0.2 ml/min. Ventilation was then changed from room air to a premixed gas (21% O_2, 5% CO_2, balanced with N_2), left atrial pressure was set to 2.0 mmHg, and flow was slowly increased from 0.2 to 2 ml/min. For hypoxic ventilation, a gas mixture containing 1% O_2, 5% CO_2, balanced with N_2 was used. Ten-minute periods of hypoxic ventilation were alternated with 15 min of normoxia.

Cell culture

Rat pulmonary artery smooth muscle cells were isolated as described (18) and cultured in M199, supplemented with 10% FCS, penicillin (50 U/ml) and streptomycin (50 μg/ml).

RhoA activation assay

Isolated buffer-perfused lungs from wild-type mice were treated with solvent or 11,12-EET (3 μmol/L, 15 min) then snap frozen in liquid N_2. Lungs were then homogenized and RhoA activity was determined using a specific G-LISA assay (Cytoskeleton, Denver, CO, USA).

Immunoblotting

Rat pulmonary artery smooth muscle cells were maintained under normoxic conditions, treated with U46619 (1 μmol/L, 10 min) or exposed to hypoxia for 30 min. Cells were then immediately treated with trichloroacetic acid (15% w/v) and frozen in liquid N_2. After 30 min on ice, the suspension was centrifuged (4°C, 14000 *g*, 30 min), and the pellet was washed 3 times with water-saturated diethyl ether. Air-dried samples were solubilized in Triton X-100 lysis buffer, and proteins in the supernatant (20 μg) were heated with SDS sample buffer, separated by SDS-PAGE (12%), and transferred to a nitrocellulose membrane. The phosphorylated form of the myosin light chain 20 (P-MLC20) and α-smooth muscle actin were detected using selective monoclonal antibodies (Cell Signaling; Danvers, MA, USA; Sigma; St. Louis, MO, USA), as described previously (6).

Immunohistochemistry

sEH

Mice were euthanized by an intraperitoneal overdose of pentobarbital sodium. After intubation, a midsternal thoracotomy was performed, catheters were placed in the pulmonary artery and the left atrium, and the vasculature was flushed with 20 ml saline at a pulmonary artery pressure of 22

cm H_2O and a tracheal pressure of 12 cm H_2O. Thereafter, the pulmonary vasculature was perfused with Zamboni's fixative for 30 min at the same pressures. After ligation of the pulmonary artery, veins, and the trachea, lungs were removed and placed in the Zamboni's fixative for a further 6 h at room temperature, followed by incubation in phosphate buffer (0.1 mol/L, 12 h, 4°C). Tissues were then dehydrated and infiltrated with paraffin in an automated vacuum tissue processor (TP1050; Leica, Bensheim, Germany). After deparaffinization and rehydration of 3-μm sections, endogenous peroxidase was blocked in 3% hydrogen peroxide. Slides were incubated with trypsin (Digest All; Zytomed, Berlin, Germany) for 10 min for retrieval of the antigen, and unspecific binding sites were blocked using horse serum (Alexis, Grünberg, Germany). The sections were incubated overnight (4°C) with a polyclonal sEH antibody (dilution 1:2000) raised against a recombinant murine sEH produced in the baculovirus expression system and then purified to apparent homogeneity by affinity chromatography. The ImmPRESS anti-rabbit IgG peroxidase kit (Vector/Linaris, Wertheim-Bettingen, Germany) was then used according to the manufacturer's protocol, and the sEH was visualized using the Nova Red chromogen kit for horseradish peroxidase (Vector, Linaris, Wertheim-Bettingen, Germany). Nuclear counterstaining was performed with hematoxylin (Zytomed).

TRPC6

Translocation of the TRPC6 channel was assessed in cultured rat pulmonary artery smooth muscle cells infected with an adenovirus encoding a V5-tagged TRPC6 fusion protein (TRPC6-V5), as described previously (19). Following stimulation, samples were fixed (4% paraformaldehyde in PBS), permeabilized with Triton X-100, and incubated with phalloidin and specific antibodies to V5 (Invitrogen, Carlsbad, CA, USA) or caveolin-1 (BD Biosciences, Heidelberg, Germany). The preparations were mounted and viewed using a confocal microscope (LSM 510 META; Zeiss, Oberkochen, Germany).

Arachidonic acid metabolism

Lung microsomes were prepared as described (20), and microsomal protein (50 μg) was incubated in 100 μl of potassium phosphate buffer (100 mmol/L, pH 7.2) containing arachidonic acid (10 μmol/L) in the absence or presence of fenbendazole (100 μmol/L) and MSPPOH (10 μmol/L) for 15 min. To determine hypoxia-induced EET generation, microsomal proteins were incubated with either a hypoxic (1% O_2) or normoxic Krebs-Henseleit buffer containing arachidonic acid (10 μmol/L) for 10 min. Reactions were started by the addition of NADPH (1 mmol/L final concentration) and terminated after 20 min by placing on ice. The reaction products were extracted twice into ethyl acetate, evaporated under nitrogen, resuspended in methanol/water (vol 1.1). To determine hypoxia-induced EET generation, intact rat pulmonary artery smooth muscle cells were treated with arachidonic acid (100 nmol/L, 5 h) before being washed extensively over 60 min and exposed to hypoxia (1% O_2, 10 min).

Reactions were terminated by placing the cells on ice, and the eicosanoid profiles generated were determined with a Sciex API4000 mass spectrometer operating in multiple reaction monitoring (MRM) mode as described (ref. 21 and Supplemental Material).

