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

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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 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 Nω-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 Rho geranylgeranylation.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 phalloidin were purchased form Sigma Chemical Corp. 2,3-Diaminonaphthalene was purchased from Molecular Probes, Inc. [γ-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

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57
transf erase (C3 TF) was obtained from List Biochemical Laborato-
ries, Inc. The rabbit polyclonal antibody recognized Thr(558) phos-
phorylation of eznin and radixin and Thr(564) phosphorylation of
moesin. The specificity of the Rho-kinase antibody has been
described. The antibody detection kit (enhanced chemilumines-
cence) and the nylon and polyvinylidene difluoride transfer mem-
branes were purchased from Amersham Corp. The Rho-kinase
inhibitor hydroxyfasudil was a gift from Asahi-Kasei Corp.

Cell Culture
Human saphenous vein endothelial cells of less than 4 passages were
 cultured as described. In some experiments, cells were pretreated
 for 5 hours with DBB (50 μM/L), hydroxyfasudil (0.1 to 100
μM/L), cytochalasin D (0.25 μM/L), or C botulinum C3 TF (25
μG/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% O2, 5%
CO2, 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 O2 concentration.
Western blotting proteins were separated on SDS/polyacrylamide
gel electrophoresis as described. Immunoblotting was performed
with the use of antibodies to Rho-kinase, phosphorylated eznin-
radixin-moesin, and a murine monoclonal antibody to human eNOS
Thr(558) phosphorylation of ezrin and radixin and Thr(564) phosphorylation of
moesin. The specificity of the Rho-kinase antibody has been
described. The antibody detection kit (enhanced chemilumines-
cence) and the nylon and polyvinylidene difluoride transfer mem-
branes were purchased from Amersham Corp. The Rho-kinase
inhibitor hydroxyfasudil was a gift from Asahi-Kasei Corp.

Northern Blotting
Equal amounts of total RNA (15 μg) were separated by 1%
formaldehyde-agarose gel electrophoresis, and hybridization and
washing were performed as described. The full-length human
endothelial eNOS DNA was labeled with random hexamer priming,
[α-32P] and Klenow (Pharmacia).

eNOS Activity Assay
Endothelial cells grown in phenol-free medium were exposed to
either 20% or 3% O2 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 MgCl2. The eNOS activity was measured by conversion of
[3H]-arginine to [3H]-citrulline.

Measurements of NO Production
NO production was determined with the use of 4,5-DAF-2DA in
DMSO. Nitrite accumulation was determined with the use of
2,3-diaminonaphthalene (1.5 mmol/L DANT in 1 mol/L HCl). 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 LNMA
(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. Bovine pulmonary endothelial cells were
infected at 80% confluence with the indicated recombinant aden-

oviruses (Ad-LacZ or Ad-DN-Rho-kinase) at a multiplicity of infec-
tion of 200. Approximately 24 hours later, cells were either exposed
to normoxia (20% O2) or hypoxia (3% O2) 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 μg of
the indicated cDNA constructs: the empty vector (pcDNA3) or a
[−1.8 kb] eNOS promoter linked to the luciferase reporter gene.
As a control for transfection efficiency, 2 μg of pmCV β-gal
plasmid was cotransfected. Preliminary results using β-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 β-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
β-galactosidase activity.

Statistical Analyses
Band intensities from Northern and Western blots were quantitated
densitometrically by the National Institutes of Health Image Pro-
gram. Results are expressed as mean±SEM. All data were ana-
lyzed 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 hy-
droxyfasudil (1, 10, and 100 μmol/L, 24 hours) increased
eNOS protein expression by 165±13%, 204±15%, and
291±23% under normoxic conditions (20% O2) (n=3,
P<0.05) (Figure 1A). The eNOS protein level was signifi-
cantly increased after 12 hours of treatment with hydroxyfas-
duil (10 μmol/L) under normoxic condition and reached a
2-fold maximal increase after 36 hours (Figure 1B). Com-
pared with normoxia, exposure to hypoxia (3% O2, 24 hours)
caused a 57±5% decrease in eNOS protein level (n=3,
P<0.01). Cotreatment with hydroxyfasudil (10 or 100
μmol/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 hypox-
ia alone) (Figure 2A). In addition, cotreatment with the
actin cytoskeleton disrupter cytochalasin D (Cyto D, 0.25
μmol/L) or C botulinum C3 TF (25 μg/mL) also reversed
hypoxia-mediated decrease in eNOS protein expression
(127±5% and 103±9%, respectively, n=3, P<0.01 com-
pared 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 hy-
droxyfasudil (10 μmol/L). Treatment with hydroxyfasudil
(10 μmol/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 μmol/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.
**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.16 The ratio of nitrite to nitrate production under our culture 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 functional15 eNOS promoter linked to the luciferase gene.15 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 dyn/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.16 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.19 Nevertheless, our results are similar to the effects of statins on eNOS expression9,12 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.20 However, in contrast to Y27632, which did not affect basal eNOS expression,20 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.21 The activation of the Rho-Rho-kinase pathway leads to the phosphorylation of MLC,9,21 which regulates smooth muscle cell contraction.10 Fasudil and hydroxyfasudil are cell-permeable and potently inhibit Rho-kinase and MLC phosphorylation in vascular smooth muscle cells.14,22 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 flow23 and prevents cerebral vasospasm after subarachnoid hemorrhage.24 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,25,26 whereas human studies indicate that eNOS expression and activity are substantially reduced.3 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-

Figure 4. Western blots (15 μg of protein/lane) showing effects of hypoxia with and without hydroxyfasudil (HFD, 10 μmol/L) on Rho-kinase expression (A) and activity (B) in bovine pulmonary artery endothelial cells. Rho-kinase activity was determined by the degree of ezrin-radixin-moesin phosphorylation. *P<0.05 vs control condition. C, Western blots (15 μg of protein/lane) of eNOS protein expression from bovine pulmonary endothelial cells that were either treated with 10 μmol/L HFD or infected with Ad-DN-Rho-kinase or Ad-LacZ under normoxic or hypoxic conditions.

Figure 5. A, Effects of hypoxia (3% O2) alone or in combination with hydroxyfasudil (HFD, 10 μmol/L) on eNOS activity as measured by arginine to citrulline conversion (citrulline, □) and DAF-2 fluorescence (DAF-2, ■) in human saphenous vein endothelial cells. *P<0.05 vs basal condition. †P<0.05 vs hypoxic condition. B, Effects of hypoxia (3% O2) alone or in combination with 0.1 to 100 μmol/L HFD on NO production as measured by LNMA-inhibitable nitrite production. *P<0.05 vs basal condition.

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tion and changes in eNOS expression become evident. Indeed, eNOS-deficient mice have progressive pulmonary hypertension and right ventricular hypertrophy after chronic hypoxia. In contrast, mice overexpressing eNOS are protected from developing hypoxia-induced pulmonary hypertension and vascular remodeling, and inhalation NO therapy is clinically effective in lowering pulmonary arterial pressure. 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.

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