Important role of endothelium-dependent hyperpolarization in pulmonary microcirculation in male mice

-Implications for hypoxia-induced pulmonary hypertension-

Shuhei Tanaka, Takashi Shiroto, Shigeo Godo, Hiroki Saito, Yosuke Ikumi, Akiyo Ito, Shoko Kajitani, Saori Sato, Hiroaki Shimokawa

Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan.

Running title: Role of EDH in pulmonary microcirculation

Total word count: 10,259 words with 7 figures

Address for correspondence:

Hiroaki Shimokawa, MD, PhD
Professor and Chairman
Department of Cardiovascular Medicine
Tohoku University Graduate School of Medicine
1-1 Seiryo-machi, Aoba-ku
Sendai 980-8574, Japan
E-mail: shimo@cardio.med.tohoku.ac.jp
ABSTRACT

Endothelium-dependent hyperpolarization (EDH) plays important roles in systemic circulation, whereas its role in pulmonary circulation remains largely unknown. Furthermore, the underlying mechanisms of pulmonary hypertension (PH) also remain to be elucidated. We thus aimed to elucidate the role of EDH in pulmonary circulation in general and in PH in particular. In isolated perfused lung using male wild-type mice, endothelium-dependent relaxations to bradykinin (BK) were significantly reduced in the presence of Nω-nitro-L-arginine (L-NNA) by ~50% as compared with those in the presence of indomethacin, and the combination of apamin plus charybdotoxin abolished the residual relaxations, showing the comparable contributions of nitric oxide (NO) and EDH in pulmonary microcirculation under physiological conditions. Catalase markedly inhibited EDH-mediated relaxations, indicating the predominant contribution of endothelium-derived hydrogen peroxide. BK-mediated relaxations were significantly reduced at day 1 of hypoxia, while thereafter remained unchanged until day 28. EDH-mediated relaxations were diminished at day 2 of hypoxia, indicating a transition from EDH to NO in BK-mediated relaxations prior to the development of hypoxia-induced PH. Mechanistically, chronic hypoxia enhanced eNOS expression and activity associated with downregulation of caveolin-1. Nitrotyrosine levels were significantly higher in vascular smooth muscle of pulmonary microvessels under chronic hypoxia than under normoxia. Similar transition of the mediators in BK-mediated relaxations was also noted in Sugen hypoxia mouse model. These results indicate that EDH plays important roles in pulmonary microcirculation in addition to NO under normoxic conditions and that impaired EDH-mediated relaxations and subsequent nitrosative stress may be potential triggers of the onset of PH.

Keywords: endothelium-dependent hyperpolarization; hypoxia; pulmonary microcirculation
This study provides novel evidence that both endothelium-dependent hyperpolarization (EDH) and nitric oxide (NO) play important roles for endothelium-dependent relaxation in pulmonary microcirculation under physiological conditions in mice and that hypoxia first impairs EDH-mediated relaxations, with compensatory upregulation of NO, before the development of hypoxia-induced pulmonary hypertension.
INTRODUCTION

Pulmonary arterial hypertension (PAH) is a progressive vasculopathy characterized by specific histological changes, including intimal and medial wall thickness, muscularization of distal pulmonary arteries, and concentric obliterative and plexiform lesions (38). These structural changes increase pulmonary arterial resistance and pressure, resulting in the development of right ventricular failure and premature death (31, 32). However, the trigger(s) of these vascular disorders still remain unclear. Endothelial dysfunction, including decreased bioavailability of prostacyclin (PGI$_2$) and nitric oxide (NO) and also increased activity of endothelin and thromboxane, has been considered a key underlying mechanism of pulmonary vascular remodeling in PAH (9, 15, 26, 34, 67). Indeed, a variety of vasodilatory therapies targeting these mediators attributable to endothelial dysfunction have been developed, including PGI$_2$ and its analogs (3, 61), inhalation of NO (30, 39), soluble guanylyl cyclase (sGC) modulators (23, 24), selective phosphodiesterase-5 (PDE5) inhibitors (20), and endothelin receptor antagonists (56). They are beneficial for some patients with PAH by reducing pulmonary arterial pressure and improving long-term survival (38). However, many patients with PAH still die or need lung transplantation even with these therapies (31). Thus, a new therapeutic target remains to be elucidated to improve long-term survival of PAH patients.

Endothelium regulates vascular tonus by synthesizing and releasing endothelium-derived relaxing factors (EDRFs), including vasodilator prostaglandins (mainly PGI$_2$), NO, and endothelium-dependent hyperpolarization (EDH) factor (59). We have previously demonstrated that the contribution of EDRFs varies depending on blood vessel size; NO plays an important role in relatively large arteries, while the importance of EDH increases as vessel size decreases (39). In contrast, PGI$_2$ has a minor but constant role regardless of vessel size (60). Thus, it is conceivable that EDH is involved in the regulatory mechanisms of arterial blood pressure and organ perfusion in systemic circulation (59). We also have previously demonstrated that endothelium-derived hydrogen peroxide (H$_2$O$_2$) is an important EDH factor in several arteries in animals and humans (43-45). While NO and EDH are well-balanced in
a distinct vessel size-dependent manner under physiological conditions, the physiological
balance between NO and EDH can be disrupted under various pathological conditions, such
as aging, dyslipidemia and hypertension, leading to enhanced vasoconstriction and the initial
step toward cardiovascular diseases (69). For example, in a chronic cardiac pressure
overload model, the disruption of the physiological balance between NO and EDH exhibited
reduced survival rate, impaired coronary flow reserve, and enhanced myocardial hypoxia in
mice in vivo (28). Thus, not only NO but also EDH plays important roles in regulating
vascular tonus and maintaining cardiovascular homeostasis in systemic circulation.
However, the role of EDH in pulmonary microcirculation remains to be clarified.

In the present study, we thus aimed to examine the role of EDH in pulmonary
microcirculation in general and in pulmonary hypertension (PH) in particular. We tested our
hypothesis that EDH plays a primary role in pulmonary microcirculation under physiological
conditions and its role is altered during the development of hypoxia-induced PH in mice in
vivo. Although it is generally known that female sex is a risk factor for PAH (66), we used
only male mice in the present study along with our previous reports in systemic circulation in
order to examine the differences between pulmonary and systemic circulations.
METHODS

Animals.
The experiments were conducted in 11- to 16-week-old male C57BL/6 mice (~25 g body weight (BW) under normoxia). This study was reviewed and approved by the Committee on Ethics of Animal Experiments of Tohoku University (2015MdA-281) based on the ARRIVE guideline. Male C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan). All animals were cared for in accordance with the rules and regulations configured by the committee, fed a normal chow, and maintained on a 12-hour light and dark cycle.