Statistical analysis

Data are expressed as the mean ± SE, and statistical evaluation was performed using Student's *t* test for unpaired data or 1-way ANOVA followed by a Bonferroni *t* test when appropriate. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Effect of sEH inhibition and 11,12-EET on acute hypoxic pulmonary vasoconstriction in isolated buffer-perfused mouse lungs

Hypoxic ventilation (FiO_2=0.01) of lungs from wild-type mice resulted in an acute increase in pulmonary artery pressure (**Fig. 1*A***). Repeated stimulation (up to 5 times) resulted in quantitatively similar responses. However, following application of the sEH inhibitor ACU to the pulmonary perfusate, the acute hypoxic vasoconstriction response was significantly augmented (Fig. 1*A, B*). A similar response was observed using a second sEH inhibitor, AEPU, which has a similar potency but markedly different physical properties (Fig. 1*B*).

To determine whether CYP-derived EETs are involved in acute hypoxic pulmonary vasoconstriction, we reassessed responses in animals treated with fenbendazole (4% in chow) for 2 wk. CYP inhibition by fenbendazole was demonstrated in murine lung microsomes by determining the conversion of arachidonic acid to EET. Fenbendazole was equally as effective as the epoxygenase inhibitor MSPPOH in attenuating the generation of 11,12- and 14,15-EET without affecting the generation of either 5,6- or 8,9-EET (Supplemental Fig. 1). Treatment of mice with fenbendazole was without significant effect on the magnitude of acute hypoxic pulmonary vasoconstriction (Fig. 1*C*) but attenuated the potentiation of vasoconstriction induced by sEH inhibition.

In a second protocol, we tested the consequences of acute CYP epoxygenase inhibition and EET antagonism on hypoxic vasoconstriction and observed that although MSPPOH and 14,15-EEZE had only a marginal effect on their own, they completely prevented the increase in constriction induced by sEH inhibition (**Fig. 2*A***). As these data indicated that EETs facilitate hypoxia-induced pulmonary vasoconstriction, we next assessed the effects of 11,12-EET and 14,15-EET. In lungs from wild-type mice, the application of 11,12-EET (10 nmol/L to 3 μmol/L) to the pulmonary perfusate rapidly and concentration-dependently increased pulmonary artery pressure while 14,15-EET (10 nmol/L to 3 μmol/L) and the solvent DMSO (0.3‰) was without significant effect (Fig. 2*B*). 11,12-DHET also failed to affect pulmonary artery pressure (data not shown). An indirect effect of 11,12-EET on interstitial/alveolar edema, which could elicit a "secondary" hypoxic vasoconstriction, could be ruled out, as we found no evidence to suggest that 11,12-EET affected endothelial cell permeability (measured by monitoring lung weight) in the isolated mouse lung (data not shown).

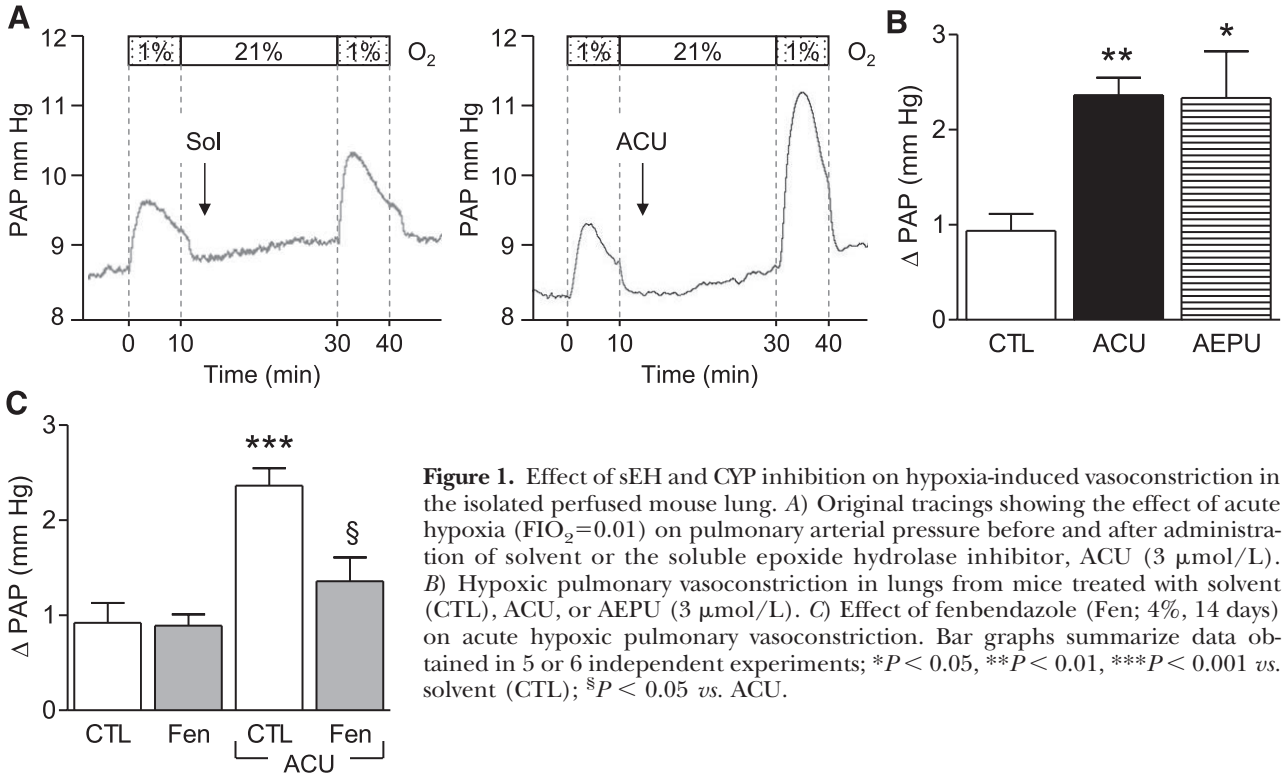

Figure 1. Effect of sEH and CYP inhibition on hypoxia-induced vasoconstriction in the isolated perfused mouse lung. *A*) Original tracings showing the effect of acute hypoxia (FIO_2=0.01) on pulmonary arterial pressure before and after administration of solvent or the soluble epoxide hydrolase inhibitor, ACU (3 μmol/L). *B*) Hypoxic pulmonary vasoconstriction in lungs from mice treated with solvent (CTL), ACU, or AEPU (3 μmol/L). *C*) Effect of fenbendazole (Fen; 4%, 14 days) on acute hypoxic pulmonary vasoconstriction. Bar graphs summarize data obtained in 5 or 6 independent experiments; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* solvent (CTL); $^{\S}P < 0.05$ *vs.* ACU.

