Vasculoprotective effect of U50,488H in rats exposed to chronic hypoxia: role of Akt-stimulated NO production

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1Department of Physiology, National Key Discipline of Cell Biology, Fourth Military Medical University, Xi’an, China; 2Department of Anesthesiology and Department of Pharmacology, Louisiana State University School of Medicine, New Orleans, Louisiana; and 3Department of Cardiac Surgery, Xijing Hospital, Fourth Military Medical University, Xi’an, China

Li J, Shi Q, Fan R, Zhang L, Zhang S, Guo H, Wang Y, Kaye AJ, Kaye AD, Bueno FR, Xu X, Yu S, Yi D, Pei J. Vasculoprotective effect of U50,488H in rats exposed to chronic hypoxia: role of Akt-stimulated NO production. J Appl Physiol 114: 238–244, 2013. First published November 8, 2012; doi:10.1152/japplphysiol.00994.2012.—Impairment of pulmonary endothelium function in the pulmonary artery is a direct result of chronic hypoxia. This study is to investigate the vasculoprotective effects of U50,488H (a selective κ-opioid receptor agonist) and its underlying mechanism in hypoxia-induced pulmonary artery endothelial functional injury. Chronic hypoxia was simulated by exposing the rats to 10% oxygen for 2 wk. After hypoxia, right ventricular pressure (RVP) and right ventricular hypertrophy index (RVHI) were measured. The pulmonary vascular dysfunction, effect of nitric oxide synthase inhibitor (L-NAME) on the relaxation of U50,488H, and level of nitric oxide (NO) were determined. In vitro, the signaling pathway involved in the anti-apoptotic effect of U50,488H was investigated. Cultured endothelial cells were subjected to simulated hypoxia, and cell apoptosis was determined by TUNEL staining. U50,488H (1.25 mg/kg) significantly reduced RVP and RVHI in hypoxia. U50,488H markedly improved both pulmonary endothelial function (maximal vasorelaxation in response to ACh: 74.9 ± 1.8%, n = 6, P <0.01 vs. hypoxia for 2 wk group) and increased total NO production (1.65 fold). U50,488H relaxed the pulmonary artery rings of the hypoxic rats. This effect was partly abolished by L-NAME. In cells, U50,488H both increased NO production and reduced hypoxia-induced apoptosis. Moreover, pretreatment with nor-binaltorphimine (nor-BNI, a selective κ-opioid receptor antagonist), PI3K inhibitor, Akt inhibitor or L-NAME almost abolished anti-apoptotic effect exerted by U50,488H. U50,488H resulted in increases in Akt and eNOS phosphorylation. These results demonstrate that pretreatment with U50,488H attenuates hypoxia-induced pulmonary vascular endothelial dysfunction in an Akt-dependent and NO-mediated fashion.

κ-opioid receptor; endothelial dysfunction; apoptosis; nitric oxide; Akt

HYPOXIA CONTRIBUTES to many pathological processes. For example, hypoxia drives angiogenesis in tumors, takes part in chronic obstructive pulmonary disease, and participates in chronic plateau disease, including hypoxic pulmonary hypertension. Studies suggest chronic hypoxia imposed endothelial injury of the pulmonary arterial, followed by the imbalance of various vasomotor factors, results in pulmonary artery contraction and the remodeling of pulmonary vessels. Hypoxia induces endothelial injury leading to increased endothelial cell apoptosis (15). Hypoxia has also been shown to induce direct apoptosis in human umbilical vein endothelial cells and glomerular endothelial cells (5, 20).

In endothelial cells, hypoxia induces the NO signaling that confers protection of EC against hypoxia-mediated damages (10). Endothelial dysfunction factors greatly in many cardiovascular risk factors and diseases including hypoxic pulmonary arterial hypertension (HPH). Endothelial dysfunction factors greatly especially in the case of impaired endothelial NO production and/or availability (1, 13).

Evidence suggests a central role for endothelial dysfunction in both the initiation and progression of pulmonary hypertension (1). A previous study has also demonstrated (9) impairment of pulmonary endothelium function in rat intrapulmonary rings as a direct result of chronic hypoxia.

