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Cytochrome P450 epoxygenase-derived 5,6-epoxyeicosatrienoic acid relaxes pulmonary arteries in normoxia but promotes sustained pulmonary vasoconstriction in hypoxia

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Abstract

Aims: The aim of the study was to investigate the role of cytochrome P450 (CYP) epoxygenase-derived epoxyeicosatrienoic acids (EETs) in sustained hypoxic pulmonary vasoconstriction (HPV).

Methods: Vasomotor responses of isolated mouse intrapulmonary arteries (IPAs) were assessed using wire myography. Key findings were verified by haemodynamic measurements in isolated perfused and ventilated mouse lungs.

Results: Pharmacological inhibition of EET synthesis with MS-PPOH, application of the EET antagonist 14,15-EEZE or deficiency of CYP2J isoforms suppressed sustained HPV. In contrast, knockdown of EET-degrading soluble epoxide hydrolase or its inhibition with TPPU augmented sustained HPV almost twofold. All EET regioisomers elicited relaxation in IPAs pre-contracted with thromboxane mimetic U46619. However, in the presence of KCl-induced depolarization, 5,6-EET caused biphasic contraction in IPAs and elevation of pulmonary vascular tone in isolated lungs, whereas other regioisomers had no effect. In patch-clamp experiments, hypoxia elicited depolarization in pulmonary artery smooth muscle cells (PASMCs), and 5,6-EET evoked inward whole cell currents in PASMCs depolarized to the hypoxic level, but not at their resting membrane potential.

Conclusions: The EET pathway substantially contributes to sustained HPV in mouse pulmonary arteries. 5,6-EET specifically appears to be involved in HPV, as it is the only EET regioisomer able to elicit not only relaxation, but also sustained contraction in these vessels. 5,6-EET-induced pulmonary vasoconstriction is enabled by PASMC depolarization, which occurs in hypoxia. The discovery of the dual role of 5,6-EET in the regulation of pulmonary vascular tone may provide a basis for the development of novel therapeutic strategies for treatment of HPV-related diseases.

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KEYWORDS
cytochrome P450 epoxygenase, epoxyeicosatrienoic acid, hypoxia, hypoxic pulmonary vasoconstriction, pulmonary arteries, soluble epoxide hydrolase

1 | INTRODUCTION

Hypoxic pulmonary vasoconstriction (HPV) is a physiological response of pulmonary arteries, which diverts blood flow from poorly ventilated areas of the lung in order to optimize pulmonary gas exchange. HPV is generally recognized as a biphasic response consisting of acute and sustained components, each relying on its own distinct mechanisms.[1] The acute (transient) phase of HPV, normally observed within the first 10-15 minutes of hypoxia, is important for lung ventilation-perfusion matching during short-term ventilatory disturbances.[2-4] In contrast, the sustained phase develops gradually after 30 minutes of hypoxic exposure and is involved in the adaptation of lung perfusion in cases of prolonged regional hypoxia, eg, in focal pneumonia or atelectasis.[1,5] However, in generalized hypoxia caused by a range of lung or neuromuscular diseases, or at high altitude, exaggerated sustained HPV contributes to the development of pulmonary hypertension.[1] The exact mechanisms underlying the sustained phase of HPV are not completely understood. According to earlier reports, it is promoted by a yet unidentified vasoconstrictor factor released in pulmonary arteries.[6,7] We hypothesized that this factor belongs to the family of epoxyeicosatrienoic acids.

Epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12- and 14,15-EET) are cytochrome P450 (CYP) epoxygenase-derived arachidonic acid metabolites that possess anti-inflammatory and analgesic properties.[8,9] These eicosanoids are known to be strong vasodilators in the systemic circulation, where they are released in response to various stimuli and elicit hyperpolarization in vascular smooth muscle cells.[9] The role of EETs in the pulmonary circulation appears to be diverse and contradictory in part. Previous studies indicate that EETs may be involved in the mediation of acute HPV. Specifically, EET levels were shown to be increased in mouse lungs during short-term hypoxia.[10] Furthermore, inhibition or deficiency of soluble epoxide hydrolase (sEH), the enzyme responsible for degradation of EETs to inactive diols, significantly potentiated HPV.[10-12] However, in these studies, pulmonary vasomotor response was assessed only during the first 5-15 minutes of a hypoxic challenge, a time range, which corresponds to the acute phase of HPV. The contribution of the EET pathway to sustained HPV was not examined. Furthermore, despite the findings mentioned above, interpreting the role of EET pathway in regulation of the pulmonary circulation is complicated by contradictory reports on their direct effects on pulmonary artery tone. In fact, application of EETs induced pulmonary vasoconstriction only in a fraction of studies. For instance, it has been shown that all four EET regioisomers contract pressurized rabbit pulmonary arteries.[13] However, only 11,12-EET caused an increase in pulmonary artery pressure (PAP) in isolated perfused mouse lungs, whereas 14,15-EET was without effect.[10] 11,12-EET also did not affect tone in porcine pulmonary arteries.[14] Furthermore, 5,6- and 11,12-EET reportedly relaxed rabbit and piglet pulmonary arteries respectively.[15,16] The reason for these discrepancies is not clear as the mechanisms underlying EET-induced vasomotor effects in pulmonary vessels are largely unknown.

Against this background, we here have examined the contribution of the EET pathway to sustained HPV and identified the mechanism responsible for EET-mediated contraction of mouse intrapulmonary arteries (IPAs).

2 | RESULTS

2.1 | Expression of CYP epoxygenases in mouse IPAs

EETs are known as autocrine and paracrine factors,[17] which implies that in order to affect pulmonary artery tone in vivo, these eicosanoids may be released in vessels by smooth muscle and/or endothelial cells as well as by cells of surrounding tissues. In view of this, we investigated whether mouse IPAs express CYP epoxygenases responsible for EET synthesis. Enzymes of CYP2C and CYP2J subfamilies are the major contributors to EET production in both human and mouse.[18] According to previous reports, CYP2C isoforms are not expressed in the mouse lung in general.[19] In contrast, CYP2J isoforms are not only expressed in the lung, but also appear to be involved in the regulation of acute HPV.[4,19] Therefore, we analysed the expression of mRNA of CYP2J proteins in isolated mouse IPAs. Quantitative PCR analysis revealed relatively high expression of CYP2J9, CYP2J11, CYP2J12 and CYP2J13 (Figure 1A). The expression of CYP2J5 and CYP2J6 was considerably lower, whereas no expression of CYP2J8 was detected. We were not able to evaluate the expression at the protein level, as isoform-specific antibodies to mouse CYP2J epoxygenases are not commercially available.