Wire myograph.
We measured isometric tensions of the first to second branch of intrapulmonary arteries (approximately 300-500 μm in external diameter) as previously described (14). After mice were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg), the intrapulmonary arteries were carefully isolated under a microscope, cut into 1-mm length rings without adventitia and mounted in a wire myograph (620M, Danish Myo Technology, Aarhus, Denmark). Each arterial ring was bathed in organ chambers filled with 5 mL Krebs-Henseleit buffer (KHB) warmed at 37 °C and aerated with 95% O₂ and 5% CO₂, and was then stretched to optimal resting tension that was determined in preliminary experiments (data not shown). After 60-min equilibration period, the rings were challenged with KCl (60 mmol/L) to test for their viability; rings that were able to generate over 1 mN of force were allowed for the following isometric tension recordings. In a preliminary study, we obtained cumulative dose response curves to U46619, a thromboxane A₂ mimetics (10⁻⁸ to 10⁻⁴.5 mol/L), and obtained the concentration of U46619 to cause 50% contraction (EC₅₀: −7.35 ± 0.08 log mol/L). After washout and 30-min recovery period, the rings were precontracted with the EC₅₀ concentration of U46619 to examine the relaxations in response to cumulative addition of acetylcholine (ACh, 10⁻¹⁰ to 10⁻⁵ mol/L). The relaxations to ACh were calculated as percentages of the precontracted levels induced by U46619. The contributions of PGI₂, NO, and EDH to ACh-induced relaxations were determined by the inhibitory effect
of indomethacin (cyclooxygenase inhibitor; Indo, $10^{-5}$ mol/L), $N^\text{\textregistered}$-nitro-L-arginine [NO synthase (NOS) inhibitor; L-NNA, $10^{-4}$ mol/L], and a combination of apamin (small-conductance calcium-activated potassium channel ($K_{Ca}$) blocker; Apa, $10^{-6}$ mol/L) and charybdotoxin (intermediate- and large-conductance $K_{Ca}$ blocker; CTx, $10^{-7}$ mol/L), respectively (28). All the inhibitors were applied to organ chambers 30 min before precontraction with U46619. The responses were continuously monitored (PowerLab 8/30 computer system; AD Instruments, Colorado Springs, CO, USA) and were analyzed by a computer-based analysis system in LabChart 7.0 software.

**Isolated perfused lung model.**

For perfusion and ventilation, an open-chest mouse lung preparation was used as previously described in detail (62, 75). Mice were pretreated intraperitoneally with heparin (100 units), then after 10 min, they were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). They were then intubated and positively ventilated with gas mixture containing with 95% $O_2$ and 5% $CO_2$ at 6 mL/kg tidal volume at a rate of 120 breaths/min (MiniVent Mouse Ventilator 845; Harvard Apparatus, Holliston, MA, USA). A sternotomy was performed and cannulas were inserted into the main pulmonary artery and the left ventricle, and the main pulmonary artery, the aorta, and the ventricles were ligated simultaneously, making the lungs completely isolated from the hearts. After cannulation, the lungs were perfused with warmed KHB containing 5% bovine serum albumin through pulmonary arterial cannula at a constant flow (0.08 mL/min/g BW) using a peristaltic pump (Minipuls 3; Gilson Medical Electronics, Inc., Middleton, WI, USA). The lungs were flushed for 9 min to remove blood and to gradually increase the target flow rate before establishing recirculation. Left atrial pressure was maintained at approximately 4 mmHg by adjusting the distance between the lung and the outlet of the cannula inserted in the left ventricle. Pulmonary arterial pressure was monitored and recorded using a pressure transducer connected to a side port of the pulmonary arterial cannula (PowerLab 8/30 computer system). We assumed the pulmonary venous pressure to be zero (1). To clarify the responses to each agonist, each lung preparation was used to study with only one dose of each agonist. After 30-min
equilibration period, the lungs were precontracted with U46619 and then vascular responses were examined. U46619 cumulative dose-response curves (10\(^{-8}\) to 10\(^{-4.5}\) mol/L) were performed and the concentration of U46619 required to produce a 50% response (EC\(_{50}\)) was used to precontract the vessels for subsequent vasorelaxations. We adjusted the degree of precontraction in each preparation to precontracted levels of the normoxic lungs in the absence of any inhibitors. To assess endothelium-dependent relaxations, bradykinin (BK, 10\(^{-5}\) mol/L) was used. The dose of BK was predetermined by the experiments that BK elicited the maximal relaxation in the control lungs (data not shown). To assess endothelium-independent relaxations, sodium nitroprusside (SNP, 10\(^{-5}\) mol/L), a NO donor, was used. Vascular responses to exogenous hydrogen peroxide (H\(_2\)O\(_2\), 10\(^{-4}\) mol/L) were examined in the presence of Indo (10\(^{-5}\) mol/L) and L-NNA (10\(^{-4}\) mol/L). The relaxations to vasodilators were calculated as percentages of the increased pressure induced by U46619. The inhibitory effects of Indo, L-NNA, the combination of Apa and CTx, and catalase (12,500 units/mL) were examined. All the inhibitors were administered from the start of perfusion. We calculated basal vascular resistance (R\(_0\)) as the value given by baseline perfusion pressure (mmHg)/flow (mL/min). Each surgical preparation before perfusion needed ~6 min and each procedure took ~2 hours. A few lungs exhibited massive edema, which were excluded.

**Hypoxia-induced PH model.**

Hypoxic exposure model was used to assess the effect of hypoxia on the relaxations of pulmonary arteries in mice (57). Briefly, 10 to 12-week-old male WT mice on a normal chow diet under a 12-hour light and dark cycle were exposed to hypoxia (10% O\(_2\)) for 1, 2, 7, 14 or 28 days. The hypoxic mice were housed in an acrylic chamber with a non-recirculating gas mixture of 10% O\(_2\) and 90% N\(_2\) by adsorption-type oxygen concentrator to utilize exhaust air (Teijin, Tokyo, Japan), while the normoxic mice were housed in room air (21% O\(_2\)). All hypoxic mice were studied within 1 hour of removal from the chamber.

**Western blotting analysis.**

We performed Western blot analysis as previously described (48). After perfusion with cold
KHB, the lungs were isolated and snap frozen. Frozen lungs were then lysed in tissue protein extraction reagent (T-PER, Thermo-Fisher, Rockford, IL, USA) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), followed by homogenate and centrifugation. The supernatants from lung homogenates were loaded on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Fairfield County, CT, USA), following blocking for 1 h at room temperature. The primary antibodies used were as follows; α-tubulin (1:10,000, Sigma-Aldrich), endothelial NOS [eNOS (1:5,000, BD transduction Laboratories, San Jose, CA, USA)], pSer1177-eNOS (1:400, BD transduction Laboratories), pThr495-eNOS (1:400, BD transduction Laboratories), pSer239-vasodilator-stimulated phosphoprotein [VASP (1:500, Abcam, Cambridge, UK), t-VASP (1:1,000, Abcam), and caveolin-1 [Cav-1 (1:1,000, Cell Signaling Technology, Danvers, MA, USA)]. The regions containing proteins were visualized by the enhanced chemiluminescence system (ECL Prime Western Blotting Detection Reagent, GE Healthcare). Densitometric analysis was performed by the ImageJ Software (NIH, Bethesda, MD, USA).

