Reciprocal actions of constrictor prostanoids and superoxide in chronic hypoxia-induced pulmonary hypertension: roles of EETs

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Abstract
Epoxyeicosatrienoic acids (EETs) are synthesized from arachidonic acid by CYP/epoxygenase and metabolized by soluble epoxide hydrolase (sEH). Roles of EETs in hypoxia-induced pulmonary hypertension (HPH) remain elusive. The present study aimed to investigate the underlying mechanisms, by which EETs potentiate HPH. Experiments were conducted on sEH knockout (sEH-KO) and wild type (WT) mice after exposure to hypoxia (10% oxygen) for three weeks. In normal/normoxic conditions, WT and sEH-KO mice exhibited comparable pulmonary artery acceleration time (PAAT), ejection time (ET), PAAT/ET ratio, and velocity time integral (VTI), along with similar right ventricular systolic pressure (RVSP). Chronic hypoxia significantly reduced PAAT, ET, and VTI, coincided with an increase in RVSP; these impairments were more severe in sEH-KO than WT mice. Hypoxia elicited downregulation of sEH and upregulation of CYP2C9 accompanied with elevation of CYP-sourced superoxide, leading to enhanced pulmonary EETs in hypoxic mice with significantly higher levels in sEH-KO mice. Isometric tension of isolated pulmonary arteries was recorded. In addition to downregulation of eNOS-induced impairment of vasorelaxation to ACh, HPH mice displayed upregulation of thromboxane A2 (TXA2) receptor, paralleled with enhanced pulmonary vasoconstriction to a TXA2 analog (U46619) in an sEH-KO predominant manner. Inhibition of COX-1 or COX-2 significantly prevented the enhancement by ~50% in both groups of vessels, and the remaining incremental components were eliminated by scavenging of superoxide with Tiron. In conclusion, hypoxia-driven increases in EETs, intensified COXs/TXA2 signaling, great superoxide sourced from activated CYP2C9, and impaired NO bioavailability work in concert, to potentiate HPH development.

Keywords
cytochrome P450, soluble epoxide hydrolase, cyclooxygenase, reactive oxygen species

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Chronic exposure to hypoxia in diseases such as chronic obstructive pulmonary disorders (COPD), sleep apnea, and mountain sickness predisposes to the development of hypoxia-induced pulmonary hypertension (HPH),\(^1,2\) attributed primarily to sustained hypoxic pulmonary vasoconstriction (HPV),\(^3\) followed by vascular dysfunction and remodeling.\(^4,5\) In this regard, HPV may serve as an initial trigger to activate HPH signaling. The physiological relevance of HPV is to acutely optimize gas exchange by shunting blood flow from hypoxic alveoli towards better ventilated areas. Also, in certain pathological conditions, the action of HPV is able to maintain systemic arterial oxygenation levels.\(^6\) In reminiscence of the physiological
relevance of acute HPV in adapting pulmonary ventilation, prolonged exposure to hypoxia followed by sustained HPV elicits pathological consequences of vascular remodeling and progressive development of HPH. To date, in addition to well-known roles of nitric oxide (NO) dysfunction in the development of PH, an alteration in arachidonic acid (AA) metabolism via cytochrome P450 (CYP) has attracted considerable attention since CYP enzymes are O₂-sensitive heme-containing proteins and CYP/epoxygenases synthesize epoxyeicosatrienoic acids (EETs) that directly constrict pulmonary arteries. Thus, hypoxia becomes one of the most critical stimuli for the activation of CYP to increase EET production. Moreover, activation of CYP produces reactive oxygen species (ROS) via a consumption of NADPH by microsomal monooxygenases, suggesting CYP as an enzymatic resource for the tissue/cellular ROS. CYP is encoded by a complex superfamily of genes, among which, CYP2C and CYP2J are key enzymes responsible for the majority of EET generation in mammals. The evidence of CYP/EETs serving as cellular mediators in activating HPV signaling was provided by studies indicating that HPV in mice was significantly attenuated by the inhibition of CYP2C9, and potentiated by overexpression of the gene of CYP2C9. Moreover, HPV was failed to be initiated in mice with genetic disruptions of either the CYP2J gene or upstream-located cytosolic phospholipase A2 (cPLA₂), the latter being an essential trigger for activating AA metabolic pathways. In vivo administration of physiological concentrations of 11,12-EET to mice elicited a significant elevation of right ventricular systemic pressure (RVSP), a response that was identical to that observed during acute exposure of mice to hypoxia. On the other hand, circulating and tissue levels of EETs are governed by, not only the synthesis of EETs by CYP/epoxygenase, but also the metabolism/dgradation of EETs by soluble epoxide hydrolase (sEH), which hydrolyzes EETs to their corresponding inactivated diols (dihydroxyeicosatrienoic acids; DHETs). In this context, we, as well as others, have demonstrated that increases in endogenous EETs, as a function of genetic deletion of the Ephx2 gene (encoding for sEH protein), or pharmacological inhibition of sEH activity significantly promote HPV in an EET-dependent manner. The downstream effect of EET-driven potentiation of HPV was evoked, at least in part, by an altered cyclooxygenase (COX) pathway that shifts prostaglandin (PG) metabolic signaling away from dilator PGs towards constrictor prostanooids to propel HPV. Notably, aforementioned studies were primarily conducted on either in vivo acute exposure of animals to hypoxia or in vitro incubation of organs/tissues in hypoxic conditions. Thus, the nature of interactions between EETs and other instigators/mediators in chronic hypoxia-driven pulmonary hypertension (HPH) has remained elusive. To this end, the present study was to define roles for cellular mediators (NO, EETs, PGs and ROS) and enzymatic resources (CYP isoforms) of ROS responsible for altered vascular function during the development of HPH.