Hypoxic pulmonary vasoconstriction in lungs from sEH$^{-/-}$ mice

Although the sEH is expressed in the carotid artery endothelium (22), this is not the case in the lung, where the enzyme was selectively expressed in vascular smooth muscle cells (**Fig. 3*A***). No sEH was detected in lungs removed from sEH$^{-/-}$ mice.

We next assessed hypoxic vasoconstriction in lungs from wild-type and sEH$^{-/-}$ mice. As before, hypoxic vasoconstriction in lungs from wild-type mice was increased by sEH inhibition. Hypoxic vasoconstriction in lungs from sEH$^{-/-}$ mice was significantly greater than that observed in lungs from wild-type mice, and responses were not affected by the sEH inhibitor (Fig. 3*B*). However, responses returned to levels observed in lungs from wild-type animals in the presence of either MSPPOH or 14,15-EEZE (Fig. 3*B*), indicating that the response to hypoxia was dependent on the activation of a CYP epoxygenase and the generation of an EET. Moreover, in microsomes prepared from the lungs of wild-type mice, hypoxia (10 min) elicited a significant increase in EET production. As expected, EET levels in microsomes from the lungs of sEH$^{-/-}$ mice were greater than those detected in samples from wild-type animals and were also increased in response to hypoxia (**Fig. 4*A–C***). In intact rat pulmonary smooth muscle cells, hypoxia (10 min) significantly increased 11,12-EET production (Fig. 4*D*) but had no effect on 8,9-EET and 14,15-EET generation (data not shown).

Effect of hypoxia and 11,12-EET on the RhoA Rho kinase pathway

Hypoxia-induced pulmonary vasoconstriction has been attributed to activation of the Rho kinase (23, 24). Given that the contraction elicited by the CYP-derived eicosanoid, 20-HETE, in the systemic circulation is linked to the Rho kinase-dependent phosphorylation of the myosin light chain (6), we compared the effects of hypoxia, ACU, and 11,12-EET on Rho kinase activity.

In pulmonary artery smooth muscle cells, hypoxia stimulated the phosphorylation of myosin light chain-20, an effect that was significantly potentiated by ACU and largely prevented by the Rho kinase inhibitor, Y27632 (**Fig. 5*A***). The exogenous application of 11,12-EET had no significant effect on myosin light chain-20 phosphorylation (not shown) but increased Rho kinase activity (measured by G-LISA) in the isolated perfused lung (Fig. 5*B*). Moreover, the increase in pulmonary artery pressure elicited by 11,12-EET was significantly inhibited by the Rho kinase inhibitor (Fig. 5*C, D*), as was the ACU-induced potentiation of hypoxic vasoconstriction (Fig. 5*D*).

Identification of TRPC6 as an effector of 11,12-EET-induced pulmonary vasoconstriction

We recently reported that 11,12-EET can affect endothelial Ca^{2+}-signaling by stimulating the intracellular translocation of TRPC6 to caveolae (19). Because TRPC6 has been linked to hypoxic pulmonary vasoconstriction (25), we analyzed the effect of 11,12-EET on pulmonary

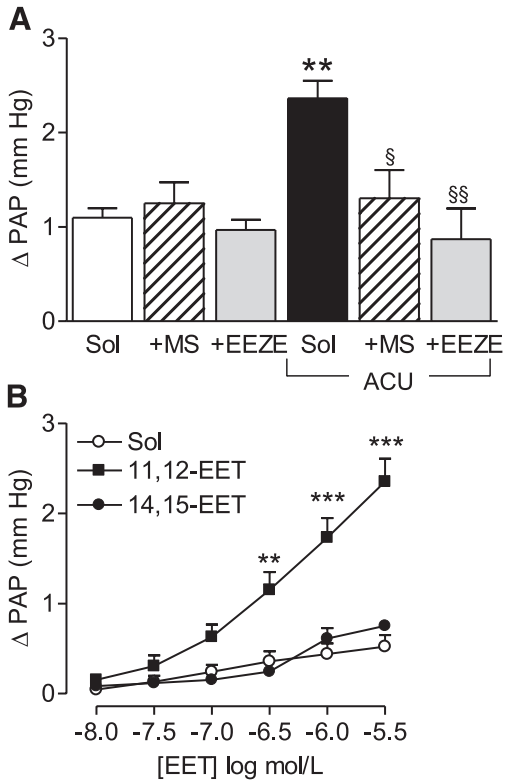

Figure 2. Comparison on the effects of sEH inhibition and exogenously applied CYP epoxygenase-derived EETs on hypoxia-induced vasoconstriction in the isolated perfused mouse lung. *A*) Hypoxic pulmonary vasoconstriction was assessed in the presence of solvent (Sol), MSPPOH (MS, 10 μmol/L), or 14,15-EEZE (EEZE, 10 μmol/L), and in the absence and presence of ACU (3 μmol/L). *B*) Effect of 11,12-EET and 14,15-EET (10 nmol/L to 3 μmol/L) *vs.* the solvent (Sol, 0.3‰) DMSO on pulmonary arterial pressure in lungs from wild-type mice. Graphs summarize data obtained using 4–6 animals/group; $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* solvent; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ *vs.* ACU alone.

arterial pressure and acute hypoxic pulmonary vasoconstriction in lungs from TRPC6$^{-/-}$ mice and their heterozygous (TRPC6$^{+/-}$) littermates.