Immunoprecipitation.

After perfusion with cold KHB, the lungs were isolated and snap frozen. Frozen lungs were lysed in tissue protein extraction reagent (T-PER, Thermo-Fisher), containing protease inhibitor cocktail (Sigma-Aldrich), followed by homogenate and centrifugation. The supernatants were incubated with anti-eNOS antibody at a dilution of 1:100 for 1 hour at 4 °C. After the incubation, 25 µL of prewashed EZ view protein G affinity gel (Sigma-Aldrich) was added to the lysates, followed by incubation for 1 hour at 4 °C. After centrifugation, the supernatants were removed. After 3-times wash with lysis buffer, 50 µL of sample buffer (10% sodium dodecyl sulfate, 30% 2-melcaptoethanol, 20% glycelol and 0.1% bromophenol blue) was added and heated at 95 °C for 5 min, followed by centrifugation. The supernatants were analyzed by immunoblotting (50).

Right heart catheterization.
After 1, 2, 7, 14 or 28 days of exposure to hypoxia (10% O₂) or normoxia, mice were
anesthetized with isoflurane (1.0 %). To examine the development of PH, we measured right
ventricular systolic pressure (RVSP) and right ventricular end-diastolic pressure (RVEDP).

For right heart catheterization, a 1.2-F pressure catheter (SciSense Inc., Ontario, Canada) was
inserted into the right ventricle through the right jugular vein to measure RVSP and RVEDP.

All data were analyzed using PowerLab 8/30 computer system and were averaged over 10
sequential beats (57).

**Histological analysis.**

After right heart catheterization, the lungs were rigorously and completely perfused with cold
KHB at physiological pressure until the color of the lungs clearly showed white. Then, they
were fixed in 10% formaldehyde solution for 24 hours on a shaker at room temperature.

After the serial steps of washing and dehydration, the whole lungs were embedded in paraffin,
and cross sections (3 μm) were prepared. Paraffin sections were stained with
Elastica-Masson. Pulmonary arteries adjacent to an airway distal to the respiratory
bronchiole were evaluated as previously reported (57). Briefly, arteries were considered
fully muscularized when they had a distinct double elastic lamina visible throughout the
diameter of the vessel cross section. The arteries were considered partially muscularized
when they had a distinct double elastic lamina visible for at least half the diameter. The
percentage of vessels with double elastic lamina was calculated as the number of
muscularized vessels per total number of vessels counted. In each section, a total of 60–80
vessels were examined by use of a computer-assisted imaging system (BX51, Olympus,
Tokyo, Japan). This analysis was performed for the small vessels with external diameters of
20–70 μm.

**Immunofluorescence analysis.**

We performed immunofluorescence analysis using paraffin-embedded sections as previously
described (35). The lungs were rigorously and completely perfused with cold KHB at
physiological pressure until the color of the lungs clearly turned white. Then, they were
fixed in 10% formaldehyde solution for 24 hours on a shaker at room temperature. After the serial steps of washing and dehydration, the whole lungs were embedded in paraffin, and cross sections (3 μm) were prepared. Paraffin sections were deparaffinized in xylene, and thereafter rehydrated and washed in ethanol and distilled water. Antigen retrieval was carried out by heating the sections in citrate buffer, pH 6.0 (Target Retrieval Solution, Dako, Glostrup, Denmark) at 120 °C for 5 min. Then, after blocking with 2% skim milk, the sections were incubated overnight at 4 °C with primary antibodies as follows; nitrotyrosine (1:200, Millipore, Bedford, MA, USA), and actin, α-smooth muscle-Cy3 (1:1,000, Sigma-Aldrich). After washing and blocking potential endogenous peroxidase, the sections were incubated at room temperature for 60 min with secondary antibody, Alexa Fluor 488 conjugated donkey anti-rabbit (1:1,000, Molecular Probes, Eugene, OR, USA), for nitrotyrosine. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Thermo-Fisher), and immunofluorescence images were obtained using a fluorescence microscope (BZ-9000, KEYENCE, Osaka, Japan). To assess the specificity of nitrotyrosine immunofluorescence, other sections were incubated with non-immune rabbit IgG instead of anti-nitrotyrosine antibody and were then processed under the same conditions. Exposure time was unified to evaluate nitrotyrosine expression, and the fluorescence intensity of vascular smooth muscle layer identified by α-smooth muscle staining was analyzed with ImageJ Software. This analysis was performed for the small vessels with external diameters of 20–70 μm and a total of ~15 vessels were examined.

Sugen/hypoxia mouse model.

Sugen/hypoxia (SuHx) mouse model was used as a more severe PH model than hypoxia-induced PH model (10). Briefly, 11-week-old male WT mice on a normal chow diet under a 12-hour light and dark cycle were exposed to hypoxia (10% O2) for 21 days and SU5416 (Sigma Aldrich) was subcutaneously injected at 0, 7, and 14 days. SU5416 was suspended in carboxymethylcellulose sodium, containing 0.9% sodium chloride, 0.4% polysorbate 80, and 0.9% benzyl alcohol in deionized water. Hypoxic exposure was performed in an acrylic chamber with a non-recirculating gas mixture of 10% O2 and 90% N2.
by adsorption-type oxygen concentrator to utilize exhaust air as well as hypoxia-induced PH model. All SuHx mice were studied within 1 hour of removal from the chamber.

**Materials.**

Charybdotoxin was obtained from Peptide Institute (Osaka, Japan), and SNP from Maruishi Seiyaku (Osaka, Japan). All the others were from Sigma Aldrich. The ionic composition of KHB was as follows (mmol/L): Na\(^+\) 144, K\(^+\) 5.9, Mg\(^{2+}\) 1.2, Ca\(^{2+}\) 2.5, H\(_2\)PO\(_4\)\(^-\) 1.2, HCO\(_3\)\(^-\) 24, Cl\(^-\) 129.7, and glucose 5.5.

**Statistical analysis.**

All results are expressed as mean ± SEM. Comparisons of means between 2 groups were performed by unpaired Student’s t-test. Comparisons of means among more than 4 groups were analyzed by one or two-way-ANOVA followed by Dunnett’s or Tukey’s test for multiple comparisons. Statistical analysis was performed using GraphPad Prism version 7.00 (GraphPad Software Inc, La Jolla, CA, USA). Results were considered to be significantly different at values of P < 0.05.
RESULTS

Endothelium-dependent relaxations of pulmonary arteries under normoxia.

