Optimization of isolated perfused/ventilated mouse lung to study hypoxic pulmonary vasoconstriction

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ABSTRACT

Hypoxic pulmonary vasoconstriction (HPV) is a compensatory physiological mechanism in the lung that optimizes the matching of ventilation to perfusion and thereby maximizes gas exchange. Historically, HPV has been primarily studied in isolated perfused/ventilated lungs; however, the results of these studies have varied greatly due to different experimental conditions and species. Therefore, in the present study, we utilized the mouse isolated perfused/ventilated lung model for investigation of the role of extracellular Ca\(^{2+}\) and caveolin-1 and endothelial nitric oxide synthase expression on HPV. We also compared HPV using different perfusate solutions: Physiological salt solution (PSS) with albumin, Ficoll, rat blood, fetal bovine serum (FBS), or Dulbecco’s Modified Eagle Medium (DMEM). After stabilization of the pulmonary arterial pressure (PAP), hypoxic (1% \(O_2\)) and normoxic (21% \(O_2\)) gases were applied via a ventilator in five-minute intervals to measure HPV. The addition of albumin or Ficoll with PSS did not induce persistent and strong HPV with or without a pretone agent. DMEM with the inclusion of FBS in the perfusate induced strong HPV in the first hypoxic challenge, but the HPV was neither persistent nor repetitive. PSS with rat blood only induced a small increase in HPV amplitude. Persistent and repetitive HPV occurred with PSS with 20% FBS as perfusate. HPV was significantly decreased by the removal of extracellular Ca\(^{2+}\) along with addition of 1 mM EGTA to chelate residual Ca\(^{2+}\) and voltage-dependent Ca\(^{2+}\) channel blocker (nifedipine 1 \(\mu M\)). PAP was also reactive to contractile stimulation by high K\(^{+}\) depolarization and U46619 (a stable analogue of thromboxane \(A_2\)). In summary, optimal conditions for measuring HPV were established in the isolated perfused/ventilated mouse lung. Using this method, we further confirmed that HPV is dependent on Ca\(^{2+}\) influx.

Key Words: hypoxia, pulmonary vasoconstriction, mouse lung, pulmonary artery, endothelial nitric oxide synthase

Hypoxic pulmonary vasoconstriction (HPV), which was first described in 1946 by von Euler and Liljestrand,[1] is an essential physiological response in the lung that optimizes the ventilation/perfusion ratio and maximizes oxygenation of venous blood. Although it has been extensively studied,[2,3] the cellular mechanism involved in HPV is still unclear. One of the hypotheses for the cellular and molecular mechanism of HPV is that the inhibition of voltage-gated K\(^+\) channels (Kv) in pulmonary arterial smooth muscle cells (PASMCs) causes membrane depolarization which subsequently leads to Ca\(^{2+}\) influx by the activation of voltage-dependent Ca\(^{2+}\) channel (VDCC).[4-6] An increase in the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{cyt}\)) in PASMCs is a key stimulus for vasoconstriction.[7] In addition, various molecules, ion channels, and signaling pathways have been suggested with regards to the oxygen sensing mechanism of HPV.[3,8-16]

To investigate the precise mechanisms of HPV, researchers have employed a variety of methods including intact animals, isolated lungs, isolated pulmonary arteries, isolated PASMCs, and endothelial cells.[4,10,11,16-21]
Therefore, in this study, we characterize the isolated perfused mouse lung method using different perfusates and pretone agents in order to define optimal conditions for studying HPV. Using our optimized conditions, we evaluated vascular reactivity in the isolated perfused ventilated mouse lung and revisited the role of extracellular \(Ca^{2+}\) and endothelial nitric oxide (NO) synthase and caveolin-1 expression in the mechanism of HPV.

