Nitric Oxide Induces Dilation of Rat Aorta via Inhibition of Rho-Kinase Signaling

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Abstract—NO induces vasodilation through cGMP-dependent protein kinase–dependent and –independent mechanisms. A recent study demonstrated that recombinant cGMP-dependent protein kinase can phosphorylate the small G protein, RhoA, thus inhibiting its activity. Additionally, sodium nitroprusside was found to reverse the phenylephrine-induced translocation of RhoA, which is further indicative of the inhibition of RhoA activity. RhoA is known to be involved in the Ca^{2+} sensitization of vascular smooth muscle through the actions of one of its downstream effectors, Rho-kinase. This study examined whether NO endogenously induces the relaxation of intact rat aorta via the inhibition of the Rho-kinase–mediated Ca^{2+}-sensitizing pathway. Endogenous Rho-kinase inhibitor activity was inhibited by the selective compound Y-27632. Treatment of endothelium-intact rat aorta with Y-27632 (1 μmol/L) resulted in an attenuation of maximal force generated in response to phenylephrine. In endothelium-denuded rings, however, 1 μmol/L Y-27632 was ineffective at inhibiting the phenylephrine-induced contraction. Additionally, 1 μmol/L Y-27632 was significantly less effective at inhibiting the phenylephrine-induced contraction of endothelium-intact rings in the presence of inhibitors of NO synthase or guanylate cyclase (N^{G}-nitro-L-arginine and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, respectively). Interestingly, sodium nitroprusside restored the ability of 1 μmol/L Y-27632 to attenuate phenylephrine-induced contraction. Rho-kinase inhibition was also found to increase the sensitivity of the endothelium-denuded aorta to sodium nitroprusside. These data demonstrate that NO inhibits Rho-kinase activity in the intact rat aorta, supporting the hypothesis that endogenous NO-mediated vasodilation occurs through the inhibition of Rho-kinase constrictor activity in the intact rat aorta. (Hypertension. 2002;39[part 2]:438-442.)

Key Words: muscle, smooth, vascular □ vasodilation □ kinase □ nitric oxide

The potent vasodilator NO is produced in the vasculature through the conversion of L-arginine to citrulline by 1 of 3 isoforms of NO synthase. NO (largely produced in endothelial cells in response to an increase in intracellular Ca^{2+}) binds to smooth muscle cell soluble guanylate cyclase, leading to an increase in cGMP and the subsequent activation of cGMP-dependent protein kinase (cGK). NO signaling has been proposed to lead to the inhibition of L-type Ca^{2+} channels, the activation of Ca^{2+}-dependent K+ channels, the promotion of plasma membrane Ca^{2+}-ATPase and Na+−Ca^{2+} exchanger activity, and the activation of sarcoplasmic reticulum Ca^{2+}-ATPases (either independent of, or directly through, cGMP/cGK signaling).1-3 cGK activation has also been found to decrease myosin light chain (MLC) phosphorylation via the telokin-mediated stimulation of MLC phosphatase.4

Recently, studies by Sauzeau et al5 and others have demonstrated the inhibition of the contraction-promoting G protein, RhoA, by NO/cGK. Recombinant RhoA was phosphorylated by cGK at Ser188, resulting in the inhibition of RhoA stress fiber formation.5,6 Additionally, sodium nitroprusside (SNP) and constitutively active cGK were demonstrated to inhibit the phenylephrine (PE)-induced or lysophosphatic acid–induced translocation of RhoA from the cytosolic to the membrane fraction in rat aortas and NIH3T3 cells, respectively, indicative of the NO/cGK-mediated inactivation of RhoA.5,6

