Nicorandil inhibits hypoxia-induced apoptosis in human pulmonary artery endothelial cells through activation of mitoK\(_{\text{ATP}}\) and regulation of eNOS and the NF-\(\kappa B\) pathway

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Abstract. Apoptosis of human pulmonary artery endothelial cells (HPAECs) is the initial step and triggering event for pulmonary hypertension (PH). However, little is known about the actions of nicorandil on HPAECs in vitro. In the present study, we investigated the anti-apoptotic effect of nicorandil on HPAECs exposed to hypoxia, and explored the underlying mechanism(s) of action. Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Annexin V and propidium iodide staining, and Hoechst 33342 staining assay were employed to detect apoptosis. In addition, the protein expression of Bax, Bcl-2, caspase-9 and -3, endothelial nitric oxide synthase (eNOS), nuclear factor-\(\kappa B\) (NF-\(\kappa B\)) and I\(\kappa B\)\(\alpha\) were determined by western blotting to investigate the possible mechanisms. We found that exposure to hypoxia for 24 h significantly decreased cell viability and increased cell apoptosis. Pretreatment with nicorandil (100 \(\mu\)M) effectively abolished the influence of hypoxia on HPAECs. However, these protective effects of nicorandil were significantly inhibited by an antagonist of mitochondrial adenosine triphosphate-sensitive potassium (mitoK\(_{\text{ATP}}\)) channels, 5-hydroxydecanoate (5-HD, 500 \(\mu\)M), and by an eNOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME, 300 \(\mu\)M). We further observed that nicorandil could upregulate the decreased protein expression of eNOS and I\(\kappa B\)\(\alpha\), and downregulate the increased protein expression of NF-\(\kappa B\), induced by hypoxia. In addition, nicorandil inhibited the enhancement of caspase-3 and -9 expression, and the increase in the Bax/Bcl-2 expression ratio, induced by hypoxia. However, these effects were also abolished by 5-HD and L-NAME. Collectively, these findings suggest that nicorandil inhibits hypoxia-induced apoptosis of HPAECs through activation of mitoK\(_{\text{ATP}}\) channels and increased eNOS expression, which in turn inhibits the NF-\(\kappa B\) pathway and the mitochondrial apoptotic pathway.

Introduction

Pulmonary hypertension (PH) is a progressive disease of various origins that results in right heart dysfunction. In all its variant presentations, this disease is estimated to affect up to 100 million people worldwide, and is associated with a poor prognosis (1). PH can be idiopathic or associated with other diseases, such as connective tissue diseases, congenital heart defects, portal hypertension, left heart disease and chronic obstructive pulmonary disease (COPD), or it can occur after long-term living in plateau environment (1). Although the exact pathogenesis of PH remains unclear, there is increasing support that pulmonary artery endothelial cell (PAEC) apoptosis induced by a variety of factors, including hypoxia, is the initial step and triggering event for PH (2-4). PAEC loss results in an abnormal overgrowth of pulmonary artery smooth muscle cells, provides conditions favoring the emergence of apoptosis-resistant hyperproliferative endothelial cells (ECs), and finally leads to advanced PH (3-5). Current therapies for chronic PH include prostanoioids, endothelin receptor antagonists and phosphodiesterase inhibitors; these therapeutic approaches mainly provide symptomatic relief and slow down disease progression (6). There is, therefore, a genuine need for novel treatments that prevent progression of PH by interfering with the pathogenesis of the disease at the initial stage. To this end, antagonism of human PAEC (HPAEC) apoptosis may be a useful approach on which to base novel treatments for PH.

Nicorandil, which is generally used for the management of angina pectoris, has a unique pharmacological profile...
that includes a nitrate-like effect to dilate peripheral coronary arteries, and an action as an agonist at mitochondrial adenosine triphosphate-sensitive potassium (mitoK\(_{\text{ATP}}\)) channels. In some cell types, nicorandil has been shown to inhibit apoptosis induced by a number of factors, through activation of mitoK\(_{\text{ATP}}\) channels (7-12). However, whether nicorandil has an anti-apoptotic effect in HPAECs remains unknown.