2.2 | Effect of inhibition and activation of EET pathway on sustained HPV

In order to examine the role of the EET pathway in sustained HPV, we investigated the effects of the CYP epoxygenase inhibitor N-methylsulfonyl-6-(2-proparglyoxyphenyl)

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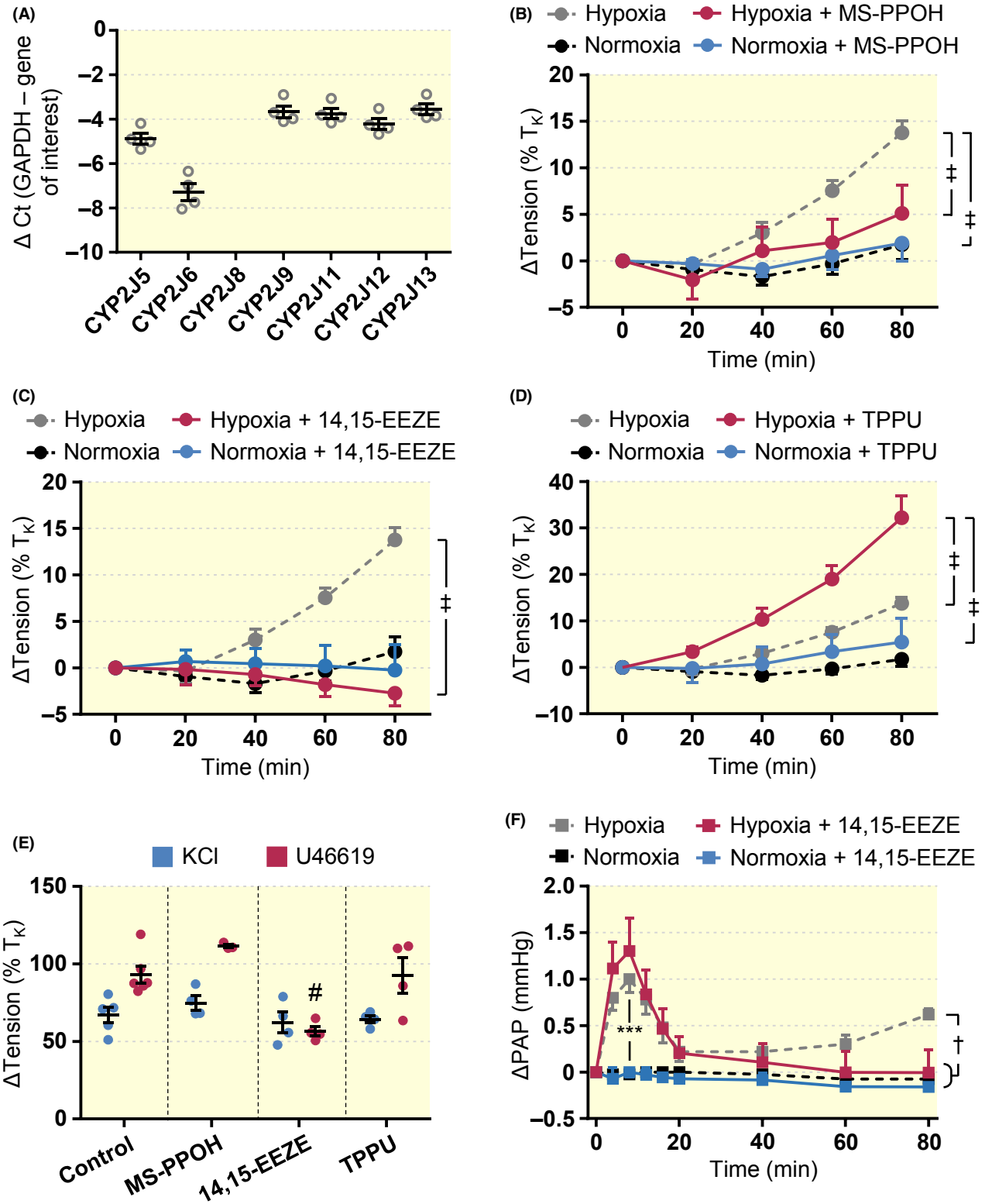

FIGURE 1 Effects of pharmacological inhibition of EET synthesis/degradation on HPV. A, Expression of mRNA of CYP2J isoforms in mouse IPAs. n = 4 for each group. B-D, Changes in isometric tension in non-pre-contracted IPAs during 80-minutes incubation in hypoxia in the presence of a P450 epoxygenase inhibitor, MS-PPOH (30 μmol/L; A), an EET antagonist, 14,15-EEZE (1.5 μmol/L; B) and a soluble epoxide hydrolase inhibitor, TPPU (3 μmol/L; C). n = 5-9 for each group. (E) The maximal contraction elicited by KCl (80 mmol/L) and U46619 (30 nmol/L) after 80-minutes incubation of IPAs in normoxia with corresponding inhibitors. n = 4-5 for each group. (F) Effect of 14,15-EEZE (10 μmol/L) on hypoxia-induced increase in PAP in isolated perfused and ventilated mouse lungs. n = 3-5 for each group. Data are expressed as means ± SEM. Multiple comparisons were performed using one-way ANOVA in (A), two-way ANOVA in (B–F). $^{\#}P < .05$ compared to control; $^{\dagger}P < .01$; $^{\ddagger}P < .001$

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hexanamide (MS-PPOH; 30 μmol/L), the EET antagonist 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE; 1.5 μmol/L) and the sEH inhibitor 1-Trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU; 3 μmol/L) on hypoxic responses in isolated mouse IPAs using wire myography. In line with our previous report,[20] hypoxia induced a monophasic, gradually developing sustained contraction in IPAs (Figure 1B) with a slow recovery after reoxygenation. MS-PPOH suppressed HPV by ~60%, and 14,15-EEZE completely abolished the hypoxic response in isolated IPAs (Figure 1B,C). In contrast, TPPU, which inhibits the sEH-catalysed hydrolysis of EETs, potentiated HPV more than twofold (Figure 1D). MS-PPOH, 14,15-EEZE and TPPU did not have any significant effect on IPA tone in normoxia. Notably, the applied inhibitors also did not affect the KCl-induced contraction in IPA, which supports the specificity of their action (Figure 1E). Moreover, only 14,15-EEZE partially reduced the response of IPAs to the synthetic thromboxane A2 mimetic U46619 (Figure 1E).

In majority of the studies, HPV in isolated vessels is elicited in the presence of pre-contraction. Some investigators suggest that under this condition, responses are more physiologically relevant as pulmonary arteries appear to maintain some basal tone in vivo.[21] Along these lines in IPAs pre-contracted with KCl (15-20 mmol/L), HPV was significantly potentiated in our study. The effects of MS-PPOH, 14,15-EEZE and TPPU in the presence of pre-contraction were similar to those in non-pre-contracted vessels (Figure S1).

We induced a knockdown of the sEH gene, *Ephx2*, in isolated IPAs using siRNA. After 3 days of incubation following transfection, expression of *Ephx2* was decreased by ~87% compared to IPAs transfected with non-targeting (NT) siRNA (control; Figure 2A). The responses to basic stimuli, such as KCl, PGF2α and acetylcholine in both sEH knockdown and control IPAs were similar to those of fresh vessels (data not shown). Sustained HPV in non-pre-contracted IPAs transfected with Ephx2 siRNA was augmented almost twofold compared to IPAs transfected with NT siRNA (Figure 2B,C). In general, the observed potentiation of HPV was similar to that induced by TPPU in fresh IPAs. No significant changes in tone of the transfected IPAs were observed during 80 minutes of normoxia.

In addition to hydrolase activity, sEH has also been shown to exhibit lipid phosphatase activity.[22] In view of this, we investigated whether the effects of sEH inhibition/downregulation on HPV may be related to this property of the enzyme. We found that geraniol (50 μmol/L), a potent inhibitor of phosphatase activity of sEH,[23] had no effect on both normoxic tone and hypoxic response in IPAs (Figure S2). Thus, we conclude that phosphatase activity of sEH does not play a significant role in HPV.

Incubation of IPAs with a mixture of siRNAs targeting CYP2J genes decreased their expression by ~30%-73% depending on the isoform (Figure 2D). As a result, HPV was inhibited by 39% in these vessels (Figure 2E). In normoxia, tension in the CYP2J siRNA transfected IPAs remained stable during an 80-minute incubation (Figure 2E).

In isolated perfused and ventilated WT mouse lungs, hypoxia induced a typical biphasic response.[2] The initial acute transient rise in PAP; (Figure 1F) was followed by a slow sustained increase, which, according to our previous report, corresponds to sustained HPV in isolated IPAs.[20] Application of 14,15-EEZE (10 μmol/L) 10 minutes prior to the onset of hypoxia had no effect on the transient phase of the hypoxic response (Figure 1F). However, the sustained phase of HPV was abolished, similar to the effect of this EET antagonist in isolated IPAs. 14,15-EEZE had no significant effect on PAP in normoxia.