Methods

Mouse model of HPH

Twelve- to fifteen-week-old male Ephx2⁻/⁻ (sEH-KO) mice and C57BL/6J mice served as wild type (WT) controls were used. As described previously, cryorecovered heterozygous (Ephx2⁺/⁻, B6.129X1Ephx2tm1Gonz/J) and WT mice were obtained from the Jackson Laboratory (Bar Harbor, ME), and homozygous sEH-KO mice were generated in the Department of Comparative Medicine, New York Medical College. Animal model of HPH was created by exposure of mice to 10% oxygen in a normobaric hypoxic chamber (cylabs, MI). WT and sEH-KO mice were kept in the hypoxic condition (10% oxygen) or room air as time controls, for three weeks. All protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conform to the guidelines of the National Institutes of Health and the American Physiological Society for the use and care of laboratory animals.

Echocardiography

Pulmonary hemodynamics were measured by echocardiography in normoxic/control mice and mice chronically exposed to hypoxia. Briefly, mice were anesthetized by inhalation of isoflurane and then placed in the supine position onto a heated echo platform with EKG leads. Transthoracic echocardiography was performed by using a 30 MHz transducer (Veo 770, Visualsonics, Toronto, Ontario, Canada). The procedure was performed in a double blinded manner. After recording left ventricle (LV) long-axis images, the transducer was rotated clockwise by 90° and short-axis views were recorded. Parasternal long-axis and short-axis views at the papillary muscle level and 2-D guided M-mode images were obtained to measure LV cardiac output (CO). After that, a parasternal short-axis view of the heart at the level of the aortic valve was obtained for correcting alignment with the pulmonary artery flow. Pulse wave doppler flow recording was used for pulmonary artery flow measurement by setting the marker parallel to the flow to obtain values of pulmonary artery acceleration time (PAAT), ejection time (ET) and velocity time integral (VTI), respectively. The lower PAAT was coincided with a higher pulmonary pressure, the ratio of PAAT/ET was therefore, used as an indicator in evaluating PH, as well as an alternative index in estimating right ventricular systolic pressure (RVSP). RV stroke volume (SV) can be calculated from VTI and RV outflow tract cross sectional area [SV = PACSA × VTI]. Therefore, VTI can be used as an indirect index of RV SV. Additionally, RV cardiac output (CO = SV × HR) and cardiac index (CI = CO/BW) were also calculated.

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\text{PACSA} = \frac{\text{SV}}{\text{VTI}}
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**Right heart catheterization**

After echocardiography analysis, anesthetized mouse was transferred to a heating plate to maintain the body temperature at 37°C. As described previously, a middle incision was made at the neck to expose the right external jugular vein. A 1.2F solid state catheter (Transonic Scisense Inc, Canada) was inserted into the jugular vein and then, was advanced into RV for monitoring RVSP. Yielding a stable ventricular pressure wave was indicative of accurate position of the catheter in RV. RVSP was recorded on PowerLab (ADInstruments, Colorado Springs, CO) and analyzed with LabChart V8 software (ADInstruments).

