Rho-Kinase Mediates Hypoxia-Induced Downregulation of Endothelial Nitric Oxide Synthase

Masao Takemoto, MD, PhD; Jianxin Sun, MD, PhD; Junko Hiroki, MD; Hiroaki Shimokawa, MD, PhD; James K. Liao, MD

Background—Hypoxia-induced pulmonary hypertension is a major cause of morbidity and mortality. Hypoxia induces pulmonary vasoconstriction, in part, by decreasing endothelial nitric oxide synthase (eNOS) expression. The mechanism by which hypoxia decreases eNOS expression is not known but may involve Rho-kinase–induced actin cytoskeletal changes in vascular endothelial cells.

Methods and Results—To determine whether hypoxia regulates eNOS expression through Rho-kinase, we exposed human saphenous and pulmonary artery endothelial cells to hypoxia (3% O₂) with and without a Rho-kinase inhibitor, hydroxyfasudil (0.1 to 100 μmol/L), for various durations (0 to 48 hours). Hypoxia increased Rho-kinase expression and activity by 50% and 74%, decreased eNOS mRNA and protein expression by 66% and 57%, and inhibited eNOS activity by 48% ± 9%. All of these effects of hypoxia on eNOS were reversed by cotreatment with hydroxyfasudil. Furthermore, inhibition of Rho by Clostridium botulinum C3 transferase or Rho-kinase by overexpression of dominant-negative Rho-kinase reversed hypoxia-induced decrease in eNOS expression. Indeed, disruption of the actin cytoskeleton, the downstream target of Rho-kinase, by cytochalasin D also upregulated eNOS expression. Hypoxia reduced eNOS mRNA half-life from 22 ± 2 to 13 ± 2 hours, which was reversed by cotreatment with hydroxyfasudil. However, neither hypoxia nor hydroxyfasudil had any effects on eNOS gene transcription.

Conclusions—These results indicate that hypoxia-induced decrease in eNOS expression is mediated by Rho-kinase and suggest that Rho-kinase inhibitors may have therapeutic benefits in patients with hypoxia-induced pulmonary hypertension. (Circulation. 2002;106:57-62.)

Key Words: endothelium • hypoxia • hypertension • nitric oxide

Pulmonary hypertension is a major cause of heart failure in individuals exposed to hypoxic conditions.¹ Reduced endothelium-derived nitric oxide (NO) production in pulmonary arterial vessels has been implicated in the pathophysiology of pulmonary hypertension.²,³ For example, individuals with pulmonary hypertension demonstrate reduced levels of pulmonary arterial endothelial NO synthase (eNOS) expression¹ and benefit clinically from inhalation NO therapy.⁴,⁵ Indeed, mutant mice lacking the eNOS gene or newborn lambs treated with the eNOS inhibitor N⁵-monomethyl-L-arginine (LNMA) developed progressive elevation of pulmonary arterial pressures and resistance.⁶,⁷

We have previously shown that HMG-CoA reductase inhibitors or statins increase eNOS expression by cholesterol-independent mechanism involving inhibition of Rhoglynylerganylation.⁸ Rho regulates myosin light chain (MLC) phosphorylation through one of its downstream targets, Rho-kinase, which inhibits MLC phosphatase.⁹ Increased phosphorylation of MLC leads to stress fiber formation, reorganization of the actin cytoskeleton, and induction in gene expression.¹⁰ Indeed, eNOS expression is regulated by changes in the actin cytoskeleton.¹¹ Although statins prevent a decrease in eNOS expression under hypoxic conditions,¹² the mechanism by which hypoxia downregulates eNOS expression is not known. The purpose of this study, therefore, was to determine whether Rho-kinase and its effect on the actin cytoskeleton mediate hypoxia-induced changes in eNOS expression.

Methods

Materials
All tissue culture reagents were purchased form Life Technologies Gibco BRL. 5,6-Dichlorobenzimidazole riboside (DRB), Triton X-100, and TRITC-labeled phallolidin were purchased form Sigma Chemical Corp. 2,3-Diaminonaphthalene was purchased from Molecular Probes, Inc. [α-³²P]CTP (3000 Ci/mmol) was purchased from New England Nuclear Life Science Products. LNMA, 4,5-diaminofluorescein diacetate (DAF), cytochalasin D, and eNOS assay kit were obtained from Calbiochem. Clostridium botulinum C3
transfase (C3 TF) was obtained from List Biochemical Laboratory, Inc. The rabbit polyclonal antibody recognized Thr(558) phosphorylation of ezrin and radixin and Thr(564) phosphorylation of moesin.\textsuperscript{13} The specificity of the Rho-kinase antibody has been described.\textsuperscript{14} The antibody detection kit (enhanced chemiluminescence) and the nylon and polyvinylidene difluoride transfer membranes were purchased from Amersham Corp. The Rho-kinase inhibitor hydroxyfasudil was a gift from Ashai-Kasei Corp.