On activation, RhoA stimulates a variety of downstream targets, including Rho-kinase, a serine/threonine kinase. The activation of RhoA, a low-molecular-weight G protein, is marked by GTP binding, the translocation of RhoA from the cytosol to the membrane, and the posttranslational addition of a geranylgeranyl phosphate onto RhoA.7-10 Numerous studies have demonstrated a role for RhoA/Rho-kinase activity in the contraction of vascular smooth muscle.7-9,11,12 Rho-kinase has been shown to phosphorylate the myosin-binding subunit of MLC phosphatase, leading to the inhibition of phosphatase activity.7-9,13 Rho-kinase has also been shown, in vitro, to phosphorylate MLC directly; however, this has yet to be demonstrated in vivo.14 The Rho-kinase–mediated inhibition of MLC phosphatase leads to the maintenance of the phosphorylated state of MLC, promoting vascular smooth muscle contraction. Numerous studies have demonstrated that the inhibition of RhoA/Rho-kinase–mediated Ca^{2+} sensitization...
induces the relaxation of vascular smooth muscle.\textsuperscript{7–9,12} It is tempting to speculate that the inhibition of RhoA/Rho-kinase-mediated contraction may play a mechanistic role in endogenous vasodilator responses. Thus, the present study was performed to test the hypothesis that endogenous NO induces vasodilation through the inhibition of Rho-kinase-mediated contraction.

**Methods**

**Animals**

Male Sprague-Dawley rats (200 to 250 g, Harlan Sprague Dawley, Indianapolis, Ind) were housed according to guidelines at the Medical College of Georgia. Animals were anesthetized with sodium pentobarbital (50 mg/kg IP), and the thoracic aortas were excised.

**In Vitro Measurement of Isometric Force Generation**

Aortas (3-mm rings) were cleaned of adherent connective tissue and hung on stainless-steel hooks attached between a force transducer and stationary mount for the measurement of isometric force generation. Endothelium was removed from some vessels by gentle rubbing of the lumen with a steel wire. Aortic rings were then placed in an isolated chamber, bathed in physiological salt solution (mmol/L: NaCl 130, KCl 4.7, KHPO<sub>4</sub> 1.18, MgSO<sub>4</sub> 1.17, CaCl<sub>2</sub> 2H<sub>2</sub>O 1.6, NaHCO<sub>3</sub> 14.9, dextrose 5.5, and CaNa<sub>2</sub> EDTA 0.03; 37°C), and gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>. Rings were set at 3-g passive tension and treated with indomethacin (1 mmol/L) to inhibit prostaglandin synthesis. After a 1-hour equilibration period, vessels were contracted with PE (0.1 mmol/L) and subsequently treated with acetylcholine (1 mmol/L) to test for the presence of endothelium.

**Relaxation Response to Various Vasodilators**

Endothelium-denuded or -intact rat aortic rings were contracted with 10 mmol/L PE or 80 mmol/L KCl to achieve a similar level of force generation. Rings were then treated with increasing concentrations of nifedipine, an L-type Ca<sup>2+</sup> channel antagonist, or diltiazem, a nonselective Ca<sup>2+</sup> channel antagonist. In other experiments, endothelium-intact or -denuded rings were contracted with 10 mmol/L PE to plateau and subsequently treated with increasing concentrations of the Rho-kinase inhibitor Y-27632 (Calbiochem). Some endothelium-denuded rings were treated with Y-27632 before contraction with 10 mmol/L PE and then relaxed with the NO donor SNP.

**Effect of Kinase Inhibition on Contraction to PE**

In experiments examining the effect of kinase inhibition on the contraction to PE, endothelium-intact or -denuded rat aortic rings were contracted with 10 mmol/L PE to plateau and then rinsed passively to baseline tension. Rings were then treated with either Y-27632 (1 to 100 mmol/L) or wortmannin (1 mmol/L) for 20 minutes (to inhibit Rho-kinase or MLCK kinase, respectively), and the contraction to 10 mmol/L PE was repeated. Some experiments were performed in the presence of Nω-nitro-L-arginine (L-NNA, 100 mmol/L) or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 mmol/L), inhibitors of NO synthase and guanylate cyclase, respectively.

**Statistical Analysis**

Data were analyzed by 1-way ANOVA followed by the Student-Newman-Keuls post hoc test or by the Student t test with Bonferroni correction as indicated. Significance was set at a level of \(P<0.05\). Data are expressed as mean±SEM.