We have found that U50,488H (trans-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl] benzeneacetaamide), a selective κ-opioid receptor agonist, relaxes rat pulmonary artery rings through κ-opioid receptor activation in the pulmonary artery (18). This effect is inhibited significantly by the NO synthase inhibitor L-NAME (19). U50,488H increases NO concentration in both blood and pulmonary tissue. Also, U50,488H decreases the level of endothelin (ET) and angiotensin II (ANG II) in both blood and pulmonary tissue during hypoxia. U50,488H depresses rat mean pulmonary artery pressure (mPAP) during a 2-wk exposure to chronic hypoxia (12). Both the vasodilatative and mPAP depression effects relate to NO. However, the possibility that U50,488H may reduce endothelial injury from chronic hypoxia has never been previously studied. By definition, endothelial cells express endothelial nitric oxide synthase (eNOS), and the eNOS is a novel Akt target. Moreover, NO, generated by the constitutive enzyme eNOS, has been shown to exert protective properties (11). A previous study demonstrated that the anti-apoptotic effect is primarily via an Akt-dependent eNOS activation pathway in coronary endothelial cells (14). So the possibility that U50,488H exerts an endothelial protective effect via some Akt-mediated eNOS activation pathway merits investigation. Therefore, the aims of the present study are twofold. The first aim is to determine whether U50,488H pretreatment preserves pulmonary vascular function and attenuates endothelial injury in rats exposed to chronic hypoxia. The second aim is to determine signaling pathways through which U50,488H may exert an anti-apoptotic effect in hypoxic endothelial cells.

Our previous studies lead us further to investigate the signaling mechanism that κ-opioid receptors use in order to protect pulmonary circulation. Also, previous studies lead us to
confirm the possible role of the NO signaling pathway in the anti-endothelial injury actions that the \( \kappa \)-opioid receptor mediates. We anticipate that the experimental results will provide novel academic perspectives in the examination of chronic hypoxia-induced pulmonary hypertension.

**MATERIALS AND METHODS**

**Animal groups and rats exposed to chronic hypoxia.** Forty-eight male Sprague-Dawley rats; 200 ± 10 g) from the animal center of the Fourth Military Medical University on Animal Care were used. This study conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health, NIH Publication No. 85–23, revised 1996. Ethical approval was also granted by the University Ethics Committee.

Rats were randomly divided into six groups. Group I served as the normoxic, normal control group. Group II served as the 2-wk hypoxia group. The rats in Group II were exposed to hypobaric and hypoxic conditions for 2 wk. Group III served as the hypoxia for 2 wk and saline group. The rats in Group III were exposed to hypobaric and hypoxic conditions for 2 wk. Also, each rat in Group III was injected intraperitoneally with 0.5 ml saline 10 min before hypoxia every other day. Group IV served as the hypoxia for 2 wk and USO,488H group. The rats in Group IV were exposed to hypobaric and hypoxic conditions for 2 wk. Also, each rat in Group IV was intraperitoneally injected with 1.25 mg/kg of USO,488H (trans-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide), the selective \( \kappa \)-opioid receptor agonist, 10 min prior to hypoxia every other day. Group V served as the hypoxia for 2 wk and nor-BNI group. The rats in Group V were exposed to hypobaric and hypoxic conditions for 2 wk. Also, each rat in Group V was intraperitoneally injected with 2.0 mg/kg of nor-BNI (nor-binaltorphimine), the selective \( \kappa \)-opioid receptor antagonist, 10 min prior to hypoxia every other day. Group VI served as the hypoxia for 2 wk, USO,488H, and nor-BNI group. The rats in Group VI were exposed to hypobaric and hypoxic conditions for 2 wk. Also, each rat in Group VI was intraperitoneally injected with both nor-BNI and USO,488H. First 2.0 mg/kg of nor-BNI was injected into each rat. Then, 15 min after each injection, each rat was subsequently injected with 1.25 mg/kg of USO,488H. Each injection was intraperitoneal. Ten minutes after USO,488H injection, each rat was exposed to hypoxia. This procedure was performed every other day. Compounds (USO,488H and nor-BNI) were dissolved in normal saline (0.85% NaCl solution) before use.