In lungs from control (TRPC6$^{+/-}$) mice, acute hypoxia elicited a vasoconstriction that was potentiated by 11,12-EET (**Fig. 6*A***). Moreover, 11,12-EET (10 nmol/L to 3 μmol/L) elicited a concentration-dependent increase in pulmonary artery pressure that was similar to that observed in wild-type mice (Fig. 6*B*). Hypoxia failed to elicit an acute increase in pulmonary pressure in lungs from TRPC6$^{-/-}$ mice (Fig. 6*A*). Moreover, in the latter animals, 11,12-EET failed to significantly potentiate the response to hypoxia or elicit an increase in pulmonary artery pressure on its own (Fig. 6*A, B*). As reported previously (25), the responsiveness of TRPC6$^{-/-}$ mice to U46619 was normal (data not shown).

To assess the consequences of 11,12-EET on the intracellular localization of TRPC6 a TrpC6-V5 fusion protein, in a double cassette together with GFP, was overexpressed in rat pulmonary artery smooth muscle cells. In cells maintained under basal conditions, the TRPC6-V5 was localized to the perinuclear Golgi apparatus as was the majority of the GFP signal. Following the application of 11,12-EET (3 μmol/L, 5 min) the channel translocated to the plasma membrane, more specifically to membrane domains enriched with the caveolae marker, caveolin-1 (**Fig. 7*A***). TRPC6-V5 translocation was also observed in response to lower concentrations of 11,12-EET (10 nmol/L to 1 μmol/L), and responses were abrogated in cells pretreated with 14,15-EEZE (data not shown). Exposure of the cells, which expressed both CYP2C11 and the sEH (Supplemental Fig. 2), to 1% O_2 (5 min) also stimulated the intracellular translocation of TRPC6-V5 to caveolae. The latter effect was not observed in cells pretreated with the EET antagonist, 14,15-EEZE (Fig. 7).

DISCUSSION

In the present study, we report that the activity of sEH plays a significant role in determining the magnitude of acute hypoxic vasoconstriction. The mechanism involved appears to be related to the generation of CYP

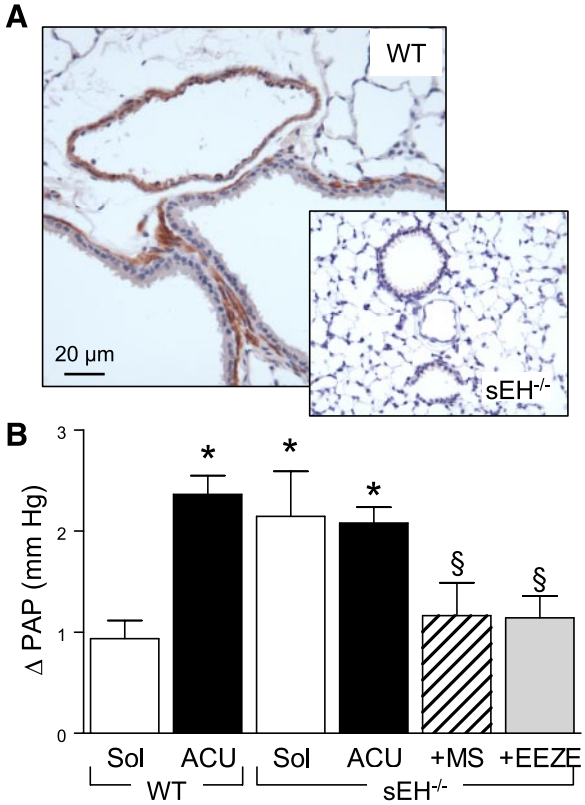

Figure 3. Hypoxic pulmonary vasoconstriction in lungs from sEH$^{-/-}$ mice. *A*) Immunohistochemical analysis showing the expression of the sEH in lungs from wild-type (WT) and sEH$^{-/-}$ mice. *B*) Acute hypoxic pulmonary vasoconstriction in lungs of WT and sEH$^{-/-}$ mice treated with solvent (Sol) or ACU (3 μmol/L) in the absence and presence of MSPPOH (MS, 10 μmol/L) or 14,15-EEZE (EEZE, 10 μmol/L). Bar graph summarizes data from 3–6 independent experiments; $^{*}P < 0.05$ *vs.* Sol WT; $^{\S}P < 0.05$ *vs.* Sol sEH$^{-/-}$.