**Chemicals**

Y-27632 was a gracious gift from Welfide Corp, Osaka, Japan. All other drugs were purchased from Sigma Chemical Co.

**Results**

**Effect of Ca<sup>2+</sup> Channel Inhibition on PE-Induced Contraction**

To determine the contribution of membrane Ca<sup>2+</sup> channels to the tonic component of agonist-induced contraction, endothelium-denuded aortic rings were first contracted with 10 mmol/L PE or 80 mmol/L KCl to achieve a similar extent of force generation and subsequently treated with a Ca<sup>2+</sup> channel antagonist (nifedipine or diltiazem). Nifedipine was a significantly less effective inhibitor of force generation in response to PE compared with KCl. At the maximum concentration used, nifedipine relaxed the vessels contracted with PE only 19±6%, whereas the same concentration of nifedipine relaxed the vessels contracted with KCl 80±4%. Diltiazem was also a poor inhibitor of PE-induced contraction, relaxing the vessels contracted with PE only 11±3% at the maximum concentration used (Figure 1, top).

**Effect of Endothelium on the Relaxation Response to Y-27632**

Next, to examine the effect of endothelium removal on the response to Rho-kinase inhibition, endothelium-intact and -denuded aortas were contracted with 10 mmol/L PE and subsequently treated with the Rho-kinase inhibitor Y-27632. (The magnitude of contraction to 10 mmol/L PE was not significantly greater \([P<0.05]\) in endothelium-denuded vessels than in endothelium-intact vessels in the present study [data not shown].) Compared with endothelium-denuded rings, endothelium-intact vessels exhibited an increased vasodilator sensitivity to Y-27632, suggesting the endogenous inhibition of Rho-kinase activity by an endothelium-derived agent (Figure 1, bottom).

**Effect of NO Removal on Response to Y-27632**

The effect of NO on the response to Y-27632 was examined through endothelium removal or by the use of inhibitors of NO synthase and guanylate cyclase. Treatment with 1 mmol/L Y-27632 resulted in a significant inhibition of force generation to 10 mmol/L PE (compared with the initial control response to 10 mmol/L PE) in endothelium-intact rat aortic rings (Figure 2, top and middle). However, 1 mmol/L Y-27632 was a completely ineffective inhibitor of the contractile response to 10 mmol/L PE in endothelium-denuded vessels (Figure 2, middle). Additionally, in the presence 100 mmol/L L-NNA or (10 mmol/L) ODQ (to inhibit NO synthase or guanylate cyclase, respectively), 1 mmol/L Y-27632 was significantly less effective at inhibiting the contractile response to 10 mmol/L PE, suggesting that endogenous NO/cGMP signaling functions to decrease Rho-kinase activity (Figure 2, middle). In endothelium-denuded rings, 10 mmol/L or 100 mmol/L Y-27632 resulted in a concentration-dependent inhibition of the contractile response to 10 mmol/L PE (Figure 2, bottom).

**Effect of Exogenous NO Addition on Response to Y-27632**

The effect of exogenous NO on the response to Y-27632 was examined through exogenous NO. In the absence of endothelium, Y-27632 (1 mmol/L) was an ineffective inhibitor of PE-
induced contraction (Figure 2, middle). However, in endothelium-denuded rings, treatment with the NO donor SNP (30 nmol/L) restored the ability of 10 μmol/L Y-27632 to inhibit significantly the response to PE (10 μmol/L), demonstrating that exogenous NO decreases Rho-kinase signaling (Figure 3, top). Treatment with 30 nmol/L SNP alone resulted in 37% inhibition of the contractile response to 10 μmol/L PE in endothelium-denuded rings.