**Cell Culture**

Human saphenous vein endothelial cells of less than 4 passages were cultured as described.\textsuperscript{12,13} In some experiments, cells were pretreated for 3 hours with DRB (50 \( \mu \)M/L), hydroxyfasudil (0.1 to 100 \( \mu \)M/L), cytochalasin D (0.25 \( \mu \)M/L), or \textit{C botulinum} C3 TF (25 \( \mu \)g/mL). For transfection studies, bovine aortic and pulmonary endothelial cells of less than three passages at 70% to 80% confluence were used. Cellular viability was determined by cell count, morphology, and trypan blue exclusion.

**Exposure to Hypoxia**

Saphenous vein, aortic, or pulmonary endothelial cells grown in 100- or 35-mm culture dishes were treated with hydroxyfasudil and placed without culture dish covers in humidified airtight chambers (Billups-Rothenberg). The chambers were gassed with 20% or 3% O\(_2\), 5% CO\(_2\), and balanced nitrogen for 10 minutes before the chambers were sealed. The chambers were maintained in a 37°C incubator for 0 to 48 hours and found to have <2% variation in O\(_2\) concentration.\textsuperscript{12,16}

Western blotting proteins were separated on SDS/polyacrylamide gel electrophoresis as described.\textsuperscript{12,16} Immunoblotting was performed with the use of antibodies to Rho-kinase, phosphorylated ezrin-radixin-moesin, and a murine monoclonal antibody to human eNOS (1:4000 dilution; Transduction Laboratories). Immunodetection was performed with the use of a goat anti-mouse secondary antibody (1:4000 dilution; Jackson ImmunoResearch) and an enhanced chemiluminescence detection kit (Amersham Corp).

**Northern Blotting**

Equal amounts of total RNA (15 \( \mu \)g) were separated by 1% formaldehyde-agarose gel electrophoresis, and hybridization and washing were performed as described.\textsuperscript{12} The full-length human endothelial eNOS DNA was labeled with random hexamer priming, \([\alpha-\text{P}]/H\text{-arginine to }[\text{H}]/L\text{-citrulline.}^{13}

**eNOS Activity Assay**

Endothelial cells grown in phenol-free medium were exposed to either 20% or 3% O\(_2\) under the indicated conditions. After 24 hours, cells were washed twice with PBS at 4°C and homogenated with 20 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1% Brij 96, and 5 mmol/L MgCl\(_2\). The eNOS activity was measured by conversion of \([\text{H}]/L\text{-arginine to }[\text{H}]/L\text{-citrulline.}^{13}

**Measurements of NO Production**

NO production was determined with the use of 4,5-DAF-2DA in DMSO.\textsuperscript{17} Nitrite accumulation was determined with the use of 2,3-diaminonaphthalene (1.5 mmol/L DANN in 1 mol/L HCl).\textsuperscript{18} The fluorescence of 1-(H)-naphthotriazole was measured with excitation and emission wavelengths of 365 and 450 nm, respectively. Standard curves were constructed with known amounts of sodium nitrite. Nonspecific fluorescence was determined in the presence of L-NMMA (5 mmol/L).

**Overexpression of Dominant-Negative Rho-Kinase**

The specificity of adenovirus containing dominant-negative Rho-kinase (Ad-DN-Rho-kinase) for inhibiting Rho-kinase has been demonstrated previously.\textsuperscript{13} Bovine pulmonary endothelial cells were infected at 80% confluence with the indicated recombinant adenoviruses (Ad-LacZ or Ad-DN-Rho-kinase) at a multiplicity of infection of 200. Approximately 24 hours later, cells were either exposed to normoxia (20% O\(_2\)) or hypoxia (3% O\(_2\)) for 24 hours before Western blotting for eNOS expression. Preliminary studies with the Ad-LacZ reporter showed that the transfection efficiency was >95%.

**Transfection Assay**

Bovine endothelial cells (70% confluent) were transfected with the use of SuperFect Transfection Reagent (Qiagen Inc.), with 6 \( \mu \)g of the indicated cDNA constructs: the empty vector (pcDNA3) or a [-1.8 kb] eNOS promoter linked to the luciferase reporter gene.\textsuperscript{13} As a control for transfection efficiency, 2 \( \mu \)g of pcMV-\( \beta \)-gal plasmid was cotransfected. Preliminary results using \( \beta \)-galactosidase staining indicate that cellular transfection efficiency was ~14%. For luciferase activity, cells were harvested 48 hours after transfection and treated with the indicated conditions. The \( \beta \)-galactosidase and luciferase activities were determined by a chemiluminescence assay (Dual-light, Tropix) with the use of Berthold L9501 luminometer. The eNOS promoter activity was expressed as the ratio of luciferase to \( \beta \)-galactosidase activity.