Emerging evidence has indicated an important role for endothelial nitric oxide synthase (eNOS) in the regulation of EC apoptosis. Reduced expression of eNOS leads to a decreased NO synthesis which is a primary factor in the pathophysiology of PH (13). In monocrotaline (MCT)-induced PH rats, nicorandil has been reported to exert a beneficial effect through an increase in eNOS expression in heart and lung tissue (14,15). Furthermore, some studies have found that the opening of mitoK\(_{\text{ATP}}\) channels could induce eNOS expression (16,17). However, it is currently unclear whether nicorandil has the ability to enhance eNOS expression via activation of mitoK\(_{\text{ATP}}\) channels in HPAECs.

Nuclear factor-κB (NF-κB) is a transcription factor that plays an important role in the regulation of the inflammatory response and apoptosis in several cells types (18). Recent studies have observed an increased expression of NF-κB in PH, and have shown that blockade of NF-κB activity could reduce the development of PH (19-21). Of note, nicorandil has been reported to reduce myocardial reperfusion injury by attenuating NF-κB activation (22), and levsimendan, a K\(_{\text{ATP}}\) channel opener, has been found to inhibit NF-κB expression in human umbilical vein endothelial cells (HUVECs) (23). However, little data exists concerning the effect of nicorandil on the NF-κB pathway in HPAECs. Furthermore, although there is accumulating evidence that eNOS or NO can mediate inhibition of NF-κB signaling and suppress NF-κB-dependent apoptosis and inflammation (24-26), little is known about the relationship between eNOS and NF-κB in HPAECs.

The aim of the present study was to determine whether treatment with nicorandil attenuates apoptosis of HPAECs exposed to hypoxia, and, if so, to investigate the mechanisms involved.

**Materials and methods**

**Reagents.** Nicorandil was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Endothelial Cell Medium (ECM), vascular endothelial growth factor (VEGF), fetal bovine serum (FBS), penicillin and streptomycin were obtained from HyClone Laboratories, Inc. (Logan, UT, USA). Antibodies against Bax, Bcl-2, caspase-3 and -9, eNOS, NF-κB and IκBα were purchased from Cell Signaling Technology (Beverly, MA, USA), and antibody against β-actin was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). 5-Hydroxydecanoate (5-HD), NG-nitro-L-arginine methyl ester (L-NAME) and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma (St. Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33342, Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) were obtained from Sigma. AnaeroPack-Anaero was purchased from Mitsubishi Gas Chemical Co. (Tokyo, Japan). All other chemicals were of the highest purity available commercially.

**Cell culture and treatment.** HPAECs were obtained from ScienCell Research Laboratories (San Diego, CA, USA). HPAECs (1x10^6/75 cm\(^2\) flask) were seeded in ECM containing 10% (v/v) FBS, 1% (v/v) VEGF, 100 U/ml penicillin and 100 U/ml streptomycin, at 37°C in a 5% CO\(_2\)-humidified atmosphere. The culture medium was replaced every 2-3 days until 80-90% confluence was achieved. HPAECs of the 3rd to 5th passages, in an actively growing condition, were used for the experiments. HPAECs were divided into 6 treatment groups: i) control group: cells were cultured under normoxic conditions for 24 h (20% O\(_2\), 5% CO\(_2\)); ii) hypoxia group: cells were cultured in the hypoxic chamber using AnaeroPack-Anaero, a disposable oxygen-absorbing and CO\(_2\)-generating agent, for 24 h; iii) hypoxia + nicorandil group: cells were pretreated with nicorandil (100 µM), and then cultured in the hypoxic chamber using AnaeroPack-Anaero for 24 h; iv) hypoxia + nicorandil + 5-HD group: cells were pretreated with nicorandil (100 µM) and 5-HD (500 µM), and then cultured in the hypoxic chamber using AnaeroPack-Anaero for 24 h; v) hypoxia + nicorandil + L-NAME group: cells were pretreated with nicorandil (100 µM) and L-NAME (300 µM), and then cultured in the hypoxic chamber using AnaeroPack-Anaero for 24 h. The concentrations of nicorandil, 5-HD, PDTC and L-NAME used in this study were selected on the basis of previously published studies (8,27,28). 5-HD, L-NAME and PDTC were added to the medium 30 min prior to the addition of nicorandil. The AnaeroPack started to absorb oxygen within 1 min, oxygen tension inside the box dropped to 1 mmHg within 1 h (O\(_2\)<1%, CO\(_2\)~5%).