To examine the contribution of EDRFs in pulmonary arteries, we performed isometric tension experiments using first- to second-branch of intrapulmonary arteries. Isolated pulmonary arterial rings from normoxic mice were precontracted with U46619 and subsequently exposed to cumulative concentrations of ACh ($10^{-10}$ to $10^{-5}$ mol/L) (Fig. 1A). The endothelium-dependent, ACh-mediated relaxations were resistant to Indo, but were highly sensitive to L-NNA, indicating that NO predominantly regulates the tonus of these large proximal intrapulmonary arteries. These results were consistent with dominant roles of NO in endothelium-dependent relaxations of relatively large vessels in systemic circulation (60). In order to explore the roles of EDH-mediated responses, we performed isolated perfused lung experiments that enabled us to examine the contributions of EDRFs in pulmonary microcirculation (4). The schema of the experimental apparatus is shown in Fig. 1B. Since relaxations were not induced by vasodilating agonists alone in these models, the lungs were precontracted with U46619 and were then exposed to vasodilating agonists (Fig. 1C). To determine the optimal tonus of precontraction, U46619 dose-response curves were obtained (Fig. 1D). The concentration of U46619 giving half-maximal response ($EC_{50}$: $–6.29 ± 0.04$ log mol/L) was used to precontract the vessels for subsequent relaxation (46). Almost full relaxations were obtained in response to BK in the absence of any inhibitor in mice under normoxia. Endothelium-dependent relaxations to BK were resistant to Indo, significantly reduced in the presence of L-NNA by $~50\%$ in the presence or absence of Indo, and were abolished by the combination of Apa and CTx (Fig. 1E), indicating the comparable contributions of NO and EDH to pulmonary microcirculation under physiological conditions. Importantly, EDH-mediated relaxations were markedly inhibited by catalase (Fig. 1E), indicating that endothelium-derived H$_2$O$_2$ mainly contributes to EDH-mediated relaxations in the pulmonary microcirculation.

Endothelium-dependent relaxations of pulmonary arteries in response to chronic hypoxia.
To assess the roles of EDRFs in the development of PH, we employed a chronic hypoxia model as a well-established model of PH (57). In the mice exposed to chronic hypoxia, as compared with those under normoxia, endothelium-dependent relaxations to BK in the absence of any inhibitor were significantly reduced, while BK-mediated relaxations in the presence of Indo were enhanced as compared with those without Indo (Fig. 2A). These results indicate that vasoconstrictor PGs might be increased by chronic hypoxia. Intriguingly, L-NNA markedly inhibited BK-mediated relaxations in the lungs exposed to chronic hypoxia as compared with normoxia, suggesting that NO plays a compensatory role for reduced EDH-mediated responses under chronic hypoxia (Fig. 2, A and B). In contrast, endothelium-independent relaxations to SNP and those to exogenous H2O2 were comparable between normoxic and hypoxic mice (Fig. 2, C and D).

**Transition of the role in BK-mediated relaxations of pulmonary microcirculation from EDH to NO during hypoxia.**

Next, we examined isolated perfused lungs using the mice exposed to hypoxia for 1, 2, 7 and 14 days in order to clarify when hypoxia causes the transition from EDH to NO as a main mediator in BK-induced relaxations. Baseline perfusion pressure was comparable between normoxic and hypoxic mice (Fig. 3A). In contrast, basal pulmonary vascular resistance was significantly increased at day 2 of hypoxia as compared with normoxia (Fig. 3B). The discrepancy between baseline perfusion pressure and basal pulmonary vascular resistance could be attributed to the adjustment of the flow rate by body weight as hypoxic exposure significantly reduced body weight (data not shown). Interestingly, although endothelium-dependent relaxations to BK were significantly reduced at day 1 of hypoxia as compared with normoxia and were then unchanged until day 28 (Fig. 3C), EDH-mediated relaxations were significantly reduced as early as day 2 of hypoxia (Fig. 3D), suggesting that the transition from EDH to NO during hypoxia occurred at day 2.

**Mechanisms of the compensatory role of NO for reduced EDH in endothelium-dependent relaxations of the pulmonary microcirculation during hypoxia.**
Western blot analyses using whole lung lysates showed that total eNOS expression was increased in the lungs at day 28 of hypoxia (Fig. 4, A and C). In the lungs from normoxic mice, eNOS phosphorylation was evident at Thr495, but to a lesser extent at Ser1177 (Fig. 4A). Hypoxia significantly dephosphorylated eNOS at Thr495 and phosphorylated at Ser1177 at day 28 (Fig. 4, A, D and E). Similarly, hypoxia significantly phosphorylated VASP at Ser239 at days 2 and 28 (Fig. 4, A and F). Furthermore, hypoxia significantly downregulated Cav-1, which negatively regulates eNOS activity by binding to eNOS oxygenase domain (25), at days 2 and 28 (Fig. 4, A and G). Immunoprecipitation of eNOS with Cav-1 showed that hypoxia had no significant effect on eNOS/Cav-1 complex (Fig. 4, B and H). Taken together, these results suggest that upregulation and activation of eNOS associated with downregulation of Cav-1 enhanced the compensatory role of NO for reduced EDH, a consistent finding with the isolated perfused lung experiments.

Pulmonary artery remodeling and development of PH after hypoxic exposure.

Elastica-Masson staining showed muscularization of distal pulmonary arteries, which was defined non-muscularized (N), partially-muscularized (P), and fully-muscularized (F) (Fig. 5A). Expectedly, the extent of muscularization was significantly accelerated at day 28 of hypoxia (Fig. 5, B and C), but morphological changes were not noted until day 2 of hypoxia. Consistent with the muscularization of distal pulmonary arteries, only the mice exposed to hypoxia for 28 days exhibited marked increase in RVSP (Fig. 5D), but not in RVEDP (Fig. 5E). These results indicate that during the development of hypoxia-induced PH, impairment of EDH-mediated relaxations precedes morphological changes of the pulmonary artery.

Increased nitrotyrosine level in vascular smooth muscle after hypoxic exposure.

We next hypothesized that the transition from EDH to NO in BK-mediated relaxations might be involved in the vascular smooth muscle remodeling in response to chronic hypoxia.

