Inhibition of Rho-Kinase Leads to Rapid Activation of Phosphatidylinositol 3-Kinase/Protein Kinase Akt and Cardiovascular Protection

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Objective—Rho-Kinase activity is increased in cardiovascular diseases and in patients with cardiovascular risk factors. However, it is not known whether inhibition of Rho-kinase could lead to cardiovascular protection and, if so, by what mechanism.

Methods and Results—In human endothelial cells, the Rho-kinase inhibitor, hydroxyfasudil (HF) (1 to 100 μmol/L), increased Akt serine-473 phosphorylation within 15 minutes, leading to a 2.2-fold and 4.0-fold increase in Akt kinase activity and nitric oxide (NO) release, respectively. Activation of Akt and eNOS by HF was completely blocked by the phosphatidylinositol 3-kinase (PI3-kinase) inhibitor, LY294002 (10 μmol/L). To determine the physiological relevance of this pathway, we used 2 models of ischemia-reperfusion (I/R) injury. Acute administration of fasudil (10 mg/kg, intraperitoneal, 1 hour before ischemia) decreased leukocyte recruitment and adhesion to the mesenteric endothelium after I/R injury in wild-type but not eNOS−/− mice. Similarly, treatment with fasudil decreased myocardial infarct size by 38% in rats subjected to transient coronary artery occlusion. Cotreatment with 2 PI3-kinase inhibitors, wortmannin and LY294002, or the eNOS inhibitor, L-NAME, blocked the cardiovascular protective effects of fasudil.

Conclusions—Inhibition of Rho-kinase leads to the activation of the PI3-kinase/Akt/eNOS pathway and cardiovascular protection. These findings suggest that Rho-kinase may play an important role in mediating the inflammatory response to I/R injury. (Arterioscler Thromb Vasc Biol. 2004;24:1842-1847.)

Key Words: endothelium • inflammation • infarction • nitric oxide • ischemia-reperfusion

The ubiquitously expressed Rho-kinase is a serine-threonine kinase, which mediates many important downstream effects of the small GTP-binding protein, Rho.1 In vascular smooth muscle cells, Rho-kinase phosphorylates the myosin binding subunit (MBS) of myosin light chain phosphatase, resulting in decreased myosin light chain phosphatase activity and increased myosin light chain phosphorylation.2,3 The activation of Rho-kinase contributes to the calcium-insensitive contraction of vascular smooth muscle1 and