**MTT cell viability assay.** Cell viability was determined by MTT incorporation. HPAECs were seeded in 96-well culture plates, cultivated, and divided into different treatment groups as described above. Following addition of the appropriate drugs, 20 µl assay medium containing 5 mg/ml MTT was added to each well before the culture was terminated. After 4 h of incubation at 37°C, the medium was aspirated and the cells were lysed by the addition of 100 µl DMSO. Following a 10-min incubation at 37°C, the optical density of each sample was measured in an ELISA microplate reader using test and reference wavelengths of 570 nm.

**Hoechst 33342 staining assay.** HPAECs were seeded in 24-well culture plates, cultivated, and divided into different treatment groups as described above. Following addition of the appropriate drugs, cells were washed twice with PBS and treated with 100 µl Hoechst 33342 (10 µg/ml) for 15 min at 37°C, in the dark. The medium was aspirated and samples were then analyzed by an Olympus fluorescence microscope using an excitation wavelength of 346 nm and an emission wavelength of 460 nm.

**Annexin V-PI analysis by flow cytometry.** Following exposure to the appropriate drugs, cells in the various treatment groups were harvested and resuspended in PBS at a density of 1x10^6 cells/ml. Following centrifugation at 1,000 x g for 5 min, 195 µl FITC-conjugated Annexin V binding buffer and 5 µl Annexin V-FITC were added. Following gentle vortexing,
the mixture was incubated for 15 min at room temperature, in the dark. Following centrifugation at 1,000 x g for 5 min, the medium was aspirated and 190 µl FITC-conjugated Annexin V binding buffer and 10 µl PI were added. Immediately after gentle vortexing, the sample was analyzed by flow cytometry. Early apoptotic cells were characterized by high Annexin V binding and low PI staining, whereas late apoptotic and necrotic cells stained strongly with both Annexin V and PI.

Western blot analysis. Cells were lysed in iced lysis buffer (97.9% RIPA, 1% PMSF, 1% phosphatase inhibitors and 0.1% protease inhibitors). Total protein (50 µg/lane) was separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). Following incubation in blocking solution (5% nonfat milk), membranes were incubated overnight at 4˚C with primary antibodies against Bax, Bcl-2, caspase-3 and -9, eNOS, NF-κB, IκBα or β-actin. Membranes were then washed. β-actin was detected directly with enhanced chemiluminescence reagents. The other proteins of interest were detected with enhanced chemiluminescence reagents following incubation with horseradish peroxidase-conjugated secondary antibody (1:8,000 dilution) for 1 h. The relative density of each protein band was normalized to that of β-actin. All results were representative of at least three independent experiments.

Statistical analysis. Data are presented as the means ± SD, derived from at least three independent experiments. Statistical significance between groups was analyzed by one-way ANOVA followed by Tukey or Dunnett’s T3 test, as appropriate, using SPSS 15.0 software. A value of P<0.05 was considered to indicate statistically significant differences.

Results

Effect of nicorandil on hypoxia-induced loss of HPAEC viability. To determine whether hypoxia can reduce HPAEC viability, HPAECs were cultured under hypoxic conditions for 0, 6, 12, 24 or 36 h, and cell viability was analyzed using MTT. HPAEC viability following hypoxia for 6 or 12 h was not significantly different (P>0.05) to that of control cells not exposed to hypoxia (Fig. 1A). However, hypoxia for 24 or 36 h decreased HPAEC viability to 67.5±3.5 and 36.7±2.1%, respectively, of the control group (P<0.05) (Fig. 1A). Based on these data, subsequent experiments were carried out using hypoxia for 24 h, unless otherwise stated. Three different concentrations (100, 500 and 1,000 µM) of nicorandil improved cell viability. Nicorandil (100 µM) itself did not have a significant effect on cell viability (Fig. 1B). However, the protective effects of nicorandil against hypoxia-induced decreases in cell viability were blunted by 5-HD or L-NAME (Fig. 1C); under hypoxic conditions, the reduction in cell viability in the presence of 5-HD or L-NAME (combined with nicorandil) was significantly greater (P<0.05) than that in their absence (i.e., nicorandil alone). In addition, nicorandil, 5-HD or L-NAME alone was without effect on HPAEC viability under normoxic conditions (data not shown). These results demonstrated that nicorandil was able to protect HPAECs against decreased cell viability induced by hypoxia, whereas 5-HD or L-NAME was found to inhibit these beneficial effects of nicorandil.