**Statistical Analyses**

Band intensities from Northern and Western blots were quantified densitometrically by the National Institutes of Health Image Program.\textsuperscript{14} Results are expressed as mean \pm SEM. All data were analyzed by means of 1 or 2-way ANOVA and Fisher’s exact test for post hoc analyses. A value of \( P<0.05 \) was considered statistically significant.

**Results**

**Regulation of eNOS Expression by Hypoxia and Rho-Kinase**

In a concentration-dependent manner, treatment with hydroxyfasudil (1, 10, and 100 \( \mu \)mol/L, 24 hours) increased eNOS protein expression by 165 ± 13%, 204 ± 15%, and 291 ± 23% under normoxic conditions (20% O\(_2\)) (\( n=3, P<0.05 \)) (Figure 1A). The eNOS protein level was significantly increased after 12 hours of treatment with hydroxyfasudil (10 \( \mu \)mol/L) under normoxic condition and reached a 2-fold maximal increase after 36 hours (Figure 1B). Compared with normoxia, exposure to hypoxia (3% O\(_2\), 24 hours) caused a 57 ± 5% decrease in eNOS protein level (\( n=3, P<0.01 \)). Cotreatment with hydroxyfasudil (10 or 100 \( \mu \)mol/L, 24 hours) not only reversed hypoxia-induced down-regulation of eNOS protein expression but increased eNOS protein expression above basal levels (150 ± 3% and 261 ± 16%, respectively, \( n=3, P<0.01 \) compared with hypoxia alone) (Figure 2A). In addition, cotreatment with the actin cytoskeleton disrupter cytochalasin D (Cyto D, 0.25 \( \mu \)mol/L) or \textit{C botulinum} C3 TF (25 \( \mu \)g/mL) also reversed hypoxia-mediated decrease in eNOS protein expression (127 ± 5% and 103 ± 9%, respectively, \( n=3, P<0.01 \) compared with hypoxia alone) (Figure 2B).

To determine whether changes in eNOS protein levels correlated with changes in eNOS steady-state mRNA levels, we performed Northern blotting on endothelial cells exposed to normoxic and hypoxic conditions with and without hydroxyfasudil (10 \( \mu \)mol/L). Treatment with hydroxyfasudil (10 \( \mu \)mol/L) increased eNOS mRNA levels (153 ± 7%, \( n=5, P<0.05 \)) under normoxic conditions. Exposure of endothelial cells to hypoxia for 24 hours reduced eNOS mRNA levels by 66 ± 2% (\( n=5, P<0.01 \) compared with normoxia) (Figure 3A). Treatment with hydroxyfasudil (10 \( \mu \)mol/L) completely reversed hypoxia-induced the downregulation of eNOS mRNA (115 ± 7%, \( n=5, P<0.05 \) compared with hypoxia.
alone). Furthermore, treatment with Cytochalasin D (0.25 μmol/L) or C3 TF (25 μg/mL) also completely reversed the decrease in eNOS expression under hypoxic conditions (172±2% and 106±7%, respectively, n=3, P<0.05 compared with hypoxia alone) (Figure 3B).

**Activation of Rho-Kinase by Hypoxia Mediates Downregulation of eNOS**

Treatment of bovine pulmonary artery endothelial cells with hydroxyfasudil (10 μmol/L) did not affect basal Rho-kinase expression. However, exposure to hypoxia increased Rho-kinase expression by 50±7% (P<0.05 compared with control, n=4) (Figure 4A). Cotreatment with hydroxyfasudil inhibited hypoxia-induced increase in Rho-kinase expression. These findings suggest that hypoxia-induced Rho-kinase expression is in part mediated by increases in Rho-kinase activity. Indeed, hypoxia increased Rho-kinase activity by 74±6% (P<0.05 compared with control, n=3), which was completely inhibited by cotreatment with hydroxyfasudil (Figure 4B).

When pulmonary endothelial cells were infected with an adenovirus containing dominant-negative Rho-kinase (Ad-DN-Rho-kinase) under normoxic conditions, eNOS protein expression was modestly increased by 40% to 45% compared with infection with an adenovirus containing LacZ (Figure 4C). Under hypoxic conditions, infection with Ad-DN-Rho-kinase or treatment with hydroxyfasudil prevented hypoxia-induced downregulation of eNOS protein expression in pulmonary artery endothelial cells. These findings indicate that Rho-kinase activity and eNOS expression are inversely related and that Rho-kinase mediates hypoxia-induced downregulation of eNOS expression.