Immunofluorescence showed the ubiquitous presence of 3-nitrotyrosine (3-NT) in lung tissues of both normoxia and hypoxia (Fig. 6A). In contrast, the lung tissues exposed to chronic hypoxia slightly but significantly exhibited the higher level of 3-NT in the vascular smooth
muscle layer than in the lungs under normoxia (Fig. 6, A and B). These results suggest that chronic hypoxia-induced NO upregulation results in nitrosylation in pulmonary artery vascular smooth muscle, implying that nitrosylation in vascular smooth muscle might be involved in pulmonary artery remodeling after chronic hypoxia.

Endothelium-dependent relaxations in Sugen/hypoxia mouse model.

To evaluate vascular reactivity in severe PH model, we performed isolated perfused lung experiments using SuHx mouse model, which is an established model of more severe PH (10). Weekly SU5416 injection, during hypoxia for 3 weeks, significantly elevated RVSP but not RVEDP as compared with normoxia (Fig. 7, A and B). RVSP tended to be elevated more in SuHx than in chronic hypoxia (SuHx: 39.4±4.2 vs. chronic hypoxia: 36.6±2.0 mmHg; P=0.57). SuHx exhibited higher baseline perfusion pressure and basal pulmonary vascular resistance as compared with normoxia (Fig. 7, C and D). In line with the results of chronic hypoxia model, endothelium-dependent relaxations to BK tended to be reduced in SuHx as compared with normoxia (P=0.07) (Fig. 7E), and Indo significantly improved but additive L-NNA markedly diminished BK-mediated relaxations in SuHx as compared with normoxia (Fig. 7E), indicating that SuHx also induces the transition from EDH to NO in BK-mediated relaxations as well as chronic hypoxia (Fig. 7F).
DISCUSSION

The major findings of the present study are as follows. First, EDH plays an important role in endothelium-dependent relaxation in pulmonary microcirculation, in addition to NO, under normoxia in mice. Second, endothelium-derived H$_2$O$_2$ plays an important role in EDH-mediated relaxations in pulmonary microcirculation under normoxia. Third, hypoxia impairs the role of EDH in endothelium-dependent relaxation as early as 2 days with resultant compensatory role of NO. Fourth, this compensatory role of NO is mediated, at least in part, by enhanced the expression and activation of eNOS associated with decreased expression of Cav-1 prior to the development of hypoxia-induced PH. Fifth, hypoxia induces nitrosylation especially in pulmonary artery vascular smooth muscle. Sixth, NO-mediated relaxations compensate for impaired EDH-mediated relaxations in SuHx PH model as in hypoxia-induced PH model. To the best of our knowledge, this is the first study that demonstrates that EDH factor/H$_2$O$_2$ plays an important role in pulmonary microcirculation in addition to NO and that the impairment of the role of EDH could be one of the initial processes of hypoxia-induced PH.

Contributions of endothelium-dependent hyperpolarization and other endothelium-derived relaxing factors in the pulmonary circulation under normoxic conditions.

In systemic vessels, there is a general consensus on the converse contribution of NO and EDH in a vessel size-dependent manner, as the contribution of EDH increases while that of NO decreases as vessel size become smaller (59). Indeed, we have previously demonstrated the crucial roles of EDH in regulating the tonus of resistance arteries, adjusting organ perfusion and blood pressure, and modulating coronary autoregulation and metabolic dilatation (64, 73, 74). The present study demonstrates that EDH contributes to endothelium-dependent relaxations of pulmonary arteries in addition to NO under normoxic conditions. Importantly, in the present study, not only the combination of Apa and CTx but also catalase significantly inhibited non-PGs- and non-NO-mediated relaxations, suggesting the important role of endothelium-derived H$_2$O$_2$ as one of EDH factors in pulmonary
circulation. NO and H$_2$O$_2$ modulate each other in a complex manner; H$_2$O$_2$ not only activates eNOS through PI3-kinase pathway (65), but also suppresses the enzyme in a redox-dependent manner via protein kinase G (PKG) modification (8). Also, NO desensitizes blood vessels to H$_2$O$_2$-induced vasodilatation, and in turn, pharmacological inhibition of sGC sensitizes blood vessels to H$_2$O$_2$-induced vasodilatation in mice (8, 29). There appears to be a physiological balance between NO and EDH/H$_2$O$_2$ under physiological conditions, and previous studies have shown that relative contributions of them to endothelium dependent vasodilatation vary depending on the vasculature, species, and experimental conditions examined (19, 59). We have previously reported that Cav-1 is one of the key factors for regulating this physiological balance between NO and EDH by binding to eNOS in systemic arteries of male mice (28, 50). In addition, both small- and intermediate-conductance K$_{Ca}$ channels (SK$_{Ca}$ and IK$_{Ca}$) in endothelial cells are involved in EDH-mediated relaxations (13, 27), and large-conductance K$_{Ca}$ channel (BK$_{Ca}$) activation leads to vascular smooth muscle hyperpolarization in response to H$_2$O$_2$ (7, 33). In the present study, both catalase-sensitive mediator and Apa/CTx-sensitive mediator contributed to EDH-mediated relaxations under physiological conditions. The detailed mechanisms of the contributions of NO, H$_2$O$_2$, SK$_{Ca}$, IK$_{Ca}$, and BK$_{Ca}$ to the pulmonary circulation remain to be fully elucidated in future studies. From another viewpoint, in pulmonary circulation, NO has an approximately half contribution to BK-mediated relaxations even in resistance vessels, which may underlie the difference in arterial functions between systemic and pulmonary circulations. Indeed, vascular responses to acute hypoxia are quite different between coronary and pulmonary arteries (12, 17). This viewpoint may provide a clue to modulate vascular responses under hypoxia.

**Effects of chronic hypoxia on endothelium-dependent relaxations in the pulmonary circulation.**

Chronic hypoxia impairs endothelium-dependent relaxations and directly affects vascular smooth muscle cells by altering substrate bioavailability for NOS (16, 71). On the other hand, acute hypoxia does not affect BK-mediated relaxations of both male or female porcine
pulmonary arteries, and male guinea pig basilar arteries (17, 54). In the present study, 
BK-mediated relaxations were impaired as early as day 1 of hypoxia, while they remained 
unchanged until day 28. Importantly, hypoxia for 2 days caused a transition from EDH to 
NO in BK-mediated relaxations, indicating the compensatory role of NO for reduced EDH in 
response to hypoxia. Furthermore, consistent with these results, exposure to chronic 
hypoxia caused ~1.5-fold increase in eNOS expression, enhanced phosphorylation at 
stimulatory Ser1177, and decreased phosphorylation at inhibitory Thr495. Similarly, 
phosphorylation of VASP at Ser239, a marker of PKG activity, was also significantly 
enhanced at day 2 of hypoxia. Interestingly, Cav-1 expression was significantly reduced 
only after 2 days of hypoxia although eNOS/Cav-1 complexes were unaltered. Cav-1 
hinders the electron transfer and inhibits NO generation by binding to eNOS and forming 
eNOS/Cav-1 complex (25, 50). Thus, the reduced levels of Cav-1 in response to hypoxia 
may cause eNOS/VASP activation, triggering the transition from EDH to NO in 
endothelium-dependent relaxations of pulmonary arteries. Although the mechanisms of the 
comparable contributions of NO and EDH under physiological conditions also remain unclear, 
the interaction between eNOS and Cav-1 is likely to be involved in hypoxia-induced 
functional alterations in BK-mediated relaxation in pulmonary arteries as well as systemic 
resistance vessels. In SuHx model, NO also played a dominant role in BK-mediated 
relaxations as well as hypoxia-induced PH model although hypoxia itself may be responsible 
for the compensatory increase of NO. Taken together, it is also conceivable that Cav-1 can 
be a therapeutic target for PH in order to maintain the physiological balance between NO and 
EDH (28).