Effect of nicorandil on hypoxia-induced apoptosis of HPAECs. To investigate whether the protective effect of nicorandil on hypoxia-induced cytotoxicity may be due to the attenuation of hypoxia-induced apoptosis, HPAEC apoptosis was determined using a Hoechst 33342 staining assay and flow cytometry.

The Hoechst 33342 staining assay demonstrated that exposure to hypoxia for 24 h increased the apoptotic ratio of cells, which showed a profile of cell shrinkage, chromatin condensation and fragmented fluorescent nuclei (Fig. 2). The number of Hoechst 33342-positive cells and the apoptotic ratio were significantly reduced by nicorandil (Fig. 2). However, the protective effects of nicorandil against hypoxia-induced cell apoptosis were blunted by 5-HD or L-NAME, as shown by the increase in the number of Hoechst 33342-positive cells and in the apoptotic ratio in the presence of 5-HD or L-NAME, compared with the hypoxia + nicorandil group (Fig. 2).

These findings were supported by the results of experiments using Annexin V and PI, analyzed by flow cytometry. Exposure to hypoxia for 24 h induced a significant increase in the number of apoptotic cells, as identified by flow cytometry, compared with normoxic culture (Fig. 3). Nicorandil markedly promoted cell survival. However, pretreatment with 5-HD or L-NAME abolished the anti-apoptotic effect of nicorandil in cultured HPAECs exposed to hypoxia.
Effect of nicorandil on activation of eNOS. To further investigate the mechanism underlying the anti-apoptotic effect of nicorandil, we examined eNOS protein expression in the various treatment groups by western blot analysis. Hypoxia decreased the expression of eNOS protein, compared with the control group, whereas nicorandil markedly increased the expression of eNOS compared with the hypoxia group (Fig. 4). However, the effects of nicorandil to enhance eNOS expression were suppressed by 5-HD or L-NAME (Fig. 4). By contrast, nicorandil, 5-HD and L-NAME alone had no influence on eNOS expression in HPAECs under normoxic conditions (data not shown).

Effect of nicorandil on inhibition of the NF-κB pathway. As NF-κB plays an important role in the regulation of cell death in some cell types, we examined its effects on hypoxia-induced HPAEC apoptosis. Hypoxia was found to increase expression of NF-κB protein compared to the control group (Fig. 5C), whereas pretreatment with PDTC markedlydownregulated the expression of NF-κB (Fig. 5C), inhibited hypoxia-induced cell apoptosis (Fig. 5A) and restored decreased HPAEC viability (Fig. 5B). By contrast, PDTC alone had no influence on HPAEC apoptosis and viability under normoxic conditions (data not shown). We next examined the effect of nicorandil on the regulation of the expression of NF-κB and IκBα (a protein that inhibits NF-κB), and found that nicorandil markedly decreased the expression of NF-κB and increased the expression of IκBα, compared with the hypoxia group. However, these effects of nicorandil were reduced by 5-HD or L-NAME (Fig. 6).
Effect of nicorandil on caspase-3 and -9 protein expression, and the Bax/Bcl-2 protein expression ratio. To explore the potential apoptotic signals involved in the protective effect of nicorandil on hypoxia-induced cell death, we examined protein expression levels of Bax, Bcl-2, caspase-3 and -9 in the various treatment groups, using western blot analysis. Hypoxia increased the Bax/Bcl-2 protein expression ratio, as well as caspase-3 and -9 protein levels, compared with the control group (Fig. 7). Nicorandil markedly decreased the Bax/Bcl-2 protein expression ratio as well as caspase-3 and -9 protein expression, compared with the hypoxia group (Fig. 7). However, these effects of nicorandil were reduced by 5-HD or L-NAME (Fig. 7).