2.3 | Effect of hypoxia on EET levels in pulmonary artery smooth muscle cells (PASMCs)

The results described above suggest that sustained HPV in IPAs is mediated by activation of EET synthesis. In order to verify this hypothesis, we measured EET levels in mouse PASMCs in normoxia and hypoxia using 11,12- and 14,15-EET/DHET ELISA kits. The assays allow to measure DHET levels before and after EET hydrolysis in samples. The difference between the obtained values represents EET levels for each sample.

DHET levels were significantly increased in hypoxia ($P < .001$ for the main effect of hypoxia in both analyses; Figure 3A,B). Furthermore, in case of 11,12-DHET/EET assay, the interaction between the factors (pO_2 and EET hydrolysis) was highly significant, which suggests that the difference between 11,12-DHET and 11,12-DHET + 11,12-EET levels in hypoxia was significantly greater than in normoxia (Figure 3A). In 14,15-DHET/EET measurements, the interaction between factors was not significant. Nevertheless, there was a tendency of an increase in the difference between 14,15-DHET and 14,15-DHET + 14,15-EET values in hypoxia as compared to normoxia (Figure 3B). In addition, we performed 11,12-DHET/EET assays in human PASMCs and found that in these cells 11,12-DHET levels were also significantly increased after 24-hour exposure to hypoxia ($P < .001$ for the main effect of hypoxia; Figure S3).

2.4 | Effects of EETs on pulmonary artery tone

The hypoxia-induced increase in EET levels appears to contribute to sustained HPV. However, it is not clear which EET regioisomers specifically can induce pulmonary

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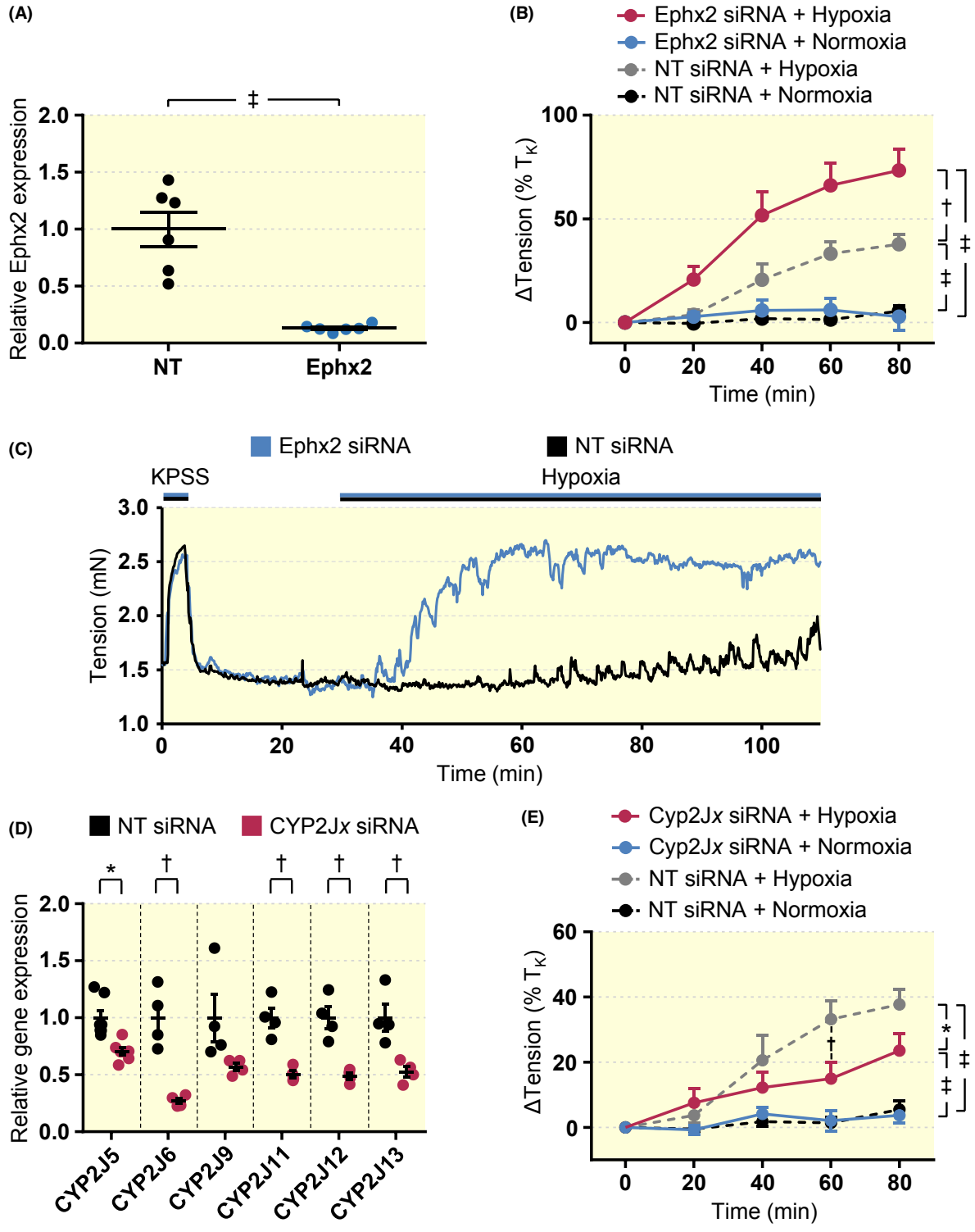

FIGURE 2 Effect of knockdown of sEH and CYP2J epoxygenase isoforms on HPV in mouse IPAs. A, Relative changes in the expression of Ephx2 mRNA in mouse IPAs transfected with non-targeting (NT) or Ephx2 siRNA. Here and in (D) results were normalized to expression of GAPDH mRNA and shown as fold change in transcript levels relative to non-targeting control. n = 6 for each group. B, HPV in IPAs transfected with Ephx2 siRNA. n = 7 for Ephx2 siRNA + Hypoxia, n = 5 for all other groups. C, Representative tension traces of hypoxic responses of IPAs treated with either Ephx2 siRNA (blue trace) or NT siRNA (black trace). D, Relative changes in the expression of mRNA of CYP2J epoxygenase isoforms in mouse IPAs transfected with a mixture of siRNAs targeting CYP2J5, CYP2J6, CYP2J9, CYP2J1, CYP2J12 and CYP2J13 mRNA (CYP2J*x* siRNA). n = 4 for each group. E, HPV in IPAs transfected with CYP2J*x* siRNA. n = 6 for Cyp2J*x* siRNA + Hypoxia/Normoxia, n = 5 for other groups. In (A, B, D) and (E) data are expressed as means ± SEM. Statistical comparison between groups in (A) and (D) was performed using unpaired Student's *t*-test, multiple comparisons in (B) and (E) were performed using two-way ANOVA. $^{*}P < .05$; $^{\dagger}P < .01$; $^{\ddagger}P < .001$