Hypoxia-induced PH in mice is an established model, and the phenotypes of PH, such as 
a rise in RVSP, pulmonary vascular remodeling and RV hypertrophy, appear after 3 to 4 weeks 
of hypoxia (57). In the present study, baseline pulmonary perfusion pressure in isolated 
perfused lung was unchanged after chronic hypoxia, an inconsistent finding with the previous 
reports (1, 18). We consider that this discrepancy between the present study and the 
previous reports was based on the fact that we adjusted perfusion flow rate by a body weight 
in the present study, since hypoxia caused a significant body weight loss and induced a
relatively low perfusion flow in hypoxic mice. Indeed, 4 weeks of hypoxia caused not only PH phenotypes in mice but also significantly increased pulmonary vascular resistance in isolated perfused lung, indicating the integrity of hypoxia-induced PH model in the present study. In addition, we consider that baseline pulmonary perfusion pressure was appropriately evaluated since it reflected the severity of PH in the SuHx model.

**Effects of hypoxia-induced compensatory NO upregulation on pulmonary artery vascular smooth muscle.**

NO regulates vascular tonus via sGC/cyclic guanyllyl monophosphate/PKG pathway (70). In the pulmonary circulation, it was previously reported that reduced NO bioavailability is involved in the pathogenesis of PH (15, 26, 53). Moreover, the importance of NO pathway in the pulmonary circulation is supported by the effectiveness of inhaled NO (30, 39), sGC modulators (23, 24), and selective PDE5 inhibitors (20) in PAH patients. However, it has been controversial whether the pathogenesis of PAH is attributed to the reduced bioavailability of NO. Although it has been reported that reduced NO bioavailability lead to the onset of PH (15, 26, 53), there also is opposite evidence that upregulated eNOS or enhanced eNOS-derived NO production could lead to the development of PH in animals and humans (42, 72, 77). From the latter viewpoint, it is conceivable that excessive NO might be involved in the pathogenesis of PH.

NO reacts with superoxide anions in extremely fast rate and forms peroxynitrite that causes protein modification via tyrosine nitration (55). It was previously reported that chronic exposure to hypoxia increases the production of superoxide anions through NADPH oxidase in male mice (41, 49). In the present study, hypoxia diminished EDH-mediated relaxations and induced compensatory NO upregulation with increased expression and activation of eNOS. Taken together, the present study suggests that the hypoxia-induced transition of vasodilators from EDH to NO resulted in peroxynitrite formation, followed by nitrosative stress in pulmonary arteries. Indeed, the present immunofluorescence data showed that 3-NT is ubiquitously present, especially in vascular smooth muscle layer of hypoxic mice, providing evidence that enhanced NO production induced by chronic hypoxia.
causes nitrosative stress in pulmonary artery vascular smooth muscle. Although it remains unclear whether nitrosylation of vascular smooth muscle causes vascular remodeling, a previous report showed that 3-NT expression is ubiquitously present in the lungs from patients with severe PH but not in those from controls (6). Similarly, inhaled NO increases both superoxide and peroxynitrite, resulting in elevated pulmonary vascular resistance and the onset of rebound PH (51). In contrast, Sheak JR et al. recently reported that chronic hypoxia does not alter the expression of 3-NT in neonatal rats regardless of enhanced NO (58). The discrepancy between this report and the present study may be attributed to the different experimental methods to evaluate 3-NT; Western blotting with whole lung homogenates from neonatal rats (58) vs. immunofluorescence in the present study. It is possible that the whole lung evaluation may underestimate the increased expression of 3-NT in vascular smooth muscle layer of chronic hypoxic lungs. Thus, although it is inconclusive whether nitrosylation in vascular smooth muscle directly causes vascular remodeling in the clinical situation, it may be an effective treatment to reduce nitrosative stress for PH patients. It should be mentioned that SNP-mediated relaxations were unaltered in response to chronic hypoxia in the present study, indicating preserved function of the pathway downstream of NO after chronic hypoxia. These results are consistent with the clinical evidence that inhaled NO, sGC modulators, and selective PDE5 inhibitors are effective to reduce pulmonary artery pressure in PAH patients (20, 23, 24, 30, 39).

**Study Limitations.**

Several limitations should be mentioned for the present study. First, although catalase-sensitive relaxations generally indicate H$_2$O$_2$-mediated relaxations, catalase may not be a specific scavenger of H$_2$O$_2$ (22). In systemic blood vessels, we have previously demonstrated that endothelium-derived H$_2$O$_2$ plays a major role as an EDH in animals and humans using dichlorodihydrofluorescein (DCF) diacetate and electron spin resonance (45, 59, 64). In contrast, several factors other than H$_2$O$_2$ have been proposed as a candidate of EDH in systemic blood vessels (21, 40), and several K$^+$ channels may also be involved in the development of PAH (2, 5, 52, 71). Further studies are needed to address this point.
Second, it remains to be elucidated whether changes in Cav-1 expression contribute to the onset of PH. It has been reported that mutant Cav-1-F92A increases NO bioavailability but does not cause PH as in the case of Cav-1 knockout male mice (36). On the contrary, it has also been reported that Cav-1 is down-regulated in PAH patients (76) and that endothelial Cav-1 exerts a protective role against spontaneous development of PH in male mice (47).

Third, although PAH is a disease with a high prevalence of females (66), we used only male mice in the present study. As regards the high prevalence of females, sex hormones, especially estrogen, are considered to be one of the possible contributors to the pathogenesis of PAH (63). Estrogen has positive roles in vascular functions of pulmonary arteries through eNOS upregulation and activation in addition to enhancing PGI₂ release and ET-1 downregulation, leading to relaxations of pulmonary arteries and inhibition of hypoxic pulmonary vasoconstriction (37). Thus, female sex may exhibit better pulmonary artery reactivity than male. However, despite the positive effects of estrogen on pulmonary vessels, the opposite evidence has also been demonstrated that exogenous estrogen treatments promoted the onset of PAH (63); these two-sided effects of estrogen are known as ‘estrogen paradox’ (68). Further studies are warranted to investigate the sex difference in endothelium-dependent relaxations in the pulmonary circulation. Fourth, the discrepancy between the slight rise in RVSP and the marked increase in basal pulmonary vascular resistance could be attributed to the development of right ventricular failure, which was not evaluated in the present isolated perfused lung model. However, according to the previous report, right ventricular contractility is preserved after 21 days of hypoxia in wild-type mice (11).