**Discussion**

There are two major findings in the present study that expand our understanding of the mechanisms of action of nicorandil in HPAECs. First, we found that nicorandil protected HPAECs from hypoxia-induced apoptosis. Second, we demonstrated that the anti-apoptotic effect of nicorandil was through activation of mitoK<sub>ATP</sub> channels and increased eNOS expression, with subsequent inhibition of the NF-κB pathway and the mitochondrial apoptotic pathway.

HPAEC apoptosis has been proposed to be the initial step and the triggering event for PH, including hypoxic PH (HPH) (2-4), and chronic hypoxia is known to be an important
cause of EC apoptosis. Date et al (8) showed that nicorandil could inhibit serum starvation-induced apoptosis in vascular endothelial cells through activation of mitoK<sub>ATP</sub> channels, and a similar result was found in oxidative stress-induced apoptosis in neurons (9). In the present study, we demonstrated that nicorandil markedly inhibited the decreased HPAEC viability and increased HPAEC apoptosis induced by hypoxia. However, this beneficial effect of nicorandil was almost completely suppressed by pretreatment with 5-HD, a mitoK<sub>ATP</sub> channel inhibitor, indicating that the effect of nicorandil on HPAECs is mediated via activation of mitoK<sub>ATP</sub> channels.

eNOS, an important enzyme in the production of NO, is present in vascular endothelial cells, and a decreased expression level or abnormality of eNOS protein has been shown to cause EC apoptosis in the early stage of PH (including HPH), leading to the development of PH (13,29-31). For example, mutant mice lacking the eNOS gene, or newborn lambs treated with an eNOS inhibitor, were found to develop progressive elevation of pulmonary arterial pressure and resistance (30,31), and individuals with PH have been shown to have reduced levels of pulmonary arterial eNOS expression (13). Hongo et al (14) reported that, in an MCT-induced PH model, nicorandil was able to protect endothelial function and enhance eNOS expression, and a similar result was found in cardiac tissue (15). Furthermore, Grossini et al (16) showed that opening mitoK<sub>ATP</sub> channels could induce eNOS expres-
sion through Akt, ERK and p38 activation, and Roth et al (17) observed that ischemic preconditioning could open mitoK$_{\text{ATP}}$ channels and subsequently activate eNOS. In our study, we found that pretreatment with nicorandil could restore decreased expression of eNOS induced by hypoxia in HPAECs, while this effect could be inhibited by 5-HD and L-NAME, an eNOS inhibitor, indicating that the effect of nicorandil to enhance eNOS expression was via activation of mitoK$_{\text{ATP}}$.

NF-κB can be activated in a variety of cells in response to various factors, and can thereby regulate cell apoptosis (27,32,33). IκBα is the inhibitory protein of NF-κB, and degradation of IκBα results in NF-κB activation. Matsushita et al (32) showed that hypoxia-induced endothelial apoptosis was through NF-κB-mediated Bcl-2 suppression. Similarly, Chen et al (33) reported that HUVECs treated with intermittent high glucose showed activation of the NF-κB pathway, which inhibited cell proliferation and induced cell apoptosis. Furthermore, Huang et al (34) found that NF-κB played a pivotal role in disrupting PAEC membrane integrity in an MCT-induced PH model. Our present study is consistent with previous studies in showing that hypoxia upregulated the expression of NF-κB protein and increased apoptosis of HPAECs, while PDTC, an inhibitor of NF-κB, significantly reduced the enhanced apoptosis of HPAECs and the increased expression of NF-κB. There have also been some reports of an interaction between NF-κB and mitoK$_{\text{ATP}}$ channels. For example, Kawamura et al (22) reported that nicorandil could attenuate NF-κB activation via opening of mitoK$_{\text{ATP}}$ channels, and thereby reduce myocardial reperfusion injury. In addition, Revermann et al (23) demonstrated that activation of K$_{\text{ATP}}$ channels could inhibit NF-κB expression in HUVECs. However, whether nicorandil could inhibit the NF-κB pathway via activation of mitoK$_{\text{ATP}}$ was previously unknown. Our data indicated that nicorandil markedly decreased the protein expression of NF-κB, while increasing the expression of IκBα in HPAECs, and that this effect was diminished by 5-HD. This, therefore, provides the first evidence that nicorandil can inhibit the NF-κB pathway in HPAECs via activation of mitoK$_{\text{ATP}}$.