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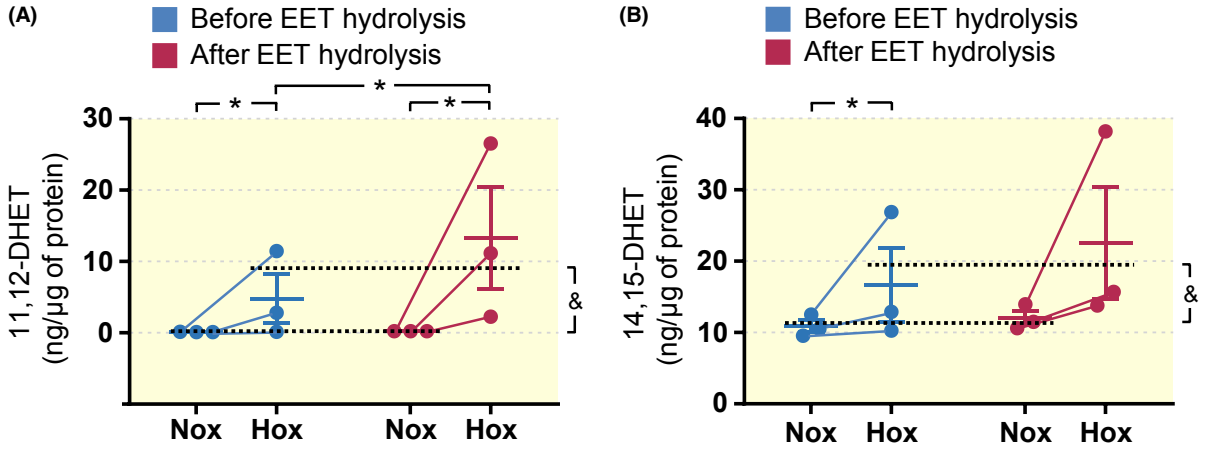

FIGURE 3 Effect of hypoxia on DHET and EET levels in mouse PASMCs. 11,12- and 14,15-DHET levels (A and B, correspondingly) were measured in PASMCs after 24-hour exposure to normoxia (Nox) and hypoxia (Hox). For each sample, DHET levels were measured before and after EET hydrolysis. The difference between the obtained values represents EET levels. Each point represents an average of three replicates of one sample. Each sample contained ~2 million PASMCs. n = 6 animals for each assay. Data are expressed as means ± SEM. Multiple comparisons were performed using non-parametric mixed factorial ANOVA. $^{*}P < .05$; $^{\&}P < .001$ for the main effect of hypoxia

vasoconstriction and what the underlying mechanism of this response is. Thus, we examined the effects of direct applications of exogenous EETs on IPA tone. All EET regioisomers in the concentration range of 0.1-3 µmol/L had no effect on tension in non-pre-contracted vessels (Table S1). Furthermore, 8,9-, 11,12- and 14,15-EET also had no effect in the presence of KCl-induced pre-contraction (Table S1). In contrast, under these conditions, 5,6-EET evoked concentration-dependent contraction (Figure S4A). At low concentrations, the response was monophasic and sustained. However, at concentrations ≥1 µmol/L, it was biphasic and consisted of a transient (with a peak at 3- minutes after application) and a sustained component (Figure S4B). In further experiments, we used the concentration of 1.5 µmol/L, as we found this to be the minimum concentration at which 5,6-EET reliably elicited both phases of vasoconstriction (Figure 4A,C). The effect of 5,6-EET was completely reversible upon washout. We also observed a prominent increase in $[Ca^{2+}]_i$ in response to 5,6-EET in PASMCs of IPAs pre-contracted with KCl but not in the absence of pre-contraction (Figure S5). Notably, 5,6-EET had no effect on tone in KCl-pre-contracted aorta and femoral artery (Figure 4F).

In IPAs pre-contracted with U46619, all EETs evoked significant sustained relaxation with a plateau reached within 12-20 minutes after the application (Figure 4B,D). The response to 5,6-EET, however, was less pronounced compared to all other regioisomers. A similar 5,6-EET-induced relaxation was also observed in IPA pre-contracted with α_1-adrenergic receptor agonist phenylephrine, which suggests that the response was not related to specific inhibition of TP receptors (Figure S6).

The EET antagonist, 14,15-EEZE, almost completely inhibited both 5,6-EET-induced contraction and relaxation (Figure S7). Furthermore, the 5,6-EET metabolite, 5,6-dihydroxyeicosotrienoic acid, had no effect on tension in KCl-pre-contracted IPA (Figure S8). Taken together, these two findings suggest that the observed responses of 5,6-EET application were a direct effect of this metabolite.

All EET regioisomers (1.5 µmol/L) did not affect PAP in isolated mouse lungs (Table S2). Addition of 20 mmol/L KCl to the perfusion buffer induced a sustained increase in PAP. In such KCl-pretreated lungs, 5,6-EET evoked a biphasic increase in PAP, which was similar to the response observed in isolated IPAs (Figure 4E). 8,9-, 11,12- and 14,15-EET had no effect (Figure 4E). Since this study examines the mechanism underlying HPV, we focused our further investigation on the properties of vasoconstrictor action of 5,6-EET.

2.5 | 5,6-EET-induced ion currents in PASMCs

The resulting response to 5,6-EET in isolated IPAs appears to be dependent on the type of pre-contraction. Considering this, we presumed that the mode of action of 5,6-EET is defined by PASMC membrane potential. Indeed, at the concentrations used for IPA pre-contraction, KCl (15 mmol/L) caused significant depolarization in PASMCs, whereas U46619 (1 nmol/L) had no effect (Figure 5A). In line with our previous report,[24] hypoxia caused depolarization of PASMCs from –36.8 ± 2.0 mV to –24.3 ± 2.0 mV (Figure 5B). Further, we investigated the effect of 5,6-EET on transmembrane ion current at holding potentials close to the membrane potentials observed in PASMCs in normoxia and hypoxia (–40 and –20mV respectively). At –40 mV, a slight decrease in current as compared to the baseline was observed in normoxia in response

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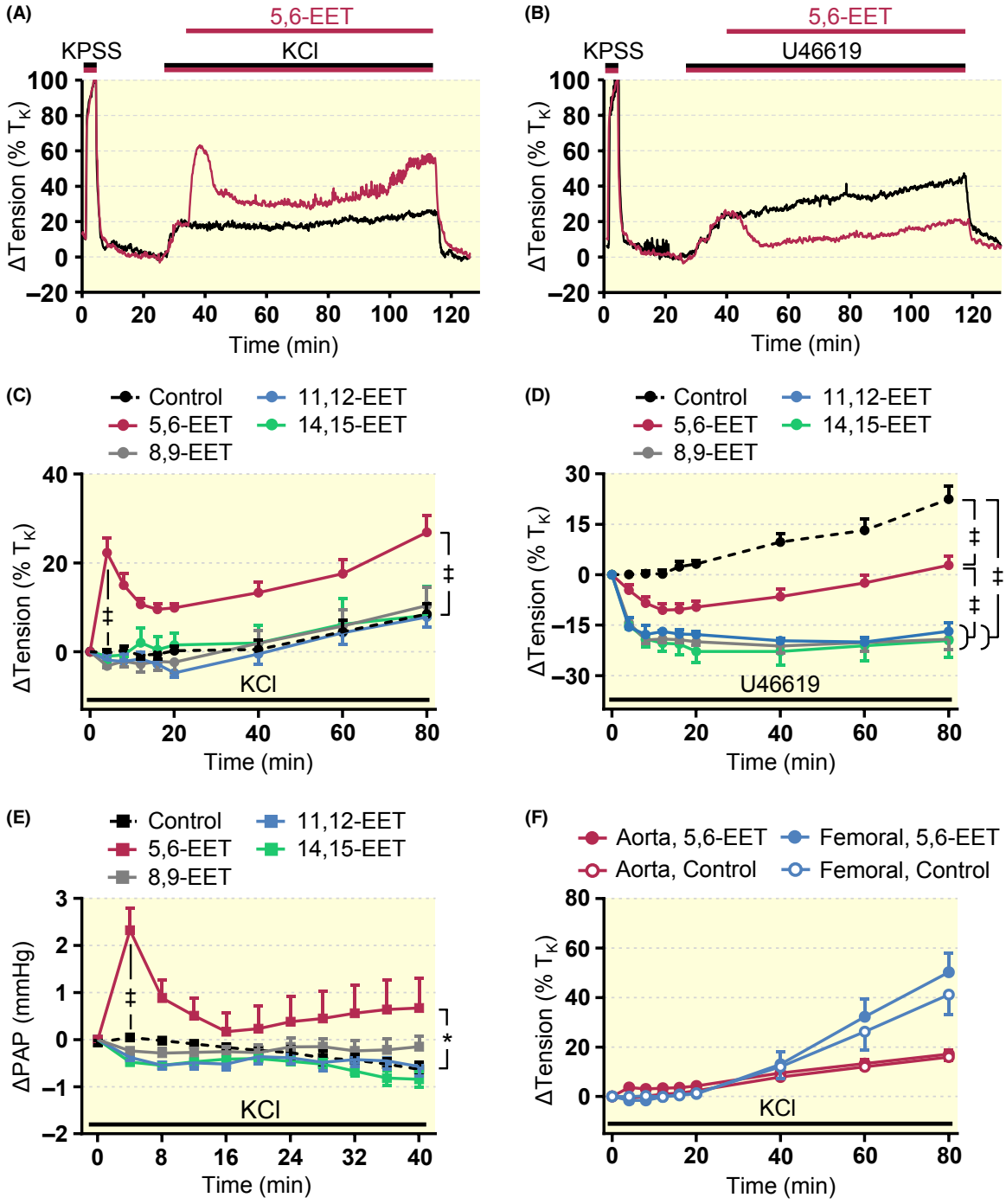