For the appropriate rats, hypoxia was induced for 8 h every day. This was done by exposing the rats to low pressure and low oxygen (air pressure 50 kPa, oxygen concentration 10%). The normoxic group of rats was kept in room air.

**Hemodynamics following hypoxia.** The appropriate rats were exposed to hypoxia. After the exposure to hypoxia, these rats were anesthetized via peritoneal injection with pentobarbital sodium (60 mg/kg ip). Supplemental doses of sodium pentobarbital were given when needed in order to maintain uniform anesthesia. A microcatheter was inserted into right ventricle through the right external jugular vein, and right ventricular pressure (RVP) was measured (17). The hearts and blood were then harvested. Each of the following was isolated in order to calculate the right ventricular hypertrophy index (RVH1): right ventricle (RV), left ventricle (LV), and septum (S). The RVH1 itself was expressed as the tissue weight ratio of RV/(LV + S). Isolation and perfusion of pulmonary arterial rings. After hypoxia, the rats were killed by pentobarbital injection. For each rat, the chest was opened and the heart and lungs were gently removed. The organs were then placed into an ice-cold and oxygenated (5% CO\(_2\) and 95% O\(_2\)) Krebs-Henseleit (K-H) buffer. The K-H buffer consists of (mmol/l) 118 NaCl, 4.75 KCl, 2.54 CaCl\(_2\), 2H\(_2\)O, 1.19 KH\(_2\)PO\(_4\), 1.19 MgSO\(_4\)•7H\(_2\)O, 25 NaHCO\(_3\), 10.0 glucose, pH = 7.4. The pulmonary artery rings were carefully isolated and cleaned of fat and connective tissue. A second grade branch of PA was picked. Then they were cut into 2-mm-long rings. The rings were then mounted onto stainless steel hooks and suspended in 5 ml tissue baths. Then they were connected to FORT-10 force transducers (made in Spain by Panlab s.l.). The force transducers facilitated the record of changes in force transduction. The computer software used was the MacLab data acquisition system.

Each experimentation system was conducted as follows. The baths were filled with 5 ml of K-H buffer and aerated with 95% O\(_2\)-5% CO\(_2\) at 37°C. The rings were then stretched to an optimal preload of 750 mg. Then the rings were allowed to equilibrate for 60 min. During this period, the K-H buffer in the tissue bath was replaced every 20 min. The optimal preload of isolated pulmonary rings (750 mg) was determined by the length-developed tension relationship as described previously (19).

After equilibration, phenylephrine (PE, 1 \( \mu \)mol/l) was added to the bath. Once the stable contraction was reached, acetylcholine (ACH), the endothelium-dependent vasodilator, was added to the bath. ACh was added in cumulative concentrations of 10\(^{-8}\), 10\(^{-7}\), and 10\(^{-6}\) mol/l. This was done in order to determine the endothelial function. The cumulative response was allowed to stabilize. The rings were then washed and allowed to equilibrate back to baseline. After equilibration, PE (1 \( \mu \)mol/l) was added to the bath. Once the stable contraction was reached, S-nitroso-N-acetylpenicillamine (SNP) (10\(^{-8}\), 10\(^{-5}\) mol/l), the endothelium-independent vasodilator, was added to the bath. This was done in order to determine vascular smooth muscle function.

After equilibration, PE at 1 \( \mu \)mol/l was added to the bath. Once the stable contraction was reached, USO,488H (70 \( \mu \)mol/l) was added to the bath. The USO,488H was added in order to determine the relaxation effect after exposure to chronic hypoxia. The response was allowed to stabilize. After response stabilization, the rings were washed and allowed to equilibrate to baseline. PE at 1 \( \mu \)mol/l induces a vasoconstriction effect. Upon stabilization, 1-NAME (100 \( \mu \)mol/l) was added to the bath. Fifteen minutes after 1-NAME addition, USO,488H (70 \( \mu \)mol/l) was administered. This methodology allowed the determination of USO,488H abolishment by 1-NAME.