**Effect of Y-27632 on NO-Induced Relaxation**

To examine the effect of Rho-kinase inhibition on the relaxation response to NO, endothelium-denuded vessels were first contracted with 10 μmol/L PE (in the presence or absence of 1 μmol/L Y-27632) and subsequently relaxed with increasing concentrations of SNP. Compared with untreated vessels, vessels treated with 1 μmol/L Y-27632 before the PE-induced contraction exhibited an increased vasodilator sensitivity to SNP (Figure 3, bottom). These data suggest that the inhibition of Rho-kinase potentiates NO-induced relaxation. It is important to note that treatment with 1 μmol/L Y-27632 did not significantly augment the contractile response to 10 μmol/L PE in endothelium-denuded rings, as reported above (Figure 2, middle).
The findings of the present study confirm the role of Rho-kinase activity in the maintenance of agonist-induced force generation and further support the hypothesis that NO induces the relaxation of vascular smooth muscle through the inhibition of RhoA/Rho-kinase-mediated contraction. Much evidence suggests that the initial phase of agonist-induced force generation is stimulated by increases in intracellular Ca$^{2+}$, whereas the maintained portion of contraction is regulated by a Ca$^{2+}$-sensitization mechanism mediated by the activity of RhoA/Rho-kinase. $^{7,8,15}$ In contrast, depolarization-induced contraction is primarily maintained by a sustained increased in intracellular Ca$^{2+}$. $^{12}$ In confirmation, we found treatment with the Ca$^{2+}$ channel antagonists diltiazem and nifedipine (a nonselective and L-type Ca$^{2+}$ channel antagonist, respectively) to have little effect on the plateau phase of PE-induced contraction compared KCl-induced contraction (Figure 1, top). These data suggest that Ca$^{2+}$ influx through membrane Ca$^{2+}$ channels does not play a significant role in the sustained contraction to $\alpha$-adrenergic agonists. However, in agreement with other reports, $^{12}$ we found that treatment with the selective inhibitor of Rho-kinase, Y-27632, completely relaxed rat aortic rings contracted with PE, indicative of the role of Rho-kinase activity in agonist-induced force generation (Figure 1, bottom). Interestingly, endothelium-intact rings exhibited an increased vasodilator sensitivity to NO-mediated Ca$^{2+}$ sensitization. Y-27632, suggesting an interaction between an endothelium-derived agent and Rho-kinase activity (Figure 1, bottom).

We hypothesized that the endothelium-derived agent is NO and that the principle endogenous mechanism of NO-mediated vasodilation in intact rat aortas is the inhibition of Rho-kinase activity. NO is known to stimulate an increase in cGMP levels, leading to the activation of cGK. Recent studies have reported recombinant cGK to phosphorylate RhoA, destabilizing the membrane binding of RhoA, thus providing a mechanism by which NO may inhibit RhoA/Rho-kinase activity. $^{5,6}$ Additionally, cGK and the NO donor SNP have been shown to inhibit the agonist-induced migration of RhoA from the cytosolic to membrane cellular fractions, indicative of the inactivation of RhoA. $^{5,6}$

In the present study, we used the Rho-kinase inhibitor Y-27632 to test the hypothesis that NO induces vasorelaxation through the inhibition of Rho-kinase–mediated Ca$^{2+}$ sensitization. Y-27632 is a pyridine derivative that acts as a competitive inhibitor of the RhoA/Rho-kinase pathway, the effect of endothelium removal on the response to SNP. Data are mean±SEM (n=4 per group) and are expressed as percentage of inhibition of PE-induced force generation after treatment with wortmannin (compared with the initial contraction to PE). P=NS (Student t test).