FIGURE 4 Effect of EETs on tone in mouse pulmonary and systemic arteries. A and B, Normalized tension traces of responses to 5,6-EET (1.5 μmol/L; red traces) in IPAs pre-contracted with KCl (15-20 mmol/L; A) or U46619 (1-3 nmol/L; B). Black traces represent control experiments where only pre-contraction was applied. C and D, The effect of 5,6-, 8,9-, 11,12- and 14,15-EET (1.5 μmol/L) on tension in IPAs in the presence of KCl-induced (B) and U46619-induced pre-contraction (C). E, Changes in PAP elicited by application of EETs (1.5 μmol/L) in isolated perfused and ventilated mouse lungs pretreated with 20 mmol/L KCl. F, Effect of 5,6-EET in KCl-pre-contracted isolated segments of aorta and femoral artery. n = 4-8 for each group. Data are expressed as means ± SEM. Multiple comparisons were performed using two-way ANOVA. $^{*}P < .05$; $^{‡}P < .001$

to 5,6-EET application (Figure 5C, Figure S9). In contrast, 5,6-EET induced a significant increase in current at –20 mV (Figure 5C). The effect of 5,6-EET on transmembrane current was slightly diminished in hypoxia. Nevertheless, this eicosanoid induced a 37% increase at −20 mV while having no effect at –40 mV (Figure 5C). Notably, 5,6-EET did not affect PASMC membrane potential, although there was a tendency to hyperpolarization in normoxia (Figure 5D). Our findings confirm that the response of PASMCs to 5,6-EET depends on their membrane potential.

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2.6 | Responses to 5,6-EET in the presence of H_2O_2 and 4-aminopyridine (4-AP)

It has been recently shown that in PASMCs, COX4i2 is responsible for the increased production of mitochondrial reactive oxygen species (ROS) in acute hypoxia. The elevation of ROS levels was found to cause plasma membrane depolarization via inhibition of voltage-gated K^+ (K_V) channels, which appears to be a crucial event for HPV.[24] We hypothesized that ROS-induced PASMC depolarization may be the