**MATERIALS AND METHODS**

**Isolated perfused mouse lung**

Experiments using the mice in this study were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago, USA. C57BL/6 mice (22-25 g) were deeply anesthetized with ketamine (100 mg/kg)/xylazine (26 mg/kg) via intra-abdominal injection. After a tracheostomy was performed, mice were ventilated with a gas mixture of 21% \(O_2\), 5% \(CO_2\) via a rodent ventilator (minivent type 845, Harvard Apparatus, USA). Respiratory rate was maintained at 80 breaths/min and tidal volume was 10 mL/kg (~250 ml). Positive end-expiratory pressure was maintained at 2 mmH\(_2\)O. End-inspiratory plateau pressure (EIP) was measured by a pressure transducer (MPX type 399/2, Hugo Sachs Elektronik-Harvard Apparatus, Germany) which was connected with a tracheal catheter. The mice were secured in the chamber of the isolated perfused lung system (IL-1 Type 839, Harvard Apparatus, USA), the chest was opened by median sternotomy, and then the pericardium, thymus, and fat tissue were carefully removed. In order to prevent blood coagulation, 20 IU heparin was injected into the right ventricle. A stainless steel catheter was inserted into the main pulmonary artery (PA) after performing a right ventriculotomy, and the PA and ascending aorta were tied together using a 6-0 black silk suture. Pulmonary arterial pressure (PAP) was measured using a pressure sensor (P75 Type 379, Hugo Sachs Elektronik-Harvard Apparatus, Germany), which was connected to the PA catheter. The other end of the catheter was connected to a tube for pulmonary perfusion. After a small incision of the left ventricle, the mitral valve was opened using a small hemostat, and the stainless steel cannula was inserted into the left atrium via a left ventriculotomy, which was drained into the reservoir. This cannula was fixed by a black silk suture, which was tied to the left ventricular wall. The pulmonary circulation was maintained in a closed circuit via a peristaltic pump (ISM 834, ISOMAT\_E, USA). The total volume of perfusate was 50 mL and the flow rate was maintained at 2 mL/min. The PAP, left atrial pressure, and EIP were monitored continuously. Before each cannula was inserted, the pressure was zeroed at the height of the heart. For data acquisition and data storage, Powerlab 8/30, Quad Bridge Amp, and LabChart (AD Instruments, Australia) were used. To maintain the humidity of the opened lung, physiological saline was applied into the thoracic cavity. Temperature was maintained at 36-38°C with a water jacket in the chamber and reservoir. After basal PAP was stabilized for 40-60 minutes, the experiments were performed.

**Hypoxic condition for HPV**

For observing HPV, mice were ventilated with a normoxic gas mixture (21% \(O_2\), 5% \(CO_2\) and balanced with \(N_2\)) and a hypoxic gas mixture (1% \(O_2\), 5% \(CO_2\) and balanced with \(N_2\)) in five-minute intervals. The amplitude of HPV was calculated from the change in PAP from basal to peak level.

**Solutions and chemicals**

The physiological salt solution (PSS) used for the perfusate consisted of the following composition (mM): NaCl 120, KCl 4.3, NaHCO\(_3\) 19, KH\(_2\)PO\(_4\) 1.1, glucose 10, CaCl\(_2\) 1.8, and MgCl\(_2\) 1.2 (pH 7.4). In order to protect prostaglandin synthesis, 3.1 mM sodium meclofenamate was added to the...
perfusion. For isotonic high-K⁺ solutions (40 mM), NaCl was replaced by an equimolar amount of KCl. A total of 1 mM EGTA was added and CaCl₂ was replaced by equimolar MgCl₂ for Ca²⁺-free solutions. To compare the effect of different perfusates on HPV, each chemical was added to the perfusate as follows: (1) 4% albumin (Product No. A7906, Sigma); (2) 4% Ficoll (Product No. F2637, Sigma); (3) DMEM with 4.5 g/L D-glucose, L-glutamine and without sodium pyruvate (Product No. 11965-092, GIBCO); and (4) fetal bovine serum (FBS) (Product No. 16000-044, GIBCO). U46619 was dissolved in DMSO and angiotensin II and nifedipine (Nif) were dissolved in distilled water. All drugs were obtained from Sigma (St. Louis, Mo., USA) and stored at -20°C.

Statistical analysis
The data are presented as mean ± standard error of the mean. Paired or unpaired Student’s t-tests and one-way analysis of variance with Bonferroni multiple range tests were used for statistical analysis. Significance level was set at P < 0.05.