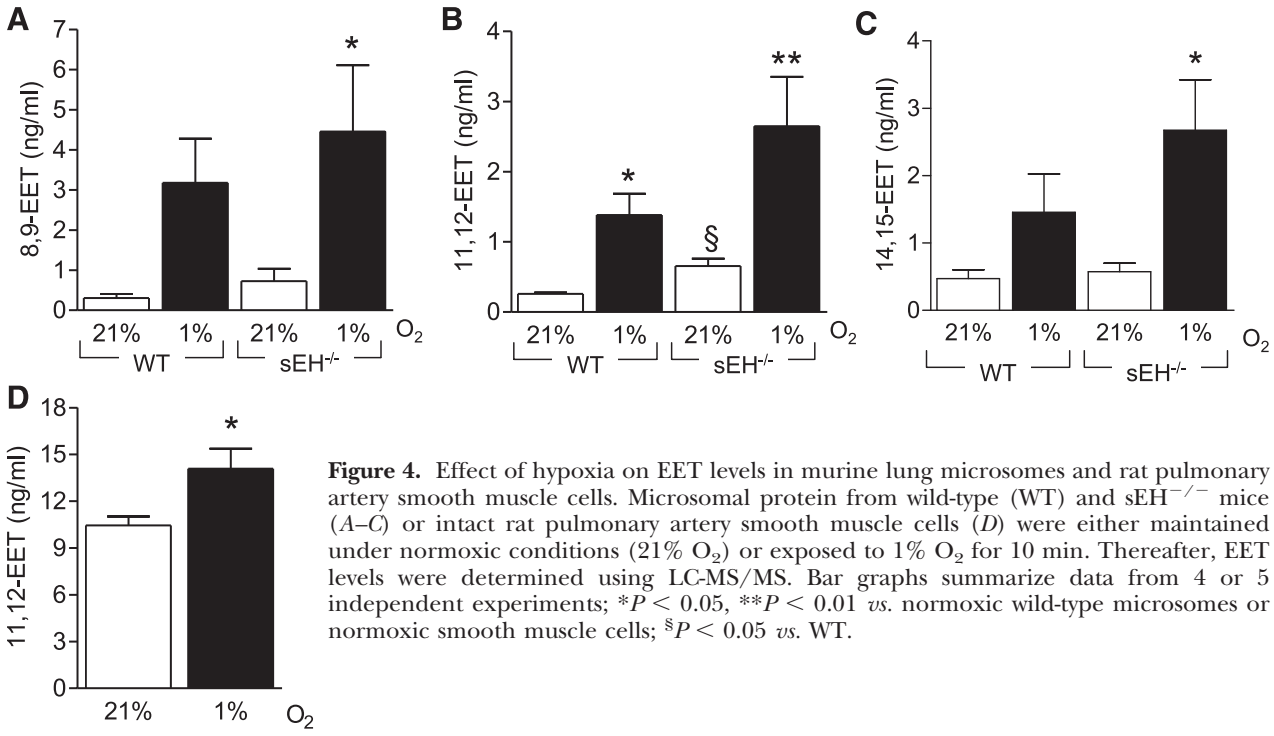

Figure 4. Effect of hypoxia on EET levels in murine lung microsomes and rat pulmonary artery smooth muscle cells. Microsomal protein from wild-type (WT) and sEH$^{-/-}$ mice (*A–C*) or intact rat pulmonary artery smooth muscle cells (*D*) were either maintained under normoxic conditions (21% O_2) or exposed to 1% O_2 for 10 min. Thereafter, EET levels were determined using LC-MS/MS. Bar graphs summarize data from 4 or 5 independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* normoxic wild-type microsomes or normoxic smooth muscle cells; $^{\S}P < 0.05$ *vs.* WT.

epoxygenase-derived epoxides because both the epoxygenase inhibitor, MSPPOH, and the EET antagonist, 14,15-EEZE, abrogated the effects of sEH inhibition and the exogenous application of 11,12-EET elicited a rapid increase in pulmonary perfusion pressure. Both hypoxia and 11,12-EET were able to elicit the activation