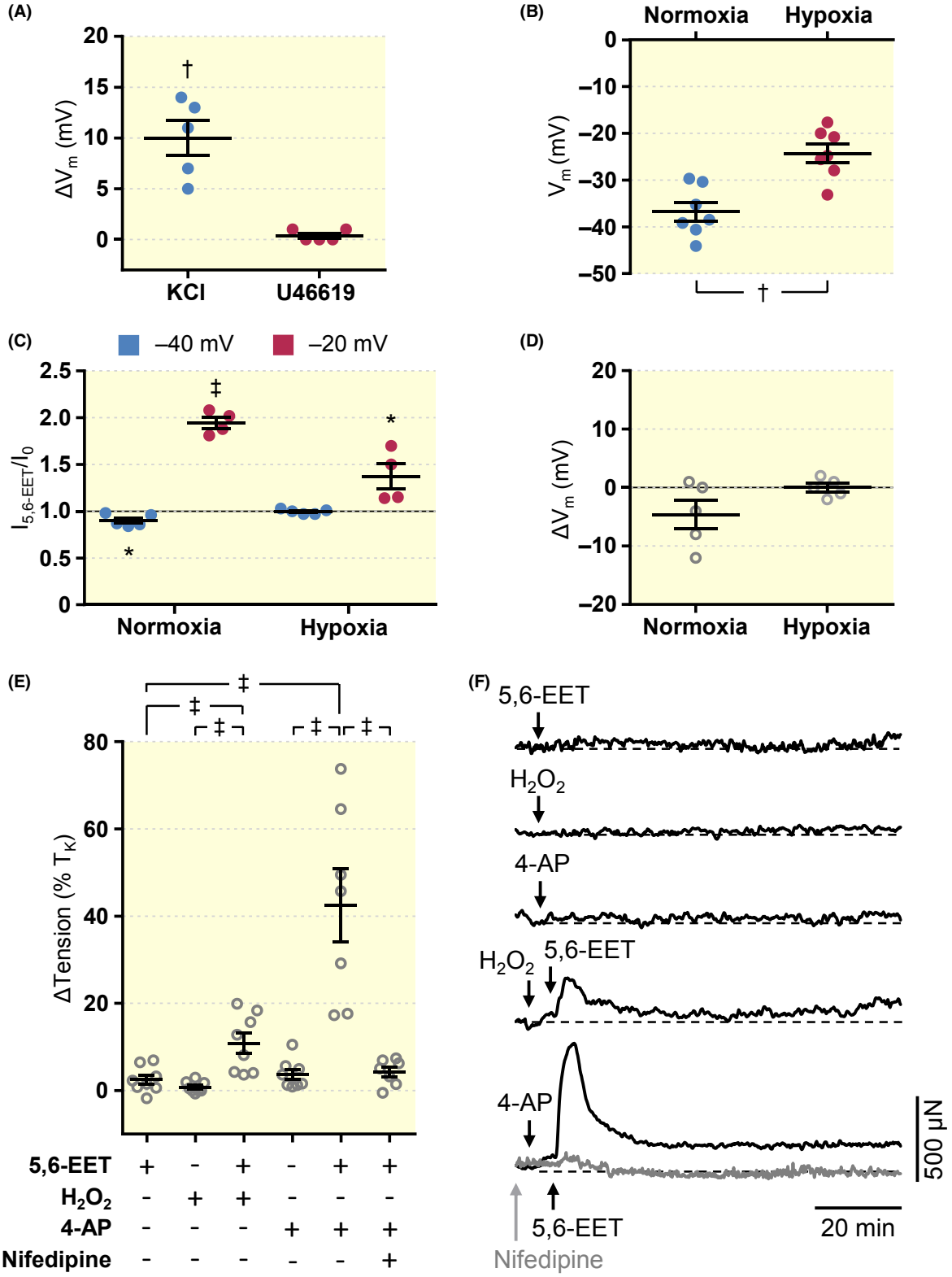

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FIGURE 5 Role of PASMC membrane depolarization in responses to 5,6-EET. A, Changes in PASMC membrane potential (V_m) in response to applications of KCl (15mM) or U46619 (1 nmol/L). B, Effect of hypoxia on membrane potential in PASMCs. C, Effect of 5,6-EET (1.5 μmol/L) on transmembrane current in normoxia and hypoxia measured in the voltage clamp-mode at holding potentials close to membrane potential values observed in normoxia and hypoxia (–40 and –20 mV respectively). The results are presented as changes relative to corresponding baseline current ($I_{5,6\text{-EET}}/I_0$). D, Changes in PASMCs membrane potential induced by 5,6-EET (1.5 μmol/L) in normoxia and hypoxia. E, Changes in isometric tension in non-pre-contracted IPAs in response to 5,6-EET (1.5 μmol/L), H_2O_2 (3 μmol/L) and K_v channel blocker 4-aminopyridine (4-AP; 1 mmol/L). The values were measured 3 minutes after the application of the substances. F, Representative tension traces of IPA responses to 5,6-EET, H_2O_2 and 4-AP. Arrows indicate the time points at which the substances were applied. Grey trace shows the response in the presence of 1 μmol/L nifedipine. Dashed lines show baseline tone. Scale is the same for all traces. n = 4-8 for each group. In (A-E), data are expressed as means ± SEM. Statistical comparisons in (A,B) were performed using paired Student's *t*-test. Multiple comparisons in (C-E) were performed using two-way ANOVA. $^*P < .05$; $^\dagger P < .01$; $^\ddagger P < .001$

factor that enables vasoconstriction to 5,6-EET in hypoxia. Thus, we investigated whether 5,6-EET-induced contraction in IPAs may be promoted by H_2O_2 or a classic K_V channel inhibitor 4-aminopyridine (4-AP). At the concentration range of 0.1-3 μmol/L, H_2O_2 did not affect tension in IPAs, whereas higher concentrations (>10 μmol/L) evoked small but irreversible contraction rendering vessels unresponsive to other stimuli (data not shown). Because of this, we used a concentration of 3 μmol/L in our further experiments. As has already been mentioned, 5,6-EET alone had no effect on IPA tone. In contrast, in the presence of H_2O_2, it induced contraction similar to that observed in KCl-pre-contracted vessels, although less pronounced (Figure 5E,F). Nevertheless, the amplitude of the response was comparable to the amplitude of HPV. 4-AP (1 mmol/L), induced only a slight increase in IPA tone, whereas application of 5,6-EET in the presence of 4-AP led to a prominent biphasic contraction (Figure 5E,F). Interestingly, this response was completely abolished by 1 μmol/L nifedipine (Figure 5E,F). In order to investigate whether the increase in ROS levels can be the cause of the hypoxia-induced increase in EET synthesis, we conducted experiments on IPAs isolated from COX4i2 knockout mice. HPV was absent in non-pre-contracted COX4i2$^{-/-}$ IPAs (Figure S10A). As it was previously shown that other oxygen sensing mechanisms in addition to COX4i2 may contribute to the membrane depolarization in PASMCs in acute hypoxia,[24] we then replaced the missing COX4i2 signal with a KCl application in order to further shift the depolarization induced by other mechanisms to a level that is sufficient for HPV. This approach allowed us to dissect the effect of ROS and membrane depolarization on EET-induced vasoconstriction in hypoxia. Accordingly, HPV was observed in KCl-pre-contracted COX4i2$^{-/-}$ vessels, and it was inhibited by MS-PPOH (Figure S10B). This finding suggests that the hypoxia-induced EET production is not dependent on ROS.

3 | DISCUSSION

The data obtained in this study allowed us to evaluate the impact of the EET pathway in sustained HPV. In our experiments, isolated mouse IPAs responded to hypoxia with a sustained, slowly developing contraction. As shown previously, this hypoxic response corresponds to the sustained phase of biphasic HPV that can be observed in isolated perfused and ventilated mouse lungs as well as in in vivo models.[20] We detected the expression of EET-producing CYP2J epoxygenases in mouse IPAs. Knockdown of these enzymes as well as pharmacological inhibition of EET synthesis specifically suppressed sustained HPV, whereas non-hypoxia-induced vasoconstriction was not altered. Although HPV was not abolished completely, its attenuation correlated very well with the expected degree of the decrease in CYP epoxygenase activity/expression. In particular, MS-PPOH reduced HPV by ~60%, which corresponds to the extent of EET synthesis inhibition produced by this agent at the applied concentration.[25] Furthermore, the treatment with siRNAs decreased mRNA levels of the most highly expressed CYP2J isoforms (CYP2J9, CYPJ11, CYP2J12, CYPJ13) in IPAs by 43%-52%, and the resulting inhibition of HPV was at a comparable level (~39%). It should be noted that the mouse genome contains a high number of CYP isoforms with similar functions.[18] It is possible that in the mouse lung, EETs are also synthesized by epoxygenases from other P450 subfamilies.

A synthetic structural analog of 14,15-EET, 14,15-EEZE, is known to block effects of EETs.[26,27] In our experiments, this antagonist completely abolished sustained HPV in isolated IPAs and lungs. Interestingly, the EET antagonist had no effect on the acute phase of the hypoxic response in isolated lungs. This finding was also previously shown by Keseru et al[10] It should be noted that, unlike other inhibitors used in our study, 14,15-EEZE attenuated vasoconstriction evoked by the thromboxane receptor agonist U46619. This effect, however, may be explained by an earlier finding suggesting that 14,15-EEZE is also a weak EET agonist and thus can mimic vasodilator effect of 14,15-EET to some extent.[28]

Soluble epoxide hydrolase is known to be specifically involved in the metabolism of EETs by converting them to the corresponding inactive dihydroxyeicosatrienoic acids (DHET). Inhibition of sEH leads to accumulation of EETs and therefore augments their effects.[17] In our study, both application of a classic sEH inhibitor TPPU and the knockdown of sEH gene *Ephx2* increased sustained HPV approximately twofold. Notably, no significant changes in tone of the Ephx2 siRNA-treated IPAs were observed in normoxia.

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We found that 11,12-/14,15-DHET and 11,12-EET levels were significantly increased in PASMCs after 24-hour incubation in hypoxia, and there was a tendency of an increase in 14,15-EET. We were not able to assess 5,6- and 8,9-EET levels, as no ELISA kits are commercially available for this purpose. Nevertheless, all EET regioisomers are known to be produced simultaneously by each of the CYP 2J proteins, although at different rates.[29] Furthermore, all regioisomers are hydrolysed by the same sEH.[30,31] Therefore, the changes in 5,6- and 8,9-EET levels, in general, should correspond to the observed changes in 11,12- and 14,15-EET levels. Notably, it has also been previously shown that EET levels are elevated during acute hypoxia in mouse lung.[3,10]

Collectively, these findings suggest that hypoxia elicits an increase in EET synthesis by CYP2J (and possibly also by other) epoxygenases in mouse IPA, and the resulting accumulation of EETs mediates sustained HPV. Hypothetically, inhibition of sEH by hypoxia could also be the cause of the increased EET levels. However, in this case the application of TPPU or *Ephx2* knockdown would rather lead to a decrease of the hypoxic response as sEH was already inhibited. Furthermore, if the decrease in sEH activity is the primary cause of HPV, then pharmacological inhibition of sEH would mimic HPV in normoxia, which could be excluded by our experiments. Taken together with previous reports where EETs were shown to modulate the acute phase of HPV,[4,10] our findings also imply that both phases of HPV (despite the essential differences in their mechanisms)[1] depend on hypoxia-induced EET synthesis in pulmonary arterial vessels.