RESULTS

HPV in PSS with 4% albumin
We compared HPV using different pretone agents in PSS with 4% albumin as a perfusate. Without the pretone agent, exposure to hypoxia negligibly affected PAP. The amplitude of acute hypoxia-induced increase in PAP, or HPV, was only 2.2% ± 0.6% of that induced by 40 mM K⁺ solution (Fig. 1A). Also, increased PAP induced by hypoxia was not observed after angiotensin II was applied (Fig. 1B). In contrast, in the presence of thromboxane A₂ analogue U46619, basal PAP and the amplitude of HPV (or alveolar hypoxia-induced increase in PAP) were dose-dependently increased. When 1 and 2 µM U46619 were applied as a pretone agent, the amplitudes of hypoxia-induced increase in PAP were 3.6 ± 0.1 mmHg (Fig. 1C) and 9.6 ± 1.1 mmHg (Fig. 1D), respectively. These data indicate that (a) acute alveolar hypoxia is unable to increase PAP or cause HPV in the isolated lung perfused with 4% albumin in the absence of a pretone agent, (b) presuperfusion of the pulmonary vasculature with certain pretone agents (e.g., the thromboxane A₂ analogue, U46619) confers HPV and dose-dependently enhances the amplitude of HPV, and (c) different pretone agents (or priming factors) affect acute HPV differently under these conditions.

HPV in PSS with 4% Ficoll
In the isolated mouse lung, several studies have used Ficoll as an oncotic agent of perfusate. We observed HPV using 4% Ficoll in PSS as a perfusate and pretone agent (angiotensin II). In the absence of angiotensin II, alveolar hypoxia alone did not increase PAP (Fig. 2A). To examine whether the inability of hypoxia to cause HPV was due to low basal PAP, we repeated the experiment when we changed the baseline perfusion rate. The increase of flow rate from 2 to 3 mL/min increased basal PAP but had no effect on HPV (Fig. 2A). However, in the isolated lung perfused with 5% Ficoll in PSS, alveolar hypoxia slightly increased PAP after angiotensin II (0.2 µg) was applied.

Figure 1: Comparison of HPV using different pretone agents in PSS perfusate with 4% albumin in the isolated perfused/ventilated mouse lung. Flow rate was regularly controlled with 2 mL/min. After stabilization of pulmonary arterial pressure (PAP) for 40-60 min, treatment of hypoxia or drug was applied. Figure 1A: Hypoxia (1% O₂) alone did not induce vasoconstriction without a pretone agent. (B) Angiotensin II (Ang-II, 0.2 µg/100 µL) did not affect the HPV response. (C and D) In the presence of U46619 (1 µM, C, or 2 µM, D), baseline PAP was dose-dependently increased and the amplitude of HPV was gradually increased. Mean ± SEM is shown as a bar graph (right panel). *Indicates statistically significant difference from the control value (P < 0.05). Lungs are isolated from two to four mice.
The amplitude of acute hypoxia-induced increase in PAP was slightly increased (by approximately 2 mmHg) but not statistically significant (P = 0.07, Fig. 2B). These data indicate that, in isolated perfused/ventilated mouse lung, perfusate which includes 4% Ficoll is insufficient for acute hypoxia to cause HPV in the absence or presence of a pretone agent (e.g., angiotensin II).

**HPV in PSS with rat blood**

To investigate the effect of blood and the PSS perfusate mixture on HPV in isolated perfused/ventilated mouse lung, we collected blood from rats that were deeply anesthetized with pentobarbital sodium (100 mg/kg). A total of 10 mL whole blood from rat was added to 40 mL PSS perfusate before the experiment. When PSS with 20% rat blood was used as perfusate, the amplitude of alveolar hypoxia-induced increase in PAP was 2.2 ± 0.1 mmHg without angiotensin II (Fig. 3A). After the application of angiotensin II, the amplitude of HPV was slightly increased (Fig. 3B) but was not significantly different from control (Fig. 3B, inset). These data indicate that perfusate comprised of 20% rat blood in PSS is sufficient for alveolar hypoxia to induce a significant increase in PAP; the acute HPV, however, was not enhanced by application of the pretone agent, angiotensin II.