**LC/MS-MS based EET and DHET measurements**

Mouse lungs were excised and snap frozen and pulverized in liquid nitrogen. As described previously, EETs and DHETs were extracted following alkali hydrolysis to release esterified EETs and DHETs and quantified with a Q-trap 3200 linear ion trap quadrupole Liquid chromatography-tandem mass spectrometry (LC/MS-MS) equipped with a Turbo V ion source operated in negative electrospray mode (Applied Biosystems, Foster City, CA). NaOH (1 mol/l, 1 ml) was added to the tissue after extraction for their dissolution. Data were analyzed using Analyst 1.6 software. The protein concentration of samples, determined by the Bradford method (Bio-Rad, Hercules, CA), was used to normalize the detected lipids. Data are presented as total EETs and DHETs that include the sum of all free and esterified four regioisomeric EETs or DHETs per µg protein.

**Western blot analysis**

Lung tissue was frozen in liquid nitrogen, and then crushed and homogenized in lysis buffer. Equal amount of total protein (25 µg) extracted from the lung homogenate was separated by 10% SDS-PAGE gel electrophoresis and transferred to a PVDF membrane. The membrane was probed with specific primary antibodies for sEH, CYP2C9, eNOS and β-actin, (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and thromboxane A2 (TXA2) receptor (Cayman Chemical Ann Arbor, MI) respectively, and appropriate secondary antibodies conjugated with horseradish peroxidase. The X-ray film was developed on X-OMAT autoradiography paper (Kodak) and scanned into a computer. Specific bands were visualized with a chemiluminescence kit and normalized to β-actin. The band densitometry was digitalized with UN-SCAN-IT gel 6.1 software.

**Isometric tension experiments in vitro**

To assess hypoxia-induced endothelial dysfunction, pulmonary arteries isolated from normal and hypoxic-treated mice were used for the isometric tension study. The detail for isometric tension experiments was described previously. Briefly, intra-lobar pulmonary arteries were isolated and cut into rings. Vessel rings were mounted using stainless steel wires on a Danish myograph (DMT620M; Danish Myo Technology, Aarhus, Denmark) and perfused with physiological salt solution (PSS) gassed with 95% air and 5% CO2 at 37°C. All rings were equilibrated in PSS for one hour. Passive tension was applied by the length tension curve, followed by the treatment of vessel rings with 120 mM KCl-PSS (high K+ solution). The endothelial function of vessel rings was evaluated by a dose-dependent relaxation to acetylcholine (ACh; 1 nM to 1 µM) under a phenylephrine (PE, 100 nM)-induced pre-contraction. After re-equilibration for additional 30 min, changes in vessel force were recorded in response to U46619 (1 nM to 1 µM; a thromboxane analog) in the control and presence of valeryl salicylate (VS 1 mM; a selective COX-1 inhibitor), NS-398 (NS 10 µM; a specific COX-2 inhibitor) and Tiron (1 mM; a superoxide scavenger), respectively.

**Superoxide measurements**

Pulmonary superoxide was measured using an established lucigenin (5 µM) chemiluminescence with scintillation counter (LS6000IC; Beckman Instruments, San Diego, CA), as described in detail previously. Briefly, prepared tissue samples were incubated in the control condition and in the presence of 6-(2-proparglyoxyphenyl) hexanoic acid (POOH 10 µM; an inhibitor of CYP/epoxygenase) for 45 minutes, and then, sample chemiluminescence was detected in the presence of 5 µM lucigenin in 1 ml Krebs solution buffered with 10 mM HEPES-NaOH (pH 7.4). Final superoxide levels were normalized to the tissue weight and expressed as counts per gram of tissue.

**Chemicals and statistics**

All chemicals, unless specified otherwise, were obtained from Sigma Chemical (St. Louis, MO). Valeryl salicylate and NS-398 were purchased from Cayman Chemical (Cayman Chemical Co., Ann Arbor, MI). Data are represented as mean ± SEM. N and n refer to the number of mice and vessels, respectively. Statistical analyses were performed using GraphPad Prism 6 software. One-way or two-way ANOVA followed by the Tukey-Kramer post hoc test was used to compare the difference among multiple groups. Unpaired t-test was used to compare the difference between two groups. Statistical significance was accepted at a level of p < 0.05.