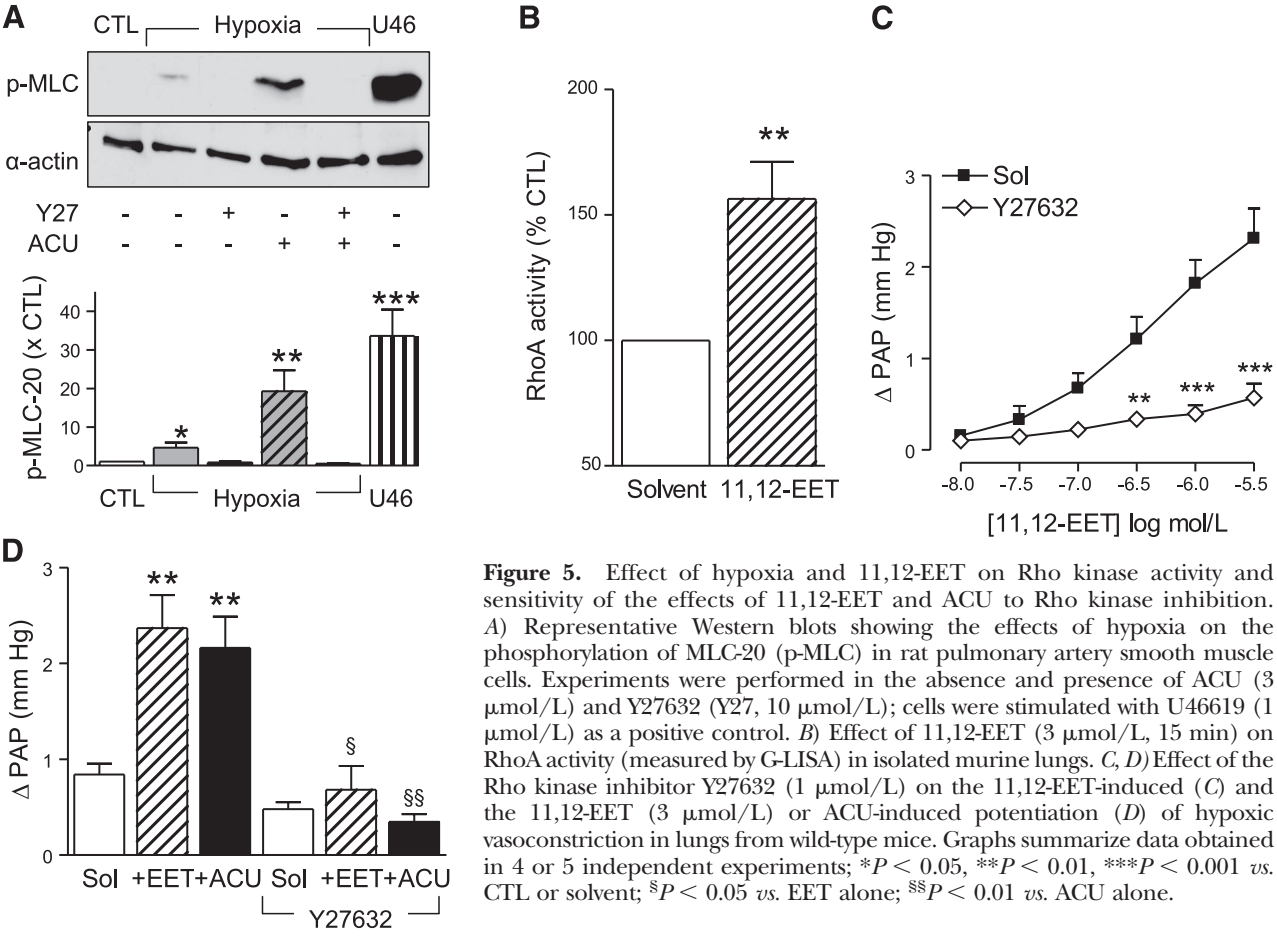

Figure 5. Effect of hypoxia and 11,12-EET on Rho kinase activity and sensitivity of the effects of 11,12-EET and ACU to Rho kinase inhibition. *A*) Representative Western blots showing the effects of hypoxia on the phosphorylation of MLC-20 (p-MLC) in rat pulmonary artery smooth muscle cells. Experiments were performed in the absence and presence of ACU (3 μmol/L) and Y27632 (Y27, 10 μmol/L); cells were stimulated with U46619 (1 μmol/L) as a positive control. *B*) Effect of 11,12-EET (3 μmol/L, 15 min) on RhoA activity (measured by G-LISA) in isolated murine lungs. *C, D)* Effect of the Rho kinase inhibitor Y27632 (1 μmol/L) on the 11,12-EET-induced (*C*) and the 11,12-EET (3 μmol/L) or ACU-induced potentiation (*D*) of hypoxic vasoconstriction in lungs from wild-type mice. Graphs summarize data obtained in 4 or 5 independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* CTL or solvent; $^{\S}P < 0.05$ *vs.* EET alone; $^{\S\S}P < 0.01$ *vs.* ACU alone.

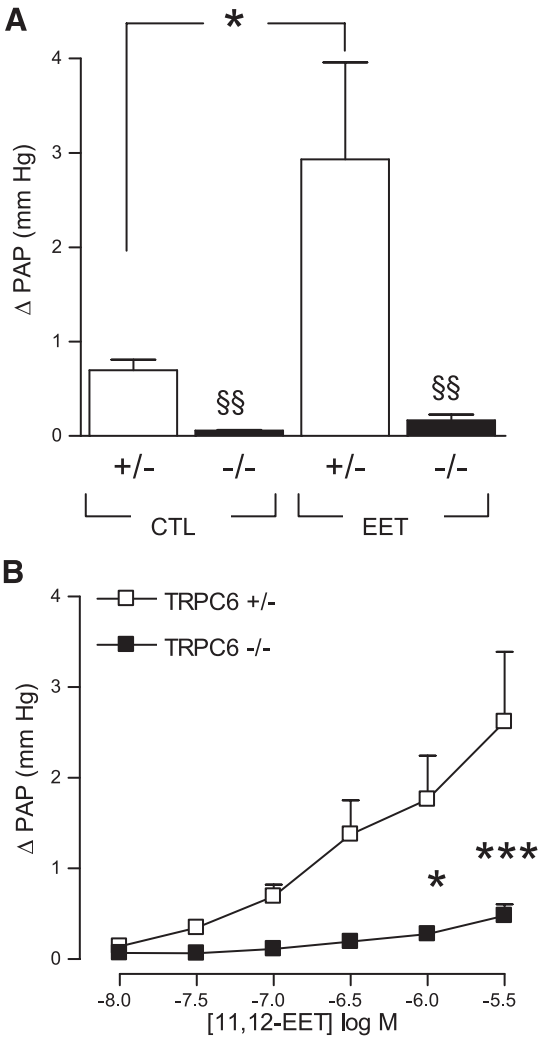

Figure 6. Identification of TRPC6 as an effector of 11,12-EET-induced pulmonary vasoconstriction. *A*) Hypoxic pulmonary vasoconstriction in buffer-perfused lungs from TRPC6$^{+/-}$ and TRPC6$^{-/-}$ mice before (CTL) and after treatment with 11,12-EET (3 μmol/L). *B*) Concentration-dependent effect of 11,12-EET on pulmonary arterial pressure (PAP) in lungs isolated from TRPC6$^{+/-}$ and TRPC6$^{-/-}$ mice. Graphs summarize data obtained in 5 independent experiments; $^{*}P < 0.05$, $^{***}P < 0.001$ *vs.* control; $^{§§}P < 0.001$ *vs.* TRPC6$^{+/-}$.

of the Rho kinase and the translocation of TRPC6 channels within pulmonary smooth muscle cells, indicating that the EET-induced activation of TRPC6 underlies the phenomenon observed.