**HPV in DMEM with FBS**

In many experiments, it is useful to repeatedly expose the lungs to hypoxic conditions in order to determine the effect of different drugs on HPV. Therefore, we performed hypoxic ventilation exposure with six trials to evaluate the persistent and repetitive HPV response to alveolar hypoxia using DMEM with 10% FBS as perfusate. During the first trial of exposure to hypoxia, strong vasoconstriction was induced; however, the amplitude of HPV was lower in the second through sixth trials. The amplitude of PAP increase by the sixth hypoxic trial was one third of that of the first hypoxic trial (Fig. 4A). Also, the amplitude of HPV did not increase after the addition of angiotensin II (Fig. 4B). These data indicate that, in isolated perfused/ventilated mouse lung, using cell culture medium (DMEM with 10% FBS) is not good enough for acute hypoxia to induce persistent HPV in the absence or presence of a pretone agent (e.g., angiotensin II).

Collectively, the results described above demonstrate that PSS with 4% albumin, 4% Ficoll, or 20% rat blood, and DMEM with 10% FBS, are not optimal perfusates for the isolated perfused/ventilated mouse lung model due to the requirement of a pretone agent and in consistent responses.

**Persistent and repetitive HPV in PSS with 20% FBS**

When PSS perfusate with 20% FBS was used in the isolated mouse lung, we applied hypoxic conditions more than six times and observed persistent and repetitive HPV. The mean
amplitude of hypoxia-induced increase in PAP was 4.4 ± 0.1 mmHg (Fig. 5A and B). The application of angiotensin II did not significantly affect HPV. The amplitude of HPV with angiotensin II was 5.1 ± 0.3 mmHg (Fig. 5A and B). We also examined the effect of relative long-term exposure to hypoxia in the isolated mouse lung. When the lung was ventilated with a hypoxic gas mixture for three hours, HPV induced a biphasic increase in PAP. PAP rapidly increased by 4.3 ± 1.5 mmHg within the first five minutes of exposure to hypoxia, and then a second increase was observed which was maintained after a slight decrease of PAP (Fig. 5C). The second phase of hypoxia-induced increase in PAP reached a plateau approximately 25-30 minutes after the initial exposure to hypoxia. These results demonstrate that persistent and repetitive HPV can be measured without the need for a pretone agent when using PSS with 20% FBS as the perfusate.

**Effects of extracellular Ca\(^{2+}\) on HPV**

Since HPV was first described in 1946,[1,2] many researchers have identified the importance of the effect of extracellular Ca\(^{2+}\) on HPV.[3,30,54,55] In the isolated perfused mouse lung, however, there is little evidence about the role of extracellular Ca\(^{2+}\).[43] Therefore, we evaluated the role of extracellular Ca\(^{2+}\) on HPV using a Ca\(^{2+}\)-free solution and a Ca\(^{2+}\)-free blocker. When a Ca\(^{2+}\)-free solution was applied, the amplitude of HPV was significantly decreased and then recovered after the removal of the Ca\(^{2+}\)-free solution (Fig. 6A). During hypoxic conditions, HPV was completely abolished by the change of perfusate from the PSS solution with 1.8 mM CaCl\(_2\) to the Ca\(^{2+}\)-free solution (Fig. 6B). Application of nifedipine, a selective blocker of VDCCs, after observing HPV three times, significantly decreased the amplitude of HPV. The amplitude of the acute hypoxia-induced increase in PAP in the presence of 1 μM Nif was 22.5% ± 0.6% of control (P < 0.01, Fig. 6C). These data provide compelling evidence that acute hypoxia-mediated pulmonary vasoconstriction is significantly dependent on a rise in cytosolic [Ca\(^{2+}\)] due to Ca\(^{2+}\) influx through VDCCs.

**Measuring HPV using isolated perfused/ventilated mouse lung: Comparisons of contractile responses in different knockout mice**

The purpose of this study was to optimize the isolated perfused/ventilated lung system to further investigate mechanisms of HPV, and therefore, we compared HPV and

![Figure 4: Effects of DMEM with 5%-10% FBS as a perfusate on HPV in the isolated perfused/ventilated mouse lung. (A) Representative trace of HPV is shown. Consistent and repetitive HPV was observed with PSS perfusate with 20% FBS. The amplitudes of HPV were increased by the application of Ang-II (0.2 mg/100 ml). (B) Bar graphs are shown as mean ± SEM. The amplitude of HPV using DMEM with 5% FBS was spontaneously decayed. After Ang-II (0.2 mg/100 ml) was applied, HPV was not significantly increased. Bar graphs are shown as mean ± SEM (right panels).](image)