**Results**

sEH deficiency exacerbates chronic hypoxia-induced impairments of pulmonary hemodynamics

Summary data of pulmonary hemodynamics including right ventricular (RV) PAAT, ET, ratio of PAAT/ET, VTI and LV CO measured by echocardiography in mice before
and after exposure to hypoxia (10% O₂) for three weeks are depicted in Fig. 1. Hemodynamics recorded under normoxic conditions were comparable between WT and sEH-KO mice, but significantly impaired by hypoxia (a–d). Specifically, chronic hypoxia elicited significant decreases in PAAT (a), ET (b), PAAT/ET ratio (c) and VTI (d) in both strains of mice, impairments that were significantly greater in sEH-KO than WT mice (a–c), revealing an EET-relevant regulatory phenomenon. Additionally, as an index of RV SV, hypoxia-induced reduction of VTI can be used to indicate a decline of RV SV, as well as RV CO since the heart rate (HR) was comparable between normoxic and hypoxic mice (CO = SV × HR). Three-week exposure to hypoxia did not affect LV CO in both strains of mice (e). In consistence with the data shown in Fig. 1, direct measurement of RVSP shows that chronic hypoxia caused significant elevation of RVSP in both groups of mice with higher incremental magnitude in the sEH-KO strain (Fig. 2).

**Cardiac hypertrophy as a consequence of HPH**

In response to increase in pulmonary pressure/RVSP, RV hypertrophy was developed. Fig. 3 shows that the hypoxia-induced RV hypertrophy, characterized as a great ratio of RV to LV with septum (LV+S) (Fulton index), was more severe in the hearts of sEH-KO than WT mice, revealing a same responsive pattern as changes in hemodynamics (Figs. 1,2). This suggests that the severity of RV hypertrophy is proportional to the degree of elevation of pulmonary pressure.

**Changes in EET metabolisms as a function of chronic hypoxia**

Changes in protein expression of sEH and CYP2C9 in response to chronic hypoxia was summarized in Fig. 4 that verifies undetectable sEH protein in lungs of sEH-KO mice and moreover, chronic hypoxia downregulated pulmonary sEH by ~50% in WT mice (a), while upregulation of CYP2C9 expression (major EET synthase in mouse lungs) in both strains of mice (b). Thus, the decreases in EET degradation by either downregulation of sEH, or the deletion of its gene, in concomitance with increases in EET production by upregulation of CYP2C9 elicited increases in pulmonary EETs. Indeed, the metabolic profile of EETs was depicted in Fig. 5 showing that in normoxic conditions, sEH-KO mice had existed with high levels of total EETs (a) and an elevated ratio of EETs/DHETs (c), as a function of lacking sEH-hydrolysis of EETs (b). Hypoxia however, initiated further increases in EETs and decreases in DHETs, which accelerates the augmented EETs/DHETs ratio in both WT and sEH-KO mice, with more dominant effect in the latter (c). Additionally, an alternative consequence of activation of CYP2C9 was revealed in Fig. 5d showing that hypoxic mice exhibited significant great superoxide formation, an enhancement that was significantly reversed by PPOH, a CYP/epoxygenase inhibitor that had no effects on basal superoxide release in normoxia condition. As such, the PPOH-reversible superoxide production confirms CYP2C9 as one of the enzymatic sources of superoxide during HPH.