In both KCl-pre-contracted IPAs and isolated perfused mouse lungs pretreated with KCl, 5,6-EET, but not other regioisomers, induced a biphasic vasoconstriction. In contrast, in the presence of U46619-induced pre-contraction, 5,6-EET, similarly to other EETs, induced vasodilation. The fact that at least one of the EET regioisomers can elicit two opposite responses in the same IPAs depending on the experimental conditions may help to finally explain the discrepancies in the previous reports on action of EETs in pulmonary vessels.[10,13-16] Interestingly, 5,6-EET was without effect in KCl-pre-contracted aorta and femoral artery segments. This observation suggests that the vasoconstrictor response to 5,6-EET is intrinsic to pulmonary but not systemic circulation. On the other hand, the ability of EETs to relax systemic arteries is well-documented.[32] It was suggested that, EETs evoke hyperpolarization in vascular endothelium that is transferred to smooth muscle cells.[9] In contrast, the possible mechanism of pulmonary vasoconstriction induced by 5,6-EET is currently not clear. It is well known that, in smooth muscle cells, elevation of $[K^+]_o$ resulting from a KCl application causes membrane depolarization and Ca^{2+} entry via voltage-dependent L-type Ca^{2+} channels. Conversely, at low concentrations, U46619 elicits vasoconstriction predominantly without activating L-type channels.[33] Indeed, we observed that U46619, unlike KCl, had no effect on membrane potential in PASMCs at the concentration used for IPA pre-contraction (Figure 4A). Thus, we presume that the difference in the responses to 5,6-EET between KCl- and U46619-pre-contracted IPAs may derive from a difference in the PASMC membrane potentials. In support of this hypothesis, we found that 5,6-EET induces a significant inward current in depolarized PASMCs, but not at their resting membrane potential. Furthermore, 4-AP, an agent that specifically induces depolarization by inhibiting K_V channels, enabled vasoconstriction to 5,6-EET similarly to KCl. Taken together, these data suggest that 5,6-EET-induced contraction in IPAs is critically dependent on PASMC membrane depolarization. This conclusion is consistent with the fact that hypoxia caused a significant increase in PASMC membrane potential. According to a recent report, acute hypoxia elicits inhibition of K_V channels in PASMCs by enhancing COX4i2-mediated ROS production.[24] Therefore, we speculate that the increase in ROS levels may represent a prerequisite for 5,6-EET-mediated pulmonary vasoconstriction in hypoxia. In support of this hypothesis, the contraction to 5,6-EET was evoked in IPAs pretreated with H_2O_2. This response was less pronounced compared to that in the presence of 4-AP, possibly because of the inhibitory effects of exogenous ROS on voltage-gated Ca^{2+} channels or components of contractile apparatus.[34-37] Nevertheless, the contraction to 5,6-EET in the presence of H_2O_2 was of a similar magnitude as compared to HPV. In addition, we found that HPV in COX4i2$^{-/-}$ IPAs, which lack hypoxia-induced ROS release but (as a result of the application of KCl) had relevant cellular membrane depolarization, still can be inhibited by MS-PPOH. Thus, we conclude that the increase in ROS levels do not directly affect EET synthesis in hypoxia, but rather enables the vasoconstrictor effect of 5,6-EET by causing PASMC depolarization. The vasodilatory effect of EETs appears to be suppressed under hypoxic conditions. Considering that EET-induced vasodilation relies on endothelium-derived hyperpolarization,[9] in hypoxia this response is apparently antagonized by intrinsic depolarization in PASMCs.

The possible mechanism of the hypoxia-induced activation of EET synthesis is obscure. NADPH is known to be a cofactor in the reaction of EET synthesis.[29,38] We found that NAD(P)H autofluorescence was significantly increased in IPAs during hypoxic challenges (Figure S11). It has been shown that elevation of NADPH levels in pulmonary arteries may result from hypoxia-induced activation of glucose-6-phosphate-dehydrogenase (G6PD).[39] Thus, we hypothesize that the activation of G6PD may be responsible for the increase in EET levels in hypoxia. Alternatively, the activation of EET synthesis may result from an increase in production of the EET precursor, arachidonic acid, by cytosolic phospholipase A_2 (cPLA$_2$). HPV was shown to be is abolished in cPLA$_{2\alpha}^{-/-}$ mice, but it could be restored with arachidonic acid administration.[40]

It is also not clear how exactly 5,6-EET evokes the increase in $[Ca^{2+}]_i$ and contraction in depolarized PASMCs. It has been

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previously suggested that hypoxia-induced inhibition of K_V channels alone without additional activation of cation currents is not enough to cause depolarization required for substantial contraction in PASMCs.[1,41] Furthermore, numerous studies showed that L-type channels in different cell types (including vascular smooth muscle cells) are inhibited by hypoxia and ROS.[34-37,42-44] Indeed, despite the fact that hypoxia and KCl caused similar changes in PASMC membrane potential within minutes, there was no immediate contraction in hypoxia in non-pre-contracted IPAs. Unlike the KCl-induced contraction, which reached plateau in 3-5 minutes, the response to hypoxia developed slowly and became significant only after 40 minutes of hypoxic challenge. This difference suggests that the increase in PASMC membrane potential may be not the sole determinant of HPV. In this study, we found that 5,6-EET alone does not cause PASMC depolarization in hypoxia. Nevertheless, the contraction in response to 5,6-EET is voltage-dependent, accompanied by an inward transmembrane current and an increase in $[Ca^{2+}]_i$, and can be abolished by nifedipine. Considering all these facts, we speculate that 5,6-EET may facilitate Ca^{2+} entry via L-type channels in hypoxia by increasing their open-state probability. Along these lines, it has been shown that L-type Ca^{2+} currents were significantly augmented in cardiac myocytes of mice overexpressing human CYP2J2 epoxygenase. This effect was inhibited by MS-PPOH.[45] Furthermore, it appears that EETs can directly interact with a site on L-type channels from porcine cardiomyocytes.[46] In our additional experiments, we found that L-type channel subunit 1D (Cav1.3) is significantly higher expressed in pulmonary arteries as compared to systemic arteries (Figure S12). Ko et al earlier reported similar findings.[47] In view of this, we hypothesize that Cav1.3 may be able to bind 5,6-EET and, thus, confers the ability to contract in response to this metabolite on PASMCs. Interestingly, in humans, a mutation in Cav1.3, which decreased the threshold of its activation and impaired inactivation, was found to cause the development of pulmonary hypertension.[48] Alternatively, 5,6-EET might also promote Ca^{2+} and Na^{+} entry in hypoxia via TRP channels. For instance, unlike other regioisomers, 5,6-EET is able to directly activate TRPV4 channel.[49,50]

This study has some limitations. We did not investigate the role of endothelium in the hypoxia-induced EET synthesis/release or EET-induced vasomotor responses. Nevertheless, in our recent study, we showed that sustained HPV in isolated mouse IPA is not dependent on the presence of functional endothelium.[20] Moreover, we showed that EET levels increase in isolated PASMCs in hypoxia. Therefore, we suggest that under hypoxic conditions, EETs may be synthesized and act in PASMCs in an autocrine manner. Lastly, in contrast to our study, Keserü et al showed that 11,12-EET induced an increase in PAP in isolated mouse lungs.[10] The reason for the discrepancy with our data is not clear. Possibly, it may be explained by strain- and/or sex-specific differences of the animals used in the studies.