In the systemic circulation, the predominant function of CYP epoxygenase-derived EETs is to elicit the hyperpolarization of endothelial and vascular smooth muscle cells and thus, vasodilatation. CYP-dependent vasoconstriction, on the other hand, is generally attributed to metabolites such as 20-HETE (26), which play an important role in the myogenic response. In the pulmonary circulation, the situation seems to be reversed, as 20-HETE is reported to dilate pulmonary arteries and inhibition of CYP4A aggravates acute hypoxic pulmonary vasoconstriction (27, 28). EETs have previously been linked with constriction in the pulmonary circulation (8, 10), and although these findings seem to be restricted to specific vessels (7), we recently reported that a CYP epoxygenase is implicated in the acute pulmonary vasoconstriction induced by hypoxia in anesthetized mice (11). In the latter study, it was also possible to demonstrate that the mediator involved was metabolized by the sEH, as inhibition of this enzyme potentiated the vasoconstrictor response. One apparent difference between the present investigation in the isolated lung the previous study in anesthetized animals (11) was the sensitivity of the hypoxic vasoconstriction *per se* to CYP epoxygenase inhibition. This difference can most likely be attributed to the methods used to assess pulmonary function as pressures measured in the isolated lung model were lower than those measured *in vivo*. The results of the present investigation, however, highlight the importance of the sEH in modulating the hypoxic vasoconstriction observed in wild-type mice, which can most probably be attributed to the metabolism of vasoconstrictor EETs. The two compounds used, ACU and AEPU, are both potent sEH inhibitors with IC_{50} values in the low nanomole per liter range for both the human and the murine recombinant enzymes. However, their structures and physical properties are quite different, and the fact that these divergent compounds led to very similar pulmonary response support the conclusion that their action was due to the inhibition of the sEH. Moreover, the acute increase in pulmonary perfusion pressure induced by hypoxia was markedly elevated in lungs from sEH$^{-/-}$ mice.

Biological activity has been attributed to all of the EET regioisomers generated in the lung. However, we observed that exposure to hypoxia acutely increased the production of 11,12-EET in murine lung microsomes and in intact rat pulmonary artery smooth muscle cells; a response also recently observed in the rabbit lung (29). Moreover, both MSPPOH and fenbendazole abrogated the effects of sEH inhibition on hypoxic vasoconstriction and attenuated the generation of 11,12- and 14,15-EET without affecting that of either 5,6- or 8,9-EET. For these reasons, we concentrated on elucidating the effects of 11,12- and 14,15-EET on the pulmonary circulation and found that 11,12-EET elicited a pronounced concentration-dependent increase in pulmonary perfusion pressure, while 14,15-EET was without effect. Generation of the corresponding diol is also unlikely to account for the observations made as 11,12-DHET had no effect on pulmonary artery pressure. Moreover, in sEH$^{-/-}$ mice, which demonstrate elevated circulating (30) and pulmonary levels of EETs and low DHET levels, neither of the sEH inhibitors tested was able to affect the acute pulmonary vasoconstriction induced by hypoxia. Thus, the sEH inhibitor-induced increase in pulmonary vasoconstriction that was dependent on the activity of a CYP epoxygenase seems to be attributable to 11,12-EET. Further support for a prominent role of an arachidonic acid product in the pulmonary vasoconstrictor response to hypoxia has been obtained using animals lacking the cytosolic phospholipase (cPL) A_2, which releases arachidonic acid

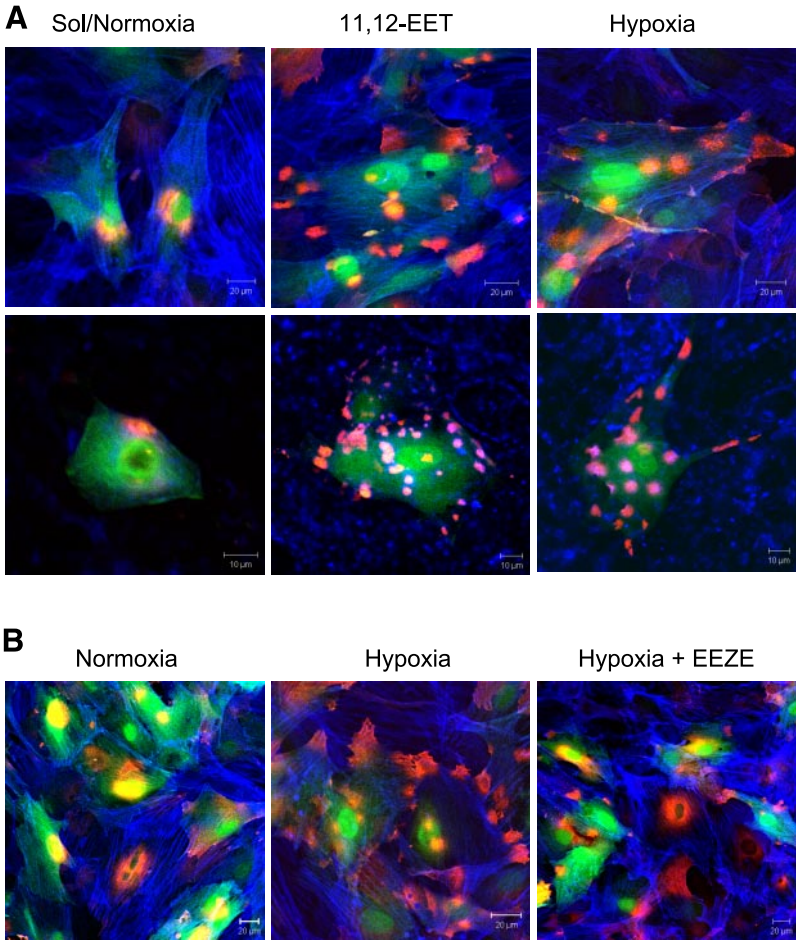

Figure 7. Effect of EETs and hypoxia on the translocation of TRPC6-V5. Rat pulmonary artery smooth muscle cells were infected with TrpC6-V5 adenoviruses 24 h prior to stimulation with solvent (Sol), 11,12-EET (3 μmol/L, 5 min), or hypoxia (1% O_2, 5 min). *A*) Comparison of the effect of 11,12-EET and hypoxia. Top panels: green fluorescent protein (GFP; green), V5 (red), phalloidin (blue). Bottom panels: GFP (green), V5 (red), caveolin-1 (blue). *B*) Effect of 14,15-EEZE (EEZE, 10 μmol/L) on the hypoxia-induced translocation of TRPC6-V5; GFP (green), V5 (red), phalloidin (blue). Identical results were obtained in four additional experiments, each using a different cell batch.

from phospholipids in cell membranes (31). In the latter study, hypoxic pulmonary vasoconstriction induced by left main stem bronchus occlusion was detectable in wild-type but not in $cPLA_2^{-/-}$ mice and could be restored in these animals by the exogenous application of arachidonic acid.