**Hypoxia alters pulmonary artery contraction and relaxation**

Changes in pulmonary artery response to vasoactive agents, as a function of hypoxia, were summarized in Figs. 6 and 7. In normoxic conditions, vascular responses to 120 mM KCl (high potassium; Fig. 6a) and U46619 (a thromboxane analog; Fig. 6b) were comparable in WT and sEH-KO mice. Chronic hypoxia, which failed to affect high K⁺-depolarization-induced force generation (Fig. 6a), significantly promoted U46619-induced increases in vessel force in both
strains of mice; this elevation, however, was significantly greater in sEH-KO vessels than in WT (Fig. 6b). As cellular mediators, the contribution of constrictor prostaglandins (PGs) to hypoxia-induced increases in vascular contraction to U46619 was assessed. As indicated by Fig. 6c, valeryl salicylate (VS) or NS-398 significantly attenuated the enhanced component by 50% in both groups of vessels. The residual portion of the increment insensitive to COX inhibitors was eliminated by additional treatment with Tiron. Furthermore, Fig. 6d provides a molecular evidence for the NS-398/VS-reversible augmentation of vessel force/contraction, indicating that as an essential downstream target for mediating COXs/constrictor PGs (PGH2 and TXA2) signaling, TXA2 receptor was dramatically upregulated in both strains of hypoxic mice. Fig. 7 shows that pulmonary arteries isolated from normoxic WT and sEH-KO mice displayed a comparable relaxation to Ach, responses that were significantly impaired in hypoxia-treated mice with a worse exacerbation in the sEH-KO strain (a), as a function of downregulation of pulmonary eNOS expression (b). Thus, an intensified COXs/TXA2 signaling (Fig. 6), concomitant with increases in superoxide (Fig. 5d) and decreases in NO synthesis and bioactivity (Fig. 7) work synergistically to impair vascular function during HPH development.

**Discussion**

The present study unravels the link that connects the activation of CYP/sEH/EETs/COXs/TXA/ROS signaling and HPH pathogenesis, as indicated by the results that (1) hypoxia initiates significant alterations in the pulmonary circulation, manifested by the reduced PAAT and ET, smaller PAAT/ET ratio, and shortened VTI (Fig. 1) that may imply a decline in RV SV and/or RV CO, coincided with greater RVSP (Fig. 2) and RV hypertrophy (Fig. 3). In this regard, the value of PAAT correlates inversely to the pulmonary artery pressure/RVSP and then, the enhanced pressure

![fig2](image2.png)

**Fig. 2.** Right ventricular systolic pressure (RVSP) via right heart catheterization in WT and sEH-KO mice in normal (normoxia) and hypoxic conditions (N = 8–10 for each group). By using one-way ANOVA with Tukey’s multiple comparison test, *indicates significant difference from their corresponding normoxic controls; **indicates significant difference from hypoxic WT mice.

![fig3](image3.png)

**Fig. 3.** Ratio of right ventricle (RV) to left ventricle with septum (LV + S) in WT and sEH-KO mice (N = 8 for each group). By using one-way ANOVA with Tukey’s multiple comparison test, *indicates significant difference from their corresponding normoxic controls; **indicates significant difference from hypoxic WT mice.

![fig4](image4.png)

**Fig. 4.** Protein expression of sEH (a) and CYP2C9 (b) in lungs of WT and sEH-KO mice in normoxia (N) and hypoxia (H) respectively. (n = 4 blots for each group). By using unpaired t-test (a) and one-way ANOVA with Tukey’s multiple comparison test (b), *indicates significant difference from their corresponding normoxic controls.
Fig. 5. Total levels of epoxyeicosatrienoic acids (EETs) (a), dihydroxyeicosatrienoic acids (DHETs) (b) and ratio of EETs to DHETs (c), and superoxide level (d) in lungs of WT and sEH-KO mice in normoxia and hypoxia respectively (N = 6 for each group). By using one-way ANOVA with Tukey’s multiple comparison test, *indicates significant difference from normoxic WT mice; #significant difference from hypoxic WT mice; @significant difference from normoxic sEH-KO mice (e).

Fig. 6. Changes in pulmonary vessel force in response to high K⁺ (a) and U46619 in the absence (b) and presence of inhibitors for COX-1 (VS) and COX-2 (NS), and Tiron (1 mM) (c) in isolated pulmonary arteries (n = 8–14 for each group); and TXA₂ receptor (TXA₂R) expression in lungs (d; n = 4 blots) of normoxic (N) and hypoxic (N) WT and sEH-KO mice. By using two-way ANOVA with Tukey’s multiple comparison test, *indicates significant difference from corresponding normoxic controls; #significant difference from hypoxic WT mice; @significant difference from their hypoxic responses without inhibitors.
leads to RV remodeling and eventually hypertrophy due to the increase in afterload. These alterations worsened significantly in sEH-KO than WT mice, suggesting the level of EETs (Fig. 5) proportional to the severity of HPH.