Discussion

The findings of the present study confirm the role of Rho-kinase activity in the maintenance of agonist-induced force generation and further support the hypothesis that NO induces the relaxation of vascular smooth muscle through the inhibition of RhoA/Rho-kinase–mediated contraction. Much evidence suggests that the initial phase of agonist-induced force generation is stimulated by increases in intracellular Ca$^{2+}$, whereas the maintained portion of contraction is regulated by a Ca$^{2+}$-sensitization mechanism mediated by the activity of RhoA/Rho-kinase. $^{7,8,15}$ In contrast, depolarization-induced contraction is primarily maintained by a sustained increase in intracellular Ca$^{2+}$. $^{12}$ In confirmation, we found treatment with the Ca$^{2+}$ channel antagonists diltiazem and nifedipine (a nonselective and L-type Ca$^{2+}$ channel antagonist, respectively) to have little effect on the plateau phase of PE-induced contraction compared KCl-induced contraction (Figure 1, top). These data suggest that Ca$^{2+}$ influx through membrane Ca$^{2+}$ channels does not play a significant role in the sustained contraction to $\alpha$-adrenergic agonists. However, in agreement with other reports, $^{12}$ we found that treatment with the selective inhibitor of Rho-kinase, Y-27632, completely relaxed rat aortic rings contracted with PE, indicative of the role of Rho-kinase activity in agonist-induced force generation (Figure 1, bottom). Interestingly, endothelium-intact rings exhibited an increased vasodilator sensitivity to NO-mediated Ca$^{2+}$ sensitization. Y-27632, suggesting an interaction between an endothelium-derived agent and Rho-kinase activity (Figure 1, bottom).

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ATP-binding site on Rho-kinase and has been shown to be ≈200 to 2000 more specific for Rho-kinase than are MLC kinase, conventional protein kinase C, and cAMP-dependent protein kinase.12,16

Measuring the isometric force generation of isolated rat aorta, we found 1 μmol/L Y-27632 to inhibit the contraction to PE ≈60% in endothelium-intact vessels (Figure 2). In the absence of NO, however, a condition in which the NO-mediated inhibition of RhoA/Rho-kinase activity is removed, we expected a higher level of agonist-induced Rho-kinase activity. Y-27632 (1 μmol/L) was indeed found to be a significantly less effective inhibitor of the PE-induced force generation in the presence of NO synthase or guanylate cyclase inhibition or in endothelium-denuded rings, supporting the presence of increased Rho-kinase activity in the absence of NO (Figure 2). It is important to note that endothelial removal had no significant effect on the response to the MLC kinase inhibition with wortmannin (Figure 4; the concentration of wortmannin used has been reported to have selectivity for MLC kinase17; however, wortmannin has also been shown to inhibit the activity of PI3 kinase).

In further support of our hypothesis, we found the exogenous addition of NO with SNP to restore the effect of 1 μmol/L Y-27632 to inhibit PE-induced contraction in endothelium-denuded rings (Figure 3, top). Moreover, an increased vasodilatory sensitivity to SNP was observed in endothelium-denuded aortic rings treated with Y-27632, further demonstrating that the inhibition of Rho-kinase promotes the relaxant effects of NO (Figure 3, bottom).

Altogether, these data support the hypothesis that the principle endogenous mechanism of NO-mediated vasodilation in intact rat aortas is the inhibition of Rho-kinase activity. This novel signaling pathway may add to our understanding of the mechanisms of vasodilation in both physiological and pathological conditions. Numerous studies have demonstrated the attenuation of endothelium-dependent vasodilation in various hypertensive models, which is often associated with a decrease in NO bioavailability.18,19 On the basis of the findings of the present study and others (supporting the hypothesis that NO inhibits RhoA/Rho-kinase constrictor activity), it is tempting to speculate that decreased NO bioavailability leads to an increase in RhoA/Rho-kinase constrictor activity. Elevated Rho-kinase activity may then mediate the increased vasoconstrictor sensitivity seen in various hypertensive animal models. Indeed, in vivo and in vitro evidence supports an increase in Rho-kinase activity in spontaneously hypertensive, deoxycorticosterone-salt, and renal hypertensive rats.12,20 Ucheta et al.12 found that the in vivo treatment of rats with Y-27632 significantly decreased blood pressure in hypertensive but not normotensive rats. Additionally, arteries from mineralocorticoid and angiotensin II hypertensive rats exhibit increased vasodilator sensitivity to Y-27632.12,20,21

In summary, the findings of the present study support the hypothesis that endogenous NO-induced relaxation occurs principally through the inhibition of RhoA/Rho-kinase activity in the intact rat aorta, providing novel insight into a prominent vasodilation pathway.

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