In summary, the obtained results indicate that sustained HPV in mouse pulmonary arteries is mediated by activation of EET synthesis. 5,6-EET specifically appears to be involved in sustained HPV, as it was the only EET regioisomer, which was able to elicit not only relaxation, but also contraction in these vessels. 5,6-EET-induced vasoconstriction is specific to pulmonary arteries and strongly dependent on the presence of depolarization in PASMCs. We conclude that PASMC depolarization, which occurs in hypoxia, enables the contraction of pulmonary arteries to 5,6-EET and this contributes to sustained HPV. The vasoconstrictor effect of 5,6-EET is possibly related to facilitation of Ca^{2+} entry via L-type channels. The exact molecular mechanisms of 5,6-EET-induced pulmonary vasoconstriction, as well as the factors contributing to the activation of EET formation in hypoxia remain to be investigated. The discovery of the binary role of 5,6-EET in the regulation of pulmonary vascular tone can provide a basis for the development of novel therapeutic strategies for treatment of HPV-related diseases. Finding a way to switch the effect of 5,6-EET under hypoxic conditions could allow the alleviation of exaggerated HPV or restoration of the diminished hypoxic response in pulmonary arteries.

4 | METHODS

For detailed experimental protocols, see the Supplementary Data.

4.1 | Animals

All animal experiments were performed according to institutional guidelines that comply with national and international regulations (EU directive 2010/63). All animal experiments were approved by governmental authorities (Regierungspraesidium Giessen). Adult male C57BL/6J mice were obtained from Charles River Laboratories. $Cox4i2^{-/-}$ mice were generated as described previously.[51] Sex- and age-matched wild-type animals from the same colony were used as controls. All animals were of similar age (8-12 weeks old).

4.2 | IPA isolation and tension measurements

IPA isolation and the following measurements of contractile responses were performed as described previously.[20] Briefly, animals were killed by cervical dislocation. Mouse intrapulmonary arteries (IPAs; 80-200 μm in diameter) were isolated and mounted in wire myograph chambers (620M, Danish Myo Technology A/S) where they were bathed in physiological saline solution at 37°C and gassed with 21% O_2, 5% CO_2, balance N_2 (pH 7.4). The vessels were stretched to a

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tension equivalent to 15 mmHg and then stimulated three times by 3-minute exposures to 80 mmol/L KCl containing physiological saline solution (KPSS). Changes in isometric tension during experiment are expressed as percentages of the maximum constriction induced by the third exposure to KPSS (% T_K). In some experiments, KCl (15-20 mmol/L) or U46619 (1-3 nmol/L), the synthetic thromboxane A2 mimetic, was applied before the onset of a hypoxic challenge or before the application of EETs. The experimental protocol for isolated segments of aorta and femoral artery was similar. The systemic vessels were stretched to a tension equivalent to 100 mmHg.

4.3 | Hypoxic protocol

When the tone in isolated IPAs reached a stable level, the gassing was switched to 1% O_2, 5% CO_2, balance N_2 in order to induce hypoxia. The partial pressure of O_2 in the bath during the hypoxic challenge was detected to be 15-22 mmHg (as measured by an optical needle-type oxygen sensor Firesting, Pyro Science). This level of pO_2 was reached within 2-3 minutes after the beginning of hypoxic gassing. The duration of hypoxia was 80 minutes.

4.4 | Gene silencing in isolated IPAs

IPAs were isolated under sterile conditions and placed into well containing 250 µL of minimal essential medium (MEM). Lipofectamine RNAiMAX (1.25 µL; Thermo Fisher Scientific) and SMARTpool siRNA targeting *Ephx2* or CYP2J genes (0.02 nmol/L; Dharmacon) were added to the wells. IPAs were incubated at 37°C for 6 hours. Afterwards the medium was changed to 3 mL MEM + 1% penicillin/streptomycin, and vessels were incubated further for 4 days. The medium was refreshed on the second day. The tension measurements were performed on the fourth day of incubation. Gene silencing was confirmed using real-time polymerase chain reaction (RT-PCR). Control experiments were conducted using a non-targeting (NT) siRNA (Dharmacon).

4.5 | Measurements of EET/DHET levels

The mouse PASMC isolation was performed as previously described.[24] Passage 1 cells obtained from each animal were split in two dishes (n = 6 animals for each assay). One of them was exposed to hypoxia (1% O_2, 5.3% CO_2, balance N_2) for 24 hours, whereas the other was kept under normoxic conditions (21% O_2, 5.3% CO_2, balance N_2). To make sure that EET/DHET levels were within detection range of the assays, each sample contained cells pooled from two dishes (~1 million cells each). Each pair of normoxic and hypoxic samples contained cells from the same animals. Human PASMCs (Lot No. 7F 3559; Lonza) were also exposed to normoxia/hypoxia for 24 hours (three samples in a group, ~1 million cells in each sample). 11,12- and 14,15-EET/DHET levels in the cells were measured using corresponding ELISA kits (Abcam) according to manufacturer's instructions. The plates were read using a Tecan Infinite M200 plate reader (Tecan).

4.6 | Patch-clamp recordings of cellular membrane potential and whole cell currents

The mouse PASMC isolation and patch-clamp recordings were performed as previously described.[24] Briefly, passage 0 PASMCs from C57BL/6J mice were used in the experiments. All patch-clamp experiments were performed in whole cell configuration using an EPC10 USB single amplifier controlled by Patchmaster software (HEKA). Cellular membrane potential was recorded in current clamp mode (I = 0). Acute hypoxia was applied by switching the perfusion from normoxic (gassed with 21% O_2, 5.3% CO_2, balance N_2) to hypoxic (1% O_2, 5.3% CO_2, balance N_2) extracellular solution. In voltage clamp experiments, K^+ currents were blocked by replacing K^+ in intra- and extracellular solutions (KCl or K^+-aspartate respectively) with equal amounts of Cs^+ (CsCl). For measurements of membrane currents, PASMCs were voltage-clamped at holding potentials of –40 mV or –20 mV. 5,6-EET (1.5 µmol/L) was dissolved in extracellular solution prior to the application and delivered to the measurement chamber via the perfusion system.

4.7 | Haemodynamic measurements in isolated perfused and ventilated mouse lungs

Mouse lungs were isolated, perfused and ventilated as described previously.[24] Briefly, the lungs were removed from the thorax while continuously ventilated and perfused with Krebs-Henseleit buffer and were freely suspended from a force transducer for the monitoring of organ weight in a temperature-equilibrated, humidified chamber. After rinsing the lungs, the perfusion circuit was closed for recirculation, left atrial pressure was set at 2 mmHg, the flow was slowly increased to 2 mL/min and the entire system heated to 37°C. Pressures in the pulmonary artery and the left atrium were registered via small diameter catheters with pressure transducers. For hypoxic ventilation, a gas mixture containing 1% O_2, 5.3% CO_2, balance N_2, was used.

4.8 | Real-time PCR

Total mRNA was isolated from homogenized mouse intrapulmonary arteries using RNeasy Mini Kit (Qiagen)

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according to the manufacturer's instructions. Isolated RNA of each sample (200 µg in 20 µL RNAse-free water) was converted to cDNA by reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). The primers were designed using NCBI PrimerBLAST tool (Table S3). Relative quantification of *Ephx2* and CYP2J gene mRNA expression was performed by real-time PCR using the iTaq SYBR Green Supermix according to the manufacturer's instructions (Bio-Rad Laboratories). The real-time PCR analyses were performed using "CFX connect" PCR system (Bio-Rad Laboratories). The results were normalized to the expression of *GAPDH* gene. Relative changes in Ephx2 and CYP2J mRNA were calculated using the $2^{-\Delta\Delta CT}$ method.

4.9 | Statistical analysis

Statistical analysis was performed using "Prism 6" (GraphPad Software Inc). All data are expressed as means ± SEM. Normal distribution of the sample sets was tested by Shapiro-Wilk normality test. Comparisons were performed using paired or unpaired Student's *t*-test, one- or two-way ANOVA with Sidak correction for multiple comparisons as appropriate. For the analyses of ELISA results, we employed non-parametric mixed factorial ANOVA on aligned rank transformed data using R package "ARTool".[52] The differences were considered statistically significant when $P < .05$.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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