(2) Hypoxia causes the downregulation of sEH and upregulation of CYP2C9 to further increase pulmonary EETs (Figs. 4 and 5), which, in turn, accelerates HPH. (3) HPH is potentially aggravated by the co-activation of COXs/contractor PGs/TAX signaling that promotes vasoconstriction/HPV, CYP2C9-derived superoxide pathway that in combination with suppression of eNOS expression, impairs NO availability/synthesis to compromise pulmonary artery relaxation (Figs. 5–7). Thus, the present study addresses two important issues: (1) the physiological presence of high EETs in sEH-KO mice seems insignificant in altering pulmonary artery function and affecting pulmonary hemodynamics and RVSP, due to in vivo presence of regulatory mechanisms including but not limited to, NO- and/or 20-HTET-mediated pulmonary vasodilatation, which counteract EET-induced PA constriction to approach an optimal balance in the hemodynamical regulation of pulmonary circulatory resistance. (2) EETs indeed, contribute significantly to the pathogenesis and development of HPH, as evidenced by the fact that hypoxia-induced impairments are significantly exacerbated in sEH-KO mice. This supports a notion of the so called “two hits” that may be required for the clinical development of PH, characterized as physiologically dampened actions of EETs in the pulmonary circulation becoming discernible in most instances, in the presence of pathological stimuli, such as hypoxia, acting as a “second hit” to instigate PH pathology.

EETs, as vasoconstrictors in the pulmonary circulation, have been believed to be correlated to female predisposition to PH in consideration of female-heritable downregulation of sEH by estrogen methylation of its (Ephx2) gene. While deeming the primary nature of acute hypoxia-induced increase in pulmonary pressure to be a HPV-based response in an EET-potentiation manner, EETs are also implicated in chronic hypoxia-induced pulmonary remodeling. The present study, therefore, aimed to test the hypothesis that changes, if any, in the expression/activity of CYP (for EET synthesis), sEH (for EET degradation), COXs/TXA signaling and nitric oxide synthase (NOS) work in concert to trigger HPH signaling via (1) promotion of EETs/COXs/PGs/TXA2 pathway, (2) augmentation of oxidative stress, and (3) impairment of NO synthesis/bioavailability.

Pathological signaling responsible for chronic hypoxia-induced pulmonary hypertension

In regards to divergent mechanisms involving pathogenesis of HPH, it has demonstrated that EET-potentiating pulmonary responsiveness to hypoxia is a multiple mechanism-engaged pathological process. During the development of HPH, each unique mechanism may be functionally connected, or interacted with the other(s) in an integral network. Moreover, vessel localization and size, as well as cell type may particularly generate different mediators and gear activation of different channels in a tissue/cell specific manner.

CYP/sEH pathway. Changes in the expressions of CYP2C9 and sEH appear to be driven by chronic hypoxia in a pulmonary-specific manner, because they were neither observed in acute hypoxia, nor presented in chronic hypoxia-subjected systemic organs, such as hearts and aorta (data not shown). Indeed, both strains of mice that had been chronically exposed to hypoxia, existed with increases in pulmonary EETs (Fig. 5) attributed to the downregulation of sEH and upregulation of CYP2C9 (Fig. 4). In this context, previous studies reported a transient increase in pulmonary CYP2C protein levels by two-fold in mice after exposure to 7% O2 for 24 hours and returning to baseline after one-week hypoxia. Alternatively, others reported significant downregulation of pulmonary CYP2C29 and
CYP2C38 mRNA associated with unchanged sEH expression after exposure of mice to hypoxia for six weeks. These controversies suggest that different regimens of hypoxic treatment may evoke divergent mechanisms and yield specific phenotypes. To date, even though the lack of consensus for whether the increase in EET synthesis and decrease in EET hydrolysis are only relevant to HPH, or universally applicable to other types of PH, our studies, as well as others have clarified hypoxia-potentiation of EET bioavailability. This provides explanations, at least in part, for undetectable sEH expression in lung samples of patients diagnosed with idiopathic pulmonary artery hypertension and significant impairment of sEH expression/activity in non-hypoxic PH and COPD as well.29,30 Moreover, in vitro exposure of vessels to hypoxia initiates upregulation of the CYP2C9 protein, along with increases in tissue EETs,31 and also, overexpression of EET synthases plays crucial roles in the hypoxia-induced cell migration and angiogenesis.13,32