**Culture of pulmonary microvascular endothelial cells.** The isolation of pulmonary microvascular endothelial cells (PMVECs) was performed as previously described (3, 21). Sprague-Dawley rats of 8–10 g were anesthetized with pentobarbital sodium via peritoneal injection. The rats were sterilized with 75% alcohol. The lungs were isolated. The organs were rinsed several times in D-Hanks solution (Ca\(^2+\) and Mg\(^2+\)-free, pH 7.4, 4°C). The pleura were discarded from the lung tissue. Then the tissues of the lung surface and edges were minced with scissors in DMEM solution. This solution contains the following: 20% fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 90 U/ml of heparin. The cells were incubated at 37°C in humidified air containing 5% CO\(_2\). After 60 h, the residue lung tissues were removed. The cells were grown in plastic tissue culture flasks. PMVECs were identified according to the following: scanning electron microscope, transmission electron microscope, factor VIII immunostaining. At confluence, cells were replated in 1.5% gelatin-coated flasks. The cells were used at 20,000 cells/cm\(^2\). Cells were used within passages 3–6 after primary culture.

**TUNEL staining.** PMVECs were divided into one normoxic group (5% CO\(_2\) at 37°C) and six hypoxic groups (2% O\(_2\), 5% CO\(_2\) and 93% N\(_2\) at 37°C). The hypoxic endothelial cells were randomly exposed to one of the following treatments: no exposure; USO,488H (70 \( \mu \)mol/l); USO,488H and nor-BNI (5 \( \mu \)mol/l); USO,488H and wortmannin (a specific inhibitor of phosphoinositide 3-kinase, PI3K inhibitor) (10 \( \mu \)mol/l); USO,488H and Akt inhibitor (1L-6-hydroxyethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecylcarbonate, Calbiochem, 5 \( \mu \)mol/l); USO,488H and 1-NAME (100 \( \mu \)mol/l). The appropriate cells were exposed to hypoxia. After 12 h of hypoxia exposure, apoptosis was determined. The determination of apoptosis was conducted with the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals). There were slight modifications in the methodology of apoptosis
Table 1. Effect of U50,488H on RVP and RV/(LV + S) in rats exposed to normoxia and chronic hypoxia for 2 wk

<table>
<thead>
<tr>
<th>Group</th>
<th>RVP, mmHg</th>
<th>RV/(LV + S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26.7 ± 0.6</td>
<td>0.27 ± 0.01</td>
</tr>
<tr>
<td>2 wk</td>
<td>35.8 ± 1.0*</td>
<td>0.37 ± 0.01*</td>
</tr>
<tr>
<td>2 wk + NS</td>
<td>37.0 ± 1.2</td>
<td>0.36 ± 0.01*</td>
</tr>
<tr>
<td>2 wk + U50</td>
<td>27.7 ± 0.0#</td>
<td>0.29 ± 0.01##</td>
</tr>
<tr>
<td>2 wk + U50 + nor-BNI</td>
<td>36.5 ± 1.1ε</td>
<td>0.35 ± 0.01ε</td>
</tr>
<tr>
<td>2 wk + nor-BNI</td>
<td>37.2 ± 1.1ε</td>
<td>0.35 ± 0.02ε</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8. Control, normoxic group; 2 wk, hypoxia for 2 wk; 2 wk + NS, hypoxia for 2 wk plus saline; 2 wk + U50, hypoxia for 2 wk plus U50,488H (1.25 mg/kg); 2 wk + U50 + nor-BNI, hypoxia for 2 wk plus nor-BNI (2.0 mg/kg) and U50,488H (1.25 mg/kg); 2 wk + nor-BNI, hypoxia for 2 wk plus nor-BNI (2.0 mg/kg). RVP, right ventricular pressure; RV/(LV + S), the tissue weight ratio of right ventricle (RV)/left ventricle (LV) + septum (S). *P < 0.05 compared with control, #P < 0.05 compared with 2 wk hypoxia; ##P < 0.05 compared with 2 wk hypoxia + NS groups. εP < 0.05 compared with 2 wk hypoxia + U50,488H group.

RESULTS

Effect of U50,488H on RVP and RV hypertrophy index in rats exposed to hypoxia. The RVP and RV hypertrophy index of rats exposed to hypoxia for 2 wk was significantly higher compared with the RVP and RV hypertrophy index of the normoxic rats. U50,488H pretreatment significantly reduced the RVP and RV hypertrophy index of the rats that were exposed to chronic hypoxia for 2 wk. The effect of the U50,488H on the RVP and RV hypertrophy index was abolished by nor-BNI (Table 1). These data suggest that rats exposed to chronic hypoxia for 2 wk experienced both κ-opioid receptor and a subsequent attenuation of both RVP and RV hypertrophy.