Conclusions.

In the present study, we were able to demonstrate that EDH plays an important role in pulmonary microcirculation in addition to NO under normoxic conditions and that impaired EDH-mediated relaxations and subsequent nitrosative stress may be potential triggers of the onset of PH.
ACKNOWLEDGEMENT

We appreciate Y. Watanabe, H. Yamashita, and A. Nishihara for their excellent technical assistance.

GRANTS

This study was supported in part by a Grant-in-Aid for Scientific Research on Innovative Areas (Signaling Functions of Reactive Oxygen Species), a Grant-in-Aid for Tohoku University Global COE for Conquest of Signal Transduction Diseases with Network Medicine, and the Grant-in-Aid for Scientific Research (16K19383), all of which are from the Ministry of Education, Culture, Sports, Science, and Technology, Tokyo, Japan.

DISCLOSURES

None
REFERENCES


31. Huertas A, Perros F, Tu L, Cohen-Kaminsky S, Montani D, Dorfmuller P,


Lahm T, Crisostomo PR, Markel TA, Wang M, Weil BR, Novotny NM, Meldrum DR. The effects of estrogen on pulmonary artery vasoreactivity and hypoxic pulmonary vasoconstriction: potential new clinical implications for an old hormone.


52. Olschewski A, Papp R, Nagaraj C, Olschewski H. Ion channels and transporters as


Sheak JR, Weise-Cross L, deKay RJ, Walker BR, Jernigan NL, Resta TC.


Shimokawa H, Yasutake H, Fujii K, Owada MK, Nakaike R, Fukumoto Y,


Yada T, Shimokawa H, Hiramatsu O, Shinozaki Y, Mori H, Goto M, Ogasawara Y, Kajiya F. Important role of endogenous hydrogen peroxide in pacing-induced


**Figure 1.** Endothelium-dependent relaxations of isolated pulmonary arteries under normoxia. 

**A:** endothelium-dependent relaxations to ACh (10^{-10} to 10^{-5} mol/L) in WT mice under normoxia. The contributions of PGI$_2$, NO, and EDH were determined by the inhibitory effect of Indo (I; 10^{-5} mol/L), L-NNA (L; 10^{-4} mol/L), Apa (A; 10^{-6} mol/L) and CTx (C; 10^{-7} mol/L), respectively. The number of rings examined was as follows: No inhibitor (n=7), I (n=6), I+L (n=5), and I+L+Apa+CTx (n=5). Results are shown as mean ± SEM. *P<0.05 vs I, analyzed by two-way ANOVA followed by Tukey’s test for multiple comparisons. 

**B:** Schematic illustration of isolated perfused apparatus. LAP, left atrial pressure; LV, left ventricle; PA, pulmonary artery. 

**C:** A representative recording in isolated perfused lung experiments. PAP, pulmonary artery pressure (mmHg). Relaxations were calculated as percentage changes in perfusion pressure from the precontracted levels with U46619. We used bradykinin (BK; 10^{-5} mol/L), sodium nitroprusside (SNP; 10^{-5} mol/L), hydrogen peroxide (H$_2$O$_2$; 10^{-4} mol/L) as vasodilating agonists. 

**D:** Dose-response curves to U46619 in WT mice under normoxia. Responses are expressed as % contraction (n=5). 

**E:** Pressure change to BK in WT mice under normoxia. Inhibitory effects of Indo (I; 10^{-5} mol/L), L-NNA (L; 10^{-4} mol/L), catalase (Cat; 12500 Units/mL), and the combination of Apa (A; 10^{-6} mol/L) and CTx (C; 10^{-7} mol/L) were examined (n=6 each). Results are shown as mean ± SEM. *P<0.05 vs I; †P<0.05 vs I+L, analyzed by two-way ANOVA followed by Tukey’s test for multiple comparisons.

**Figure 2.** Endothelium-dependent relaxations in pulmonary circulation after chronic hypoxia. 

**A:** Endothelium-dependent relaxations to BK in the absence or presence of Indo (10^{-5} mol/L) and/or L-NNA (10^{-4} mol/L) in the lungs from normoxic and 28 days of hypoxic mice (n=6 each). Results are shown as mean ± SEM. *P<0.05 vs Normoxia; †P<0.05 vs. Indo in each group, analyzed by two-way ANOVA followed by Tukey’s test for multiple comparisons. 

**B:** The changes in contributions of NO and EDH in BK-mediated relaxations between normoxic and hypoxic mice for 28 days. The contributions were calculated as
relative degree to the relaxations in ‘no inhibitor’ in mice under normoxia.  

C:
Endothelium-independent relaxations to SNP (10\(^{-5}\) mol/L) (n=6 each). Results are shown as mean ± SEM and were analyzed by unpaired t-test.  

D: Endothelium-independent relaxations to exogenous H\(_2\)O\(_2\) (10\(^{-4}\) mol/L) in the presence of Indo and L-NNA. (n=6 each). Results are shown as mean ± SEM and were analyzed by unpaired t-test.

**Figure 3.** Time-course of endothelium-dependent relaxations in pulmonary circulation in response to chronic hypoxia.  

A: Baseline perfusion pressures in normoxic and hypoxic mice.  

PAP: pulmonary artery pressure (mmHg) (n=6 each).  

B: Basal pulmonary vascular resistance (R\(_0\)) in normoxic and hypoxic mice (n=6 each).  

C: Endothelium-dependent relaxations to BK in the absence of inhibitor in normoxic and hypoxic mice (n=6 each).  

D: Endothelium-dependent relaxations to BK in the presence of Indo and L-NNA (EDH-type relaxations; n=6 each). All results are shown as mean ± SEM. *P<0.05 vs. Normoxia, analyzed by one-way ANOVA followed by Dunnett’s test for multiple comparisons.

**Figure 4.** Effects of hypoxia on NO-mediated signaling in normoxic and hypoxic mice.  

A: Representative Western blots for p\(^{Thr495}\)-eNOS, p\(^{Ser1177}\)-eNOS, t-eNOS, p\(^{Ser239}\)-VASP, t-VASP, Cav-1, and \(\alpha\)-tubulin (for loading control), using whole lung lysates from the mice under normoxia and hypoxia (for 1, 2, and 28 days) (n=6 each). All the phosphorylated proteins were detected prior to the total proteins.  