![Figure 5: Effects of PSS with 20% FBS as a perfusate on HPV in isolated perfused/ventilated mouse lung. (A) Representative trace of HPV is shown. Consistent and repetitive HPV was observed with PSS perfusate with 20% FBS. The amplitudes of HPV were increased by the application of Ang-II (0.2 mg/100 ml). (B) Bar graphs are shown as mean ± SEM. The amplitude of HPV using DMEM with 5% FBS was spontaneously decayed. After Ang-II (0.2 mg/100 ml) was applied, HPV was not significantly increased. Bar graphs are shown as mean ± SEM (right panels).](image)
Involvement of extracellular Ca$^{2+}$ influx through VDCC in HPV 

**Figure 6:** 

(A) Removal of extracellular Ca$^{2+}$ (Ca-free) significantly decreased the basal PAP and inhibited HPV. After restoration of extracellular Ca$^{2+}$ in the perfusate to 1.8 mM Ca$^{2+}$ again, HPV was recovered as before (left panel). Bar graphs are shown as mean ± SEM (right panel). **P < 0.01 vs. open and grey bars. (B) Application of Ca$^{2+}$-free (-Ca) solution during hypoxic conditions (1% O$_2$) decreased the amplitude of the hypoxia-induced increase in PAP (left panel). 1 mM EGTA was included in the Ca$^{2+}$-free solution. Bar graphs are shown as mean ± SEM (right panels). +Ca, solution including 1.8 mM Ca$^{2+}$. **Indicates statistically significant difference from the control value (P < 0.01). Lungs are isolated from three to six mice. (C) Effect of Nif on basal PAP and HPV was shown as a representative trace (left panel). After the 3rd challenge of hypoxic exposure, Nif (1 mM) was applied in the perfusate. The 4th to 6th challenges of hypoxia were performed in the presence of Nif. Application of Nif decreased amplitudes of HPV. Bar graphs are shown as mean ± SEM (right panel). **Indicates statistically significant difference from the control value (P < 0.01). Lungs are isolated from six mice.

40K$^{+}$-induced contraction and basal PAP in NOS3$^{-/-}$/Cav1$^{-/-}$ mice was 301.2% ± 45.5% and 147.8% ± 7.8% of control, respectively. However, HPV was not significantly enhanced by eNOS or eNOS/Cav1 deletion, although the basal PAP was significantly increased in NOS3$^{-/-}$ and NOS3$^{-/-}$/Cav1$^{-/-}$ mice in comparison to wild-type (WT) controls (Fig. 7B and C). Cav1 deletion had no effect on HPV, basal PAP, or 40K$^{+}$-induced contraction.

Last, biphasic vasoconstrictions were induced by hypoxic treatment for 90 minutes (also see Fig. 5C) in all types of mice (Fig. 7D). In NOS3$^{-/-}$ and NOS3$^{-/-}$/Cav1$^{-/-}$ mice, basal PAP and the transient increase in PAP induced by acute hypoxia were not significantly changed in comparison to WT control mice, whereas the second phase of HPV was actually enhanced and accelerated (Fig. 7E). These data indicate that deletion of eNOS enhances and accelerates the second phase of HPV in isolated perfused/ventilated lungs, while deletion of eNOS and Cav1 further enhanced the acute hypoxia-induced increase in PAP.

**DISCUSSION**

In the present study, we determined optimal conditions for isolated perfused/ventilated mouse lung perfusion in order to study HPV. The application of PSS with 20% FBS as perfusate was shown to be ideal for generating persistent and repetitive HPV, whereas hypoxic ventilation exposure did not increase PAP using other perfusate solutions such as PSS containing 4% albumin, 4% Ficoll, or rat blood, and DMEM containing 5-10% FBS.

With the PSS solution with 4% albumin or 4% Ficoll as a perfusate, the amplitude of HPV was under 1 mmHg. This amplitude of HPV was similar with results from other researchers who compared transgenic and WT mice using PSS, Ficoll, or rat blood, and repetitive HPV when applied to PSS perfusate containing 4% albumin, 4% Ficoll, or rat blood, and DMEM containing 5-10% FBS.