Contraction of smooth muscle cells occurs *via* Ca^{2+}-dependent mechanisms, requiring an increase in intracellular Ca^{2+}, as well as *via* Ca^{2+}-independent mechanisms related to the activation of the Rho kinase (32, 33). Hypoxic vasoconstriction has been linked to Rho kinase activation in a number of studies (23, 24), as has the 20-HETE-induced contraction of coronary arteries (6). Given that the actions of 11,12-EET and 20-HETE seem to be reversed in the lung (34, 35), we determined whether hypoxia, sEH inhibition, and/or 11,12-EET were able to affect the activity of the Rho kinase. Our results clearly demonstrate that sEH inhibition increases the hypoxia-induced phosphorylation of MLC-20 and that 11,12-EET is able to stimulate Rho kinase activity in the murine lung. Moreover, the Rho kinase inhibitor Y27632 prevented not only the sEH inhibitor-induced potentiation of hypoxic vasoconstriction but also the increase in pulmonary artery pressure elicited by exogenous 11,12-EET.

Although the Rho kinase can be activated in the absence of a sustained increase in intracellular Ca^{2+}, there is a wealth of evidence indicating that Ca^{2+} plays a pivotal role in pulmonary vasoconstriction. While the activation of several types of Ca^{2+} channels can affect $[Ca^{2+}]_i$ in smooth muscle cells, a lot of attention has been focused on the role played by the TRP channel family. To identify the potential mechanism of the hypoxia-induced, EET-mediated pulmonary vasoconstriction, we concentrated on the TRPC6 channel. EETs can affect the activity of at least 2 different classes of TRP channel (TRPV4 and TRPC6), but there were several reasons for singling out TRPC6 as an effector for 11,12-EET. First, although CYP epoxygenases can modulate the activity of TRPV4, a channel implicated in mechanotransduction, these effects have been attributed to 5,6- and 8,9-EET but not 11,12-EET (36). Second, while TRPC6 is unaffected by shear and hypoosmotic stress, as well as by 5,6-EET (unpublished observation), both its translocation and Ca^{2+} influx are stimulated by 11,12-EET (19). Thus the spectrum of EET regioisomers generated in response to a given stimulus would be expected to determine which TRP channels can be affected. Indeed, TRPC6 can also be activated by CYP-derived 20-HETE in HEK cells overexpressing the channel (37). Moreover, although relatively few studies have addressed the role of hypoxia in the regulation of TRP channels, the expression of TRPC6 and store- as well as receptor-operated Ca^{2+} entry into pulmonary artery smooth muscle cells is elevated in response to chronic (3 wk) hypoxia (38),

and acute hypoxic vasoconstriction is almost abolished in the lungs of TRPC6$^{-/-}$ mice (25). The results obtained in the present study demonstrated that neither hypoxia nor 11,12-EET were able to stimulate an increase in pulmonary perfusion pressure in lungs from TRPC6$^{-/-}$ mice. The lack of responsiveness to hypoxia and 11,12-EET appears to be a specific phenomenon, as the responsiveness of the lungs from TRPC6$^{-/-}$ mice to U46619 was normal (25), and aortic rings from these animals even demonstrate an elevated contractile response to phenylephrine (16). Given that 11,12-EET has been found to increase Ca^{2+} entry and elicit the translocation of the TRPC6 channel from the perinuclear Golgi apparatus to caveolae (19), we determined whether hypoxia could induce the translocation of the TRPC6 channel in pulmonary smooth muscle cells and whether this was an EET-dependent process. Indeed, we found that hypoxia stimulated the membrane translocation of a TRPC6-V5 fusion protein, more specifically to membrane domains enriched with caveolin-1, an effect that was mimicked by 11,12-EET and markedly attenuated in the presence of the EET antagonist.

Taken together, the results of the present study demonstrate that the activity of the sEH is an important determinant of the magnitude of hypoxic pulmonary vasoconstriction by inactivating vasoconstrictor CYP-derived EETs. These eicosanoids appear to be important modulators of pulmonary vascular tone and can elicit contraction by targeting TRPC6 channels to the plasma membrane, as well as by activating the Rho kinase. Although the sequence of these events was not addressed in detail in the present study, it has previously been reported that a TRPC6-dependent increase in cytosolic calcium is required to activate the Rho kinase in pulmonary artery endothelial cells (39). The exact molecular mechanisms by which EETs induce the membrane translocation of TRPC6 channels also remains to be elucidated, although protein kinase A may be implicated in this process (19). Given that hypoxia modulates the expression of TRPC6 channels (38), as well as that of the CYP epoxygenases that are involved in their regulation (11, 21, 40), it will be interesting to determine whether the signaling cascade outlined here plays a role in human pathology associated with alveolar hypoxia in the lung. Another aspect that is important to highlight is that based on experimental data showing that sEH inhibitors attenuate hypertension in spontaneously hypertensive rats (41) and in angiotensin II-treated mice (22), as well as protect stroke-prone spontaneously hypertensive rats from cerebral ischemia (42), sEH inhibitors are currently being developed for the treatment of human hypertension and inflammation/atherosclerosis. However, sEH deletion reduces survival after cardiac arrest (43), and genetic variation in the sEH gene (EPHX2) has been linked to a higher incidence of stroke in rats, as well as in humans (44, 45). Moreover, our results indicate that this therapeutic approach is likely to compromise ventilation/perfusion adaptation in the lung. Given the apparent involvement of CYP epoxygenases in pulmonary remodeling, in particular, in response to hypoxia (11), these compounds may even promote the development of pulmonary hypertension. FJ

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