Effect of U50,488H on endothelial dysfunction in rats exposed to hypoxia. Endothelial dysfunction serves as one of the earlier pathological indicators of chronic hypoxia. To clarify whether pretreatment with U50,488H may protect the endothelium against chronic hypoxia injury, we studied the effects of U50,488H on endothelium-dependent vasorelaxation in isolated pulmonary artery segments. As shown in Fig. 1, chronic hypoxia resulted in a significant endothelial dysfunction. This dysfunction was demonstrated through decreased vasorelaxation in response to ACh (maximal relaxation: 42 ± 9.8%, n = 6, P < 0.01 vs. 89 ± 2.51% in normoxic group). U50,488H pretreatment before hypoxia significantly improved the pulmonary arterial vasodilatory response to ACh (maximal relaxation: 75 ± 1.8%, n = 6, P < 0.01 vs. hypoxia for 2 wk group) (Fig. 1). All three groups had a normal response to an endothelium-independent vasodilator, SNP. These data provide direct evidence that U50,488H reduces chronic hypoxia-induced endothelial dysfunction of the pulmonary artery.

Effect of NO-synthase inhibitor on U50,488H relaxation. U50,488H at a concentration of 70 µmol/L induced a significant relaxation effect in the pulmonary artery rings of the rats that
were exposed to chronic hypoxia. The maximal relaxation effect was 57 ± 5.4% (n = 8, Fig. 2). Preincubation of the pulmonary artery rings with the NOS inhibitor l-NAME at 100 μmol/l for 20 min attenuated U50,488H-induced vasorelaxation. The maximal vasorelaxation was 35 ± 4.9%. These results demonstrate NO mediation in U50,488H-induced vasorelaxation in the pulmonary artery.

Effect of U50,488H on NO content in rats exposed to hypoxia.
We aimed to determine the extent to which decreased NO content was responsible for the endothelial dysfunction that was observed in the pulmonary artery from rats exposed to chronic hypoxia. In order to achieve this goal, we measured the total NOx content in the plasma. As illustrated in Fig. 3, hypoxia resulted in a significant decrease in NO plasma compared with the NO plasma in the normoxic group. U50,488H pretreatment (1.25 mg/kg ip) significantly restored plasma NO content. The U50,488H effect was abolished by nor-BNI (2.0 mg/kg). These results suggest both that U50,488H increases NO content and that this the κ-opioid receptor exerts influence on this effect.

U50,488H pretreatment significantly reduces endothelial apoptosis via Akt-stimulated NO content. In vivo results clearly demonstrate the significant attenuation by U50,488H of chronic hypoxia-induced endothelial dysfunction. The results also demonstrate the influence of U50,488H in improved NO content. The mechanisms for these demonstrations remain unknown. We infer that U50,488H attenuates endothelial injury via the stimulation of NO content. The role of NO in U50,488H’s anti-apopotic effect was investigated. In order to address this critical question, cultured PMVECs were exposed to hypoxia in vitro. As shown in Figs. 4 and 5, the simulated hypoxia resulted in significant decrease in both NO content by endothelial cells (68.8 ± 4.9 μmol/l vs. 101.5 ± 6.6 μmol/l in normal culture, n = 6, P < 0.01) and also significant endothelial apoptosis. Treatment with U50,488H at 70 μmol/l led both to a significant increase in NO content (98.7 ± 6.5 μmol/l, P < 0.01 vs. hypoxia for 12 h) and also a reduction in endothelial apoptosis (1.2 ± 0.4% vs. 11.8 ± 0.8% in hypoxia for 12 h, n = 9, P < 0.01) (Fig. 5). The pretreatment of endothelial cells with nor-BNI (5 μmol/l), wortmannin (a PI3K inhibitor, 10 nmol/l) (11), the selective Akt inhibitor (5 μmol/l), l-NAME (a nonselective NOS inhibitor, 100 μmol/l) offered notable results. The effects of U50,488H on NO content were significantly blocked (72.6 ± 5.2 μmol/l, 69.1 ± 5.4 μmol/l, 70.3 ± 5.4 μmol/l, 74.6 ± 4.2 μmol/l, respectively, n = 6, P < 0.05 vs. 98.7 ± 6.5 μmol/l in control group). Also the anti-apoptotic effect of U50,488H was abolished in cultured PMVECs exposed to hypoxia (10.2 ± 0.7%, 10.4 ± 1.2%, 9.6 ± 0.7%, and 10.8 ± 1.0%, respectively, n = 9, P < 0.01 vs. 1.2 ± 0.4% in U50,488H group). Neither l-NAME treatment nor treatment by the Akt inhibitor alone exerted an effect on TUNEL-positive staining (data not shown). These results demonstrate that U50,488H exerts its endothelial protective effect via an Akt-dependent, NO-mediated mechanism.