In isolated perfused/ventilated rat lung experiments, some researchers have used a mixture of rat blood and PSS solution as a perfusate.[21,46,66,67] When whole blood was applied in the perfusate, the amplitude of HPV increased more than two-fold, potentially because of the increased contractile responses in lungs from knockout mice. Some researchers have suggested that endothelial nitric oxide synthase (eNOS) activity is directly suppressed by caveolin-1 (Cav1),[56,57] and Cav1 knockout (Cav1$^{-/-}$) mice eventually develop pulmonary hypertension due to pathologic eNOS hyperactivity.[58-61] Also, eNOS is one of the primary signaling proteins thought to play a role in HPV.[62-65] Therefore, we compared 40K$^{+}$-induced contraction, basal PAP, HPV, and hypoxic treatment for 90 minutes in the isolated perfused/ventilated mouse lung from mice genetically deficient in Cav1, eNOS, or eNOS/Cav1. The amplitudes of 40K$^{+}$-induced contraction and basal PAP in NOS3$^{-/-}$ (eNOS deletion) and NOS3$^{-/-}$/Cav1$^{-/-}$ mice were significantly higher than those of the control mice (Fig. 7A and C). The amplitude of the
in viscosity or presence of vasoreactive molecules in blood,[21,23,42,68,69] although the absolute amplitude of HPV or alveolar hypoxia-induced increase in PAP was still small (Fig. 3).

In contrast to HPV in response to PSS with 4% albumin or Ficoll or rat blood, in the first challenge of hypoxic exposure, the absolute amplitude of the HPV in DMEM with 10% FBS increased by 9.3 mmHg. This amplitude of HPV is similar to results from previous studies in rats.[21,44,68] However, after the first trial, HPV decreased and the amplitude of the sixth trial was 2.0 mmHg (Fig. 4A). The spontaneously declining HPV did not recover upon application of angiotensin II (Fig. 4B).

To evaluate the effect of drugs or treatments on HPV, one must be able to measure consistent and repetitive HPV responses. Persistent HPV was observed when PSS with 20% FBS was perfused in the isolated mouse lung (Fig. 5A and B). From the first to the sixth challenge, the absolute amplitudes of HPV were about 4.5 mmHg in absence of pretone agent. To our knowledge, this is the first demonstration of persistent HPV responses in the isolated mouse lung perfused with PSS containing 20% FBS.
PSS or RPMI media was also used in many other studies as a perfusate in the isolated perfused lung, while albumin, Ficoll, or rat blood was added to the perfusate as an oncotic agent\([26,35,44,52,53]\). In the present study, the sustained HPV was only demonstrated with PSS with 20% FBS as a perfusate. Use of PSS containing 20% FBS as perfusate was suitable to measure acute hypoxic pressor responses of the lung vasculature to alveolar hypoxia for a few minutes as well as for chronic hypoxic exposure for more than two to three hours. When the exposure to hypoxia for three hours was applied, HPV was shown as a biphasic increase in PAP, a transient increase in PAP (which lasted approximately 10 minutes), followed by a gradual increase that reached a plateau about 30 minutes after initial hypoxic exposure (Figs. 5c and 7d, left panel). This was similar to previous results using different perfusates in the isolated perfused/ventilated lung and PA from different animals\([18,26,30,54,70]\).

Several researchers mentioned that the first peak in five minutes reflected an acute HPV. The second phase, which was prolonged vasoconstriction, might be related to the loss of endothelium-dependent vasorelaxation or an increase of vasoconstrictor production and release, which are involved in the development of pulmonary hypertension\([15,45,55,70]\). In the present study, however, our data demonstrated that the second phase of HPV, or hypoxia-induced increase in PAP, was actually enhanced and accelerated in isolated perfused/ventilated lungs of NOS3\(-/-\) and NOS3\(-/-\)/Cav1\(-/-\) mice (Fig. 7d and e). Furthermore, the proposed perfusate condition used in this study to measure HPV for long periods might be useful for the further investigation, in which it is required to identify PAP responses to prolonged hypoxia and their mechanisms.