**Figure 1.** Western blots (15 μg of protein/lane) and corresponding densitometric analyses (relative intensity) showing concentration-dependent (A) and time-dependent (B) effects of hydroxyfasudil (HFD, 10 μmol/L) on eNOS protein levels under normoxic conditions (20% O₂) in saphenous vein endothelial cells. *P<0.05 vs basal or untreated condition.

**Figure 2.** Western blots (15 μg of protein/lane) and corresponding densitometric analyses (relative intensity) showing concentration-dependent effects of hydroxyfasudil (HFD, 0.1 to 100 μmol/L) (A) and effects of cytochalasin D (CytoD, 0.25 μmol/L) or Clostridium botulinum C3 TF (25 μg/mL) (B) on eNOS protein levels under hypoxic conditions (3% O₂) in saphenous vein endothelial cells. N indicates Normoxia; H, hypoxia; and HFD, hydroxyfasudil. *P<0.05 vs normoxic condition. †P<0.01 vs hypoxic condition.
Effects of Rho-kinase Inhibition on eNOS Activity

Under normoxic conditions, treatment with hydroxyfasudil (10 μmol/L) increased eNOS activity as determined by arginine to citrulline conversion and DAF-2 fluorescence by 1.7-fold and 1.4-fold, respectively (n=5, P<0.05) (Figure 5A). Exposure of endothelial cells to hypoxia for 24 hours decreased citrulline conversion and DAF-2 fluorescence by 48±9% and 63±6%, respectively (n=5, P<0.05). Treatment with hydroxyfasudil (10 μmol/L, 24 hours) not only reversed the hypoxia-induced decrease in citrulline conversion and DAF-2 fluorescence but resulted in 2.9-fold and 3.5-fold increases in these parameters (n=5, P<0.05 compared with hypoxia alone).

The NO produced by eNOS was assessed by measuring the LNMA-inhibitable nitrite accumulation from human endothelial cells. The ratio of nitrite to nitrate production under our conditions was ≈5:1 and was similar for hypoxic and normoxic conditions (data not shown). Basal nitrite production at 20% O2 was 0.95±0.14 nmol/500 000 cells per 24 hours. Exposure of endothelial cells to hypoxia for 24 hours decreased nitrite production by 74±10% (0.25±0.08 nmol/500 000 cells per 24 hours, n=8, P<0.05) (Figure 5B). In a concentration-dependent manner, treatment with hydroxyfasudil (1, 10, and 100 μmol/L, 24 hours) reversed hypoxia-induced decrease in nitrite production (1.08±0.12, 1.86±0.55, and 3.87±0.30 nmol/500 000 cells per 24 hours, respectively, n=6, P<0.05). Treatment with hydroxyfasudil (10 μmol/L) also increased nitrite production by 2.5-fold under normoxic conditions (0.95±0.14 to 2.4±0.6 nmol/500 000 cells per 24 hours, n=6, P<0.05).

Effects of Rho-Kinase Inhibition on eNOS Gene Transcription and mRNA Stability

To determine whether hydroxyfasudil affects eNOS gene transcription, bovine aortic endothelial cells were transfected with a functional eNOS promoter linked to the luciferase gene. This promoter construct contains putative cis-acting elements for activator protein-1 and activator protein-2, sterol regulatory element-1, retinoblastoma control element, shear stress response element, nuclear factor-1, and cAMP response element. Laminar flow, which is known to increase eNOS gene transcription, was produced in a parallel chamber and served as positive control. Exposure of transfected endothelial cells to laminar flow (12 dyne/cm²) produced an 8.2-fold increase in eNOS promoter activity (n=6, P<0.01). However, neither hypoxia nor hydroxyfasudil (10 μmol/L) stimulated eNOS promoter activity (n=6, P>0.05) (Figure 6A).

To determine whether the increase in eNOS expression was due to posttranscriptional mechanisms, endothelial cells were treated with hydroxyfasudil in the presence of the mRNA synthase inhibitor DRB. Hypoxia decreased eNOS mRNA half-life from 22±2 hours to 13±2 hours (n=4, P<0.05) (Figure 6B). Treatment with hydroxyfasudil (10 μmol/L) increased eNOS mRNA half-life to 28±8 hours and 26±4 hours under normoxic and hypoxic conditions, respectively (n=3, P<0.05 for hypoxic condition). These results suggest that Rho-kinase mediates hypoxia-induced eNOS mRNA destabilization.