B: Representative Western blots showing the association of Cav-1 with eNOS immunoprecipitated from whole lung lysates of normoxic and hypoxic mice (for 1, 2, and 28 days) (n=6 each).  

C–H: Each bar graph shows the expressions or phosphorylated levels of each protein. Protein expressions were normalized to \(\alpha\)-tubulin, and phosphorylated levels were normalized to total protein expressions. Whole lung lysates were applied in the same order in all the blots. Results are shown as mean ± SEM of relative value with normoxia as 1. *P<0.05 vs. Normoxia, analyzed by one-way ANOVA followed by Dunnett’s test for multiple comparisons.

**Figure 5.** Morphological and hemodynamic changes in response to hypoxia in mice.  

A:
Representative Elastica–Masson (EM) stainings of the distal pulmonary arteries. N, non-muscularized vessels; P, partially-muscularized vessels; F, fully-muscularized vessels. 

Scale bars, 100 μm.  **B:** Representative EM stainings of the distal pulmonary arteries in normoxic and hypoxic (for 1, 2, and 28 days) mice. Scale bars, 100 μm.  **C:** Musclearization of distal pulmonary arteries with a diameter of 20 to 70 μm in normoxic and hypoxic mice (n=3 each). In each section, a total of 60–80 vessels (20–70 μm in external diameters) were examined. The percentages of the muscularized vessels were calculated as the number of muscularized vessels per total number of the vessels counted. Results are shown as mean ± SEM. §P<0.05, analyzed by one-way ANOVA followed by Dunnett’s test for multiple comparisons.  **D, E:** Right ventricular systolic pressure (RVSP; **D**) and right ventricular end-diastolic pressure (RVEDP; **E**) in normoxic and hypoxic mice measured by right heart catheterization (n=7 each). Results are shown as mean ± SEM. *P<0.05 vs. Normoxia, analyzed by one-way ANOVA followed by Dunnett’s test for multiple comparisons.

**Figure 6.** Nitrotyrosine expression in the lungs from normoxic and chronic hypoxic mice.  
**A:** Representative immunofluorescence for nitrotyrosine (3-NT; green), α-smooth muscle actin (α-SMA; red), and 4’,6-diamidino-2-phenylindole (DAPI; blue) of lung sections from normoxic and chronic hypoxic mice. To assess the specificity of nitrotyrosine immunofluorescence, non-immune rabbit IgG was used in the place of anti-nitrotyrosine antibody. Scale bars, 100 μm.  **B:** Analysis of the immunofluorescence intensity in pulmonary artery smooth muscle layer of lung sections from normoxic and chronic hypoxic mice using ImageJ Software. This analysis was performed for a total of ~15 small vessels with external diameters of 20–70 μm from each group (n=3), and the results are shown as mean ± SEM of relative intensity with normoxia as 1. *P<0.05 vs. Normoxia, analyzed by unpaired t-test.

**Figure 7.** Endothelium-dependent relaxations in Sugen/hypoxia mouse model.  
**A, B:** Right ventricular systolic pressure (RVSP; **A**) and right ventricular end-diastolic pressure
(RVEDP, B) measured by right heart catheterization in normoxic and Sugen/hypoxia (SuHx) mice (n=7 each). Results are shown as mean ± SEM. *P<0.05 vs. Normoxia, analyzed by unpaired t-test. C: Baseline perfusion pressures in normoxic and SuHx mice. PAP:

*p<0.05 vs. Normoxia, analyzed by unpaired t-test. D: Basal pulmonary vascular resistance (R₀) in normoxic and SuHx mice (n=6 each). Results are shown as mean ± SEM and were analyzed by unpaired t-test. E: Endothelium-dependent relaxations to BK in the absence or presence of Indo (10⁻⁵ mol/L) and/or L-NNA (10⁻⁴ mol/L) in the lungs from normoxic and SuHx mice (n=6 each). Results are shown as mean ± SEM. *P<0.05 vs. Normoxia; †P<0.05 vs. Indo in each group; §P<0.05 vs. No inhibitor in SuHx group, analyzed by two-way ANOVA followed by Tukey’s test for multiple comparisons. F: The changes in contributions of NO and EDH to BK-mediated relaxations between normoxic and SuHx mice. The contributions were calculated as relative degree to the relaxations in ‘no inhibitor’ in mice under normoxia.
Figure 1

A. Relaxation (%) of the vessel as a function of ACh concentration (-log M).

B. Diagram of the experimental setup showing the main PA, LV, transducer, reservoir, and pump.

C. Graph showing the change in PAP (mmHg) over time.

D. Graph showing the % contraction as a function of U46619 concentration (log mol/L).

E. Bar chart showing the pressure change to BK (%) with different inhibitors.
Figure 2

A. BK-mediated relaxations

<table>
<thead>
<tr>
<th></th>
<th>Indo</th>
<th>L-NNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Hypoxia 28 d</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Pressure change to BK (%)

+ + + + +

B. Contribution (arbitrary unit)

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Hypoxia 28 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C. SNP-mediated relaxations

Pressure change to SNP (%)

Normoxia
Hypoxia 28 d

NS

D. H₂O₂-mediated relaxations

Pressure change to H₂O₂ (%)

Normoxia
Hypoxia 28 d

NS
Figure 3

A

Hypoxia (d)

PAP (mmHg)

B

Hypoxia (d)

R0 (mmHg/mL/min)

C

Hypoxia (d)

Pressure change to BK (%)

D

Hypoxia (d)

Pressure change to BK (%)
Figure 5

A

B

C

D

E

NFP Normoxia 212 8
Hypoxia (d)

Percent of total vessel count (%)

RVSP (mmHg)

RVEDP (mmHg)

Downloaded from www.physiology.org/journal/ajpheart by ${individualUser.givenNames} ${individualUser.surname} (130.034.173.069) on March 21, 2018. Copyright © 2018 American Physiological Society. All rights reserved.
Figure 6

A

Normoxia

Hypoxia 28 d

Negative Control (Rabbit IgG)

B

Relative intensity (ratio)

3-NT

Normoxia

Hypoxia 28 d

*
Figure 7

A

B

C

D

E

F

RVSP (mmHg)

RVEDP (mmHg)

PAP (mmHg)

Rv (mmHg/mL/min)

Pressure change to BK (%)

Contribution (arbitrary unit)

Normoxia

SuHx

Normoxia

SuHx

Normoxia

SuHx

Indo

L-NNA

NO

EDH

Downloaded from www.physiology.org/journal/ajpheart by ${individualUser.givenNames} ${individualUser.surname} (130.034.173.069) on March 21, 2018.
Copyright © 2018 American Physiological Society. All rights reserved.