Several previous studies have used whole blood or a mixture of blood and PSS as the perfusate for the isolated perfused/ventilated rat lung model\([21,23,67]\); however, few studies use rat blood in the perfusate for mouse studies. The blunted HPV we see when using PSS plus 20% rat blood could be due to differences in the composition of rat blood versus mouse blood or possibly due to an immune response. The use of PSS plus 20% FBS in our studies gave the optimal response over a long period of time. It is advantageous that the perfusate composition be as simple as possible in order for the investigation of basic mechanisms of HPV by avoiding the addition of complex molecules in the perfusate.\([26]\) The addition of 20% FBS to the perfusate may provide the necessary nutrients and growth factors required to maintain the integrity of vascular function during the extended experimental time. Additionally, the FBS may contain basal pretone or priming factors similar to the blood that are necessary for the vessels to maintain their function in an experimental setting.

The goal of these studies was to optimize the isolated perfused/ventilated mouse lung model for the study of HPV. In the literature, several studies have reported very different levels of response to hypoxia, with a hypoxic-induced change in PAP ranging from 1 to 8 mmHg\([26,71]\). A portion of the differences in hypoxic-induced change in PAP could be due to the use of different strains of mice.\([72]\) It has been reported that there is a greater than two-fold difference in the hypoxic-induced change in PAP between BALB/c and C57BL/6 mice under the same conditions.\([26]\) The differences in the response to hypoxia are also seen between species. Using the rat isolated perfused/ventilated lung model, a range in hypoxic-induced change in PAP from 4 to 14 mmHg has been reported.\([21,23,73,76]\) Variable degrees of pulmonary vascular reactivity are seen in other species as well. Cattle are hyper-reactors to hypoxic stress, while sheep are hyporeactors.\([75]\) These examples emphasize the importance of using an optimized perfusion solution in order to obtain the most accurate and comparable results.

One of the major mechanisms responsible for inducing HPV is an increase in cytosolic $[\text{Ca}^{2+}]$ in PASMCs. Regulating resting membrane potential ($E_{\text{m}}$) in PASMCs is mediated by the activity of K$^{+}$-channels\([76,77]\) and Na$^{+}$/$K^{+}$-ATPase. Although K$^{+}$ channels have been mainly studied as a determinant of resting $E_{\text{m}}$\([28,77]\), two-pore domain channels (TASK)\([78-80]\) and KCNQ channels\([81]\) were also suggested to contribute to the regulation of resting $E_{\text{m}}$. When the PA was exposed to acute hypoxia, the inhibition of these K$^{+}$ channels induced membrane depolarization and the opening of VDCC, which increased $\text{Ca}^{2+}$ influx and induced vasoconstriction\([5,54]\). In several cases, the role of $\text{Ca}^{2+}$ has been proven using isolated pulmonary arteries, cells, or lungs from other species\([81-83]\) but not in the isolated perfused/ventilated mouse lungs. Our results demonstrate that HPV was nearly abolished in the absence of extracellular $\text{Ca}^{2+}$ (using a $\text{Ca}^{2+}$-free solution as a perfusate), and that Nif-sensitive VDCCs play a major role in the acute hypoxia-induced increase in PAP (Fig. 6).

To confirm the optimal conditions in the isolated perfused/ventilated mouse lung, we compared vasoconstrictive responses using mice deleted selectively for eNOS, Cav1, and eNOS/Cav1. Increased basal PAP in eNOS null mice was similar to previous studies using a NOS inhibitor.\([37,44,65]\) Interestingly, 40K$^{-}$-induced contraction and basal PAP were enhanced in the NOS3$^{-/-}$/Cav1$^{-/-}$ mice, but the HPV was not changed. Since Cav1 expression is known to regulate eNOS activity and ROS generation,\([57,84]\) further investigation is required to elucidate the combined effect of eNOS and Cav1 on PAP and HPV.

Taken together, we established and optimized conditions for measuring HPV in the isolated perfused/ventilated mouse lung. Using PSS with 20% FBS as a perfusate, a repetitive and persistent HPV, with an amplitude of about 5 mmHg, is obtained without any pretone agents. HPV in the isolated perfused/ventilated mouse lung is dependent on cytosolic...
Ca$$^{2+}$$, which is similar to other data from isolated arteries, and lungs from other species. We suggest that this design is useful for defining mechanisms of HPV and related pathogenic diseases via comparison with transgenic and WT mice.

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