We wished further to confirm the influence of U50,488H on the anti-apoptotic mechanism through the Akt-eNOS-NO signaling pathway. We performed an additional experiment. We observed the effect of U50,488H on expression of both Akt phosphorylation and eNOS phosphorylation. In the cultured PMVECs that were exposed to hypoxia, U50,488H treatment led to noteworthy results. Akt phosphorylation increased 3.3 fold. Endothelial nitric oxide synthase (eNOS) phosphorylation increased 1.9 fold. Each of these increases was abolished by both the κ-opioid receptor antagonist nor-BNI and the PI3K inhibitor (wortmannin). l-NAME treatment exerted a significant effect on neither Akt phosphorylation nor eNOS phosphorylation (Fig. 6). In vitro U50,488H pretreatment stimulates an Akt-stimulated and eNOS-NO-dependent pathway that inhibits PMVEC hypoxia-induced apoptosis.

DISCUSSION
Two novel findings have been made in the present study. First, U50,488H exerts a significant vasculoprotective effect...
that is seen in the reduction of endothelial dysfunction in a pulmonary artery afflicted with chronic hypoxia. Second, in cultured PMVECs subjected to hypoxia, we have shown directly that NO production increases and endothelial apoptosis decreases because of κ-opioid receptor stimulation with U50,488H. An ancillary finding that supports the second finding is that U50,488H induces the enhanced expression of phospho-Thr308-Akt and phospho-eNOS.

In our present study, we have demonstrated that endothelial NO production is markedly decreased in the plasma of rats that are exposed to chronic hypoxia. As a result, the decreased level of NO is responsible for the attenuated endothelium-dependent vasorelaxation in the pulmonary arteries of rats that are exposed to chronic hypoxia. Our present study also has shown for the first time that in vivo U50,488H treatment significantly preserves endothelium-dependent vasorelaxation in an isolated pulmonary artery. ACh-induced vasorelaxation in an isolated pulmonary artery is determined by three factors: the aggregate NO production by each endothelial cell, the total number of endothelial cells in a pulmonary artery segment, and the responsiveness of smooth muscle cells to NO. In this study, we found that the level of NO was decreased, while it was upregulated by U50,488H during hypoxic conditions. Increased NO levels were responsible for the conservation of endothelium-dependent vasorelaxation. The effect of U50,488H on the responsiveness of smooth muscle cells to NO is unknown. Further study is warranted.

The interrelationship between NO production and the anti-apoptotic effect of U50,488H was studied. The model was an in vitro culture of PMVECs. Our results demonstrated that U50,488H treatment preceding hypoxia significantly reduced endothelial apoptosis. It is noteworthy that this anti-apoptotic effect of U50,488H was abolished by pretreatment with either an Akt inhibitor or an eNOS inhibitor. This result provides strong evidence that κ-opioid receptor stimulation by U50,488H exerts an anti-apoptotic effect through Akt-eNOS signaling.