**CYP/PG pathway.** In comparison with acute hypoxia where an EET-favorable elevation of RVSP and HPV were primarily dependent of dysregulation of COX-1-mediated prostaglandin H$_2$/thromboxane A$_2$ signaling without significant impacts from COX-2,8,9,23 chronic hypoxia-induced activation of COX-2 has been well documented.33,34 The contribution of constrictor PGs/TXA$_2$ to the promotion of pulmonary vascular responses to U46619 was indicated by approximately 40% increases in expression of TXA$_2$ receptor (Fig. 6d). The upregulated TXA$_2$ receptor tremendously intensifies actions of its ligand-bound analogue(s), yielding a significant augmentation of U46619 (a TXA$_2$ receptor analogue)-elicited vasocontraction (Fig. 6b), a response that was prevented by blocking either of its upstream-located COX isoforms (Fig. 6c). These findings hierarchically explain the enhanced U46619-dependent vasoconstriction in a VS- or NS-398-reversible manner, confirming crucial roles of constrictor PGs/TXA$_2$ signaling in the mediation of hypoxic responses. Unlike in acute hypoxia, where the expression of CYP and sEH, as well as TXA$_2$ receptor remained unchanged and inhibition of COX-1 specifically abolished the greater component of HPV in sEH-KO vessels, but failed to affect the hypoxic force of WT vessels.23 To this end, we interpret our findings to mean that a switch of PG metabolic pathways away from the dilator axis towards the constrictor axis could be driven by increases in EETs due to either an innate (genetic/or epigenetic manipulation) or an acquired (hypoxia) adaptation of CYP and/or sEH expression. Such elevations of EETs to certain threshold level may act as substrates for COXs to possess functional connections between EETs and COXs,35 a signaling that was circularly amplified by upregulation of TXA$_2$ receptor.

**CYP/superoxide/NO pathway.** During the hypoxic process, activated CYP2C9 serves as a source of ROS/superoxide that further, exacerbates HPH. Inconsistence with our previous findings that in vessels deficient of eNOS, activation of endothelial CYP2C9 by shear stress elicited a significant increase in vascular superoxide production, a response that was prevented by a CYP inhibitor but unaffected by the inhibition of sEH or NADPH oxidase,16 the present study also demonstrates a PPOH-reversible great production of superoxide (Fig. 5d) in mice with overexpression of CYP2C9 (Fig. 4b), verifying CYP2C9 as an enzymic source of superoxide.15,36 Moreover, functional evidence indicates a Tiron-reversible enhancement of vessel force to U46619 in chronic hypoxia-treated mice (Fig. 6c), confirming that superoxide contributes significantly to HPH via either serving as a sensor to potentially trigger HPV or inactivating NO in combination of downregulation of eNOS (Fig. 7b) to impair NO-mediated vasorelaxation (Fig. 7a). Indeed, ROS have now been recognized as signaling moieties in the mediation of a wide range of pulmonary pathogeneses37 and are recognized as hypoxic signals in the pulmonary circulation because they can instigate HPV and HPH.38,39 Additionally, an alternative pathway involving interactions between EETs and superoxide has also been revealed. As reported, there is an EET-mediated increase in Thr495 phosphorylation of eNOS40 that facilitates eNOS uncoupling to shift enzymatic release of NO tipped in favor of the generation of superoxide,41 which functionally supports the notion of negative feedback control between inhibition of NO by EETs, or vice versa.42–46

In conclusion, HPH is a multiple mechanism-engaged pathological process, during which each etiologic factor operates in affecting one another to converge forming a highly interconnected network that governs HPH pathogenesis and progression. As illustrated in Fig. 8, hypoxia initiates HPV and dysregulation of EET production and degradation to increase EETs, which potentiates pulmonary vasoconstriction and re-sets COXA$_2$ towards the predisposition of constrictor PGs/TXA$_2$. Additionally, hypoxia downregulates eNOS expression and impairs NO production.

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**Fig. 8.** Schematic illustration for hypothesized EET-related interactions in HPH development. Arrow and flat heads indicate a promotion and inhibition, respectively.
bioavailability by intensifying oxidative stress via CYP-dependent and -independent mechanisms. All changes participate reciprocally in launching HPH, a pathological event that eventually leads to right heart failure.

**Author contributions**
SK, MSW, DS and AH designed research; SK, NA and DS performed experiments; SK, DS and AH analyzed data, interpreted results and prepared figures; SK and AH drafted manuscript; SK, NA, MSW, DS and AH edited and approved final version of manuscript.

**Conflict of interest**
The author(s) declare that there is no conflict of interest.

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