We therefore further explored the link between eNOS and NF-κB. A study on HPAECs found that NO could inhibit hyperoxia-induced NF-κB activation (25). Moreover, Greco et al (35) reported that inhibiting the expression of eNOS was associated with enhanced NF-κB activation and a significant increase in infarct volume in a cerebral ischemia/reperfusion rat model. A recent study reported that elevated cardiac NO levels following eNOS gene transfer in mice led to a decrease in nuclear translocation of NF-κB in a model of myocardial ischemia (24). Our study is the first to show that the effects of nicorandil to inhibit the NF-κB pathway could be diminished by L-NAME in HPAECs, indicating a relationship between eNOS and NF-κB in these cells, such that upregulation of eNOS expression could inhibit the NF-κB pathway.

The apoptotic signals involved in the protective effect of nicorandil on hypoxia-induced HPAEC death were also investigated in our study. We had already shown that nicorandil could act as a mitoK$_{\text{ATP}}$ channel opener of HPAECs induced by hypoxia, this result indicated that nicorandil may exert an influence on the mitochondrial apoptotic pathway. There are at least two broad pathways that lead to apoptosis, an ‘extrinsic’ and an ‘intrinsic’ pathway, with apoptosis in mitochondria being the best known intrinsic apoptotic pathway. Several different causes, including hypoxia, reduce the expression ratio of Bcl-2/Bax (which is an important sign of mitochondrial cell apoptosis), leading to the loss of mitochondrial inner membrane potential (Δψm) and the triggering of a release of mitochondrial cytochrome c into the cytosol. There is also activation and upregulation of caspase-9 and -3, and eventually induction of cell apoptosis. Opening mitoK$_{\text{ATP}}$ channels can increase Δψm (36), maintain the expression ratio of Bcl-2/Bax (11) and inhibit cell apoptosis. In the present study, we found that nicorandil could inhibit the increased Bax/Bcl-2 expression ratio, and the enhanced caspase-3 and -9 expression, induced by hypoxia in HPAECs. This effect of nicorandil could be diminished both by 5-HD and L-NAME, indicating that the anti-apoptotic effect of nicorandil was through inhibition of the mitochondrial apoptotic pathway, and that this action involved an increase in the expression of eNOS via activation of the mitoK$_{\text{ATP}}$ channel.

There are some limitations to the present study. First, whether it is nicorandil’s action as an NO donor that is involved in the protection of HPAECs against apoptosis remains unknown. In the current study, the protective effect of nicorandil could be almost completely inhibited by 5-HD. Furthermore, Harada et al (37) showed that nicorandil, acting as an NO donor, stimulated PKC-δ and then opened mitoK$_{\text{ATP}}$ channels. This leads us to the hypothesis that the nitrate-like effect of nicorandil is indeed involved in the protection against HPAEC apoptosis, although opening of the mitoK$_{\text{ATP}}$ channel also contributes to it. Second, our study has shown that eNOS could mediate enhanced expression of NF-κB protein; however, whether there is feedback between eNOS and NF-κB is not known, and further studies are required to clearly define the relationship between eNOS and NF-κB.

In summary, the present study shows a working hypothesis that nicorandil suppresses hypoxia-induced apoptosis of cultured HPAECs through activation of mitoK$_{\text{ATP}}$ and increased eNOS expression, which subsequently acts to inhibit the NF-κB pathway and the mitochondrial apoptotic pathway (Fig. 8). This provides strong evidence that nicorandil may be useful as an alternative drug for the treatment of HPH.
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