![Graph showing NO content in supernatants of PMVECs.](image1)

**Fig. 4.** NO content in supernatants of PMVECs. Data of NO content were presented as means ± SE; n = 6 independent experiments. **P < 0.01 compared with Con. ***P < 0.05 vs. 12 h. τP < 0.05 vs. 12 h + U50. ττP < 0.01 vs. 12 h + U50. Con, control; 12 h, hypoxia for 12 h; 12 h + U50, hypoxia for 12 h + U50; 12 h + U50 + N, hypoxia for 12 h + U50,488H + nor-BNI; 12 h + U50 + W, hypoxia for 12 h + U50,488H + wortmannin; 12 h + U50 + A, hypoxia for 12 h + U50,488H + Akt inhibitor; 12 h + U50 + L, hypoxia for 12 h + U50,488H + L-NAME.

![Photomicrograph showing TUNEL-positive nuclei.](image2)

**Fig. 5.** The anti-apoptotic effect of U50,488H on PMVECs during hypoxia through Akt-NO signaling pathway. A: representative photomicrograph of cultured PMVECs subjected to hypoxia receiving different treatments. Green fluorescence represents TUNEL-positive nuclei. Total endothelial cells are depicted by blue fluorescence (×20 objective). 12 h, hypoxia for 12 h; L, L-NAME; A, Akt inhibitor; B: quantitative analysis of endothelial cell apoptosis (%), n = 9 independent experiments. 12 h + U50, hypoxia for 12 h + U50,488H; 12 h + U50 + N, hypoxia for 12 h + U50,488H + nor-BNI pretreatment; 12 h + U50 + W, hypoxia for 12 h + U50,488H + wortmannin pretreatment; 12 h + U50 + A, hypoxia for 12 h + U50,488H + Akt inhibitor pretreatment; 12 h + U50 + L, hypoxia for 12 h + U50,488H + L-NAME pretreatment. **P < 0.01 vs. 12 h, ###P < 0.01 vs. 12 h + U50.
Other opioid receptor stimulations are known to elicit Akt signaling either for apoptosis reduction or antinociception amplification. One pathway includes the activation of the μ-opioid receptor by N-desmethylclozapine. The activation of the μ-opioid receptor by N-desmethylclozapine counteracts apoptosis induced by oxidative stress through the PI3K-Akt signaling pathway (16). The activation of the μ-opioid receptor by morphine may stimulate the nNOS/NO/KATP channel antinociceptive pathway through the PI3K/Akt signaling pathway. The PI3K/Akt signaling pathway may be located on primary nociceptive neurons (4).

Studies suggest that chronic hypoxia leads to the sustained endothelial injury of the pulmonary arteriole. After this occurs the imbalance of various vasomotor factors. The imbalance contributes both to pulmonary artery contraction and pulmonary vessel remodeling. The altered production of these endothelial vasoactive mediators has been increasingly recognized in patients with PH: NO, prostacyclin, endothelin-1 (ET-1), serotonin, and thromboxane (1). Our previous study showed the simultaneous reduction of NO levels and the significant increases of ET-1 and ANG II levels during hypoxia. U50,488H pretreatment significantly increases NO concentration. U50,488H pretreatment decreases the concentrations of both ET and ANG II in the blood and in pulmonary tissue (12). In this study, we found that U50,488H through μ-OR activation upregulated NO during hypoxia. The data further provide evidence that U50,488H may counteract the changes from hypoxia.

In summary, we have demonstrated the Akt-dependent, NO-mediated attenuation by U50,488H of hypoxia-induced endothelial apoptosis. This anti-apoptotic effect enhances the number of viable endothelial cells in the pulmonary vessels of rats that are exposed to chronic hypoxia. This effect conserves the pulmonary vasodilatory response to endothelium-dependent vasodilators, such as ACh. Theses NO-stimulating and pulmonary vasculoprotective properties of U50,488H may contribute to the clinical treatment of hypoxic pulmonary hypertension. The results of the present study demonstrate that U50,488H pretreatment both preserves pulmonary vascular function and attenuates endothelial injury in rats exposed to chronic hypoxia. We have outlined the signaling pathways through which U50,488H may exert an anti-apoptotic effect in hypoxic endothelial cells. Our study begins the process of identification of the signaling mechanism that κ-opioid receptors use in order to protect pulmonary circulation. Our study elucidates the possible role of the NO signaling pathway in the anti-endothelial injury actions that the κ-opioid receptor mediates.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).
AUTHOR CONTRIBUTIONS


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