Discussion

We have shown that hypoxia increases Rho-kinase expression and activity and that Rho-kinase mediates hypoxia-induced downregulation of eNOS expression. The mechanism by which Rho-kinase inhibits eNOS expression was not due to decreases in eNOS gene transcription but to the destabilization of eNOS mRNA. In contrast to previous studies, we did not find that hypoxia decreased eNOS gene transcription. This discrepancy may be due to differences between transfection with a limited eNOS promoter compared with nuclear run-on assay and to evaluating eNOS expression in partially confluent compared with confluent endothelial cells. Indeed, eNOS expression and activity are higher during cell growth. Nevertheless, our results are similar to the effects of statins on eNOS expression and suggest that Rho-kinase mediates the downstream effects of statins on eNOS expression during hypoxia. Thus, it is interesting to speculate that some of the cholesterol-
independent effects of statins may be mediated through inhibition of Rho-kinase.

Our findings are consistent with the results of a previous study showing that another Rho-kinase inhibitor, Y27632, also prevented thrombin-induced downregulation of eNOS expression. However, in contrast to Y27632, which did not affect basal eNOS expression, we found that treatment with hydroxyfasudil alone increased basal eNOS expression without apparently affecting basal Rho-kinase activity. These findings suggest that hydroxyfasudil may have additional Rho-kinase–independent effects on eNOS expression. Indeed, treatment with hydroxyfasudil alone did not induce eNOS promoter activity nor prolong eNOS mRNA half-life, suggesting an additional posttranslational mechanism of eNOS upregulation, which is independent of Rho-kinase.

There is growing evidence implicating the involvement of Rho-kinase in cardiovascular diseases. The activation of the Rho-Rho-kinase pathway leads to the phosphorylation of MLC, which regulates smooth muscle cell contraction. Fasudil and hydroxyfasudil are cell-permeable and potently inhibit Rho-kinase and MLC phosphorylation in vascular smooth muscle cells. The phosphorylation of MLC is required for its interaction with actin filaments, forming actin stress fibers and focal adhesion complexes. Indeed, inhibition of Rho-kinase by hydroxyfasudil enhances cerebral blood flow and prevents cerebral vasospasm after subarachnoid hemorrhage. Our finding with cytochalasin D, however, suggests that Rho-kinase–induced actin cytoskeletal reorganization mediates hypoxia-induced downregulation of eNOS expression. Thus, hypoxia-induced vasoconstriction may not only be due to Rho-kinase–induced vascular smooth muscle contraction but to Rho-kinase–induced decrease in eNOS activity in endothelial cells.

The effect of hypoxia on eNOS expression remains somewhat controversial. Some animal studies demonstrate that NO production is increased in hypoxia-induced pulmonary hypertension, whereas human studies indicate that eNOS expression and activity are substantially reduced. These discrepancies may be due in part to the different causes and stages in the development of pulmonary hypertension and to differences between in vitro and in vivo models. For example, pulmonary hypertension in humans is typically diagnosed at a later stage of the disease, whereas animal studies have mainly focus on earlier stages. It is possible that counterregulatory mechanisms during early stages of hypoxia could prevent the downregulation of eNOS. With prolonged hypoxia and elevated pulmonary pressures, endothelial dysfunc-
tion and changes in eNOS expression become evident. Indeed, eNOS-deficient mice have progressive pulmonary hypertension and right ventricular hypertrophy after chronic hypoxia.6,27 In contrast, mice overexpressing eNOS are protected from developing hypoxia-induced pulmonary hypertension and vascular remodeling,28 and inhalation NO therapy is clinically effective in lowering pulmonary arterial pressure.3 Thus, agents such as statins or Rho-kinase inhibitors, which upregulate eNOS activity, may have therapeutic benefits in pulmonary hypertension. Supporting data from large clinical trials, however, are needed before such recommendations can be made.

Acknowledgments

This work was supported by National Institutes of Health grants HL-52233, HL-70274, and HL-62602 and the American Heart Association Bugher Foundation Award. Dr Liao is an Established Investigator of the American Heart Association. Dr Takemoto is a recipient of the Banyu-Merck Fellowship in Lipid Metabolism and Atherosclerosis.

References

Rho-Kinase Mediates Hypoxia-Induced Downregulation of Endothelial Nitric Oxide Synthase
Masao Takemoto, Jianxin Sun, Junko Hiroki, Hiroaki Shimokawa and James K. Liao

Circulation. 2002;106:57-62; originally published online June 17, 2002;
doi: 10.1161/01.CIR.0000020682.73694.AB
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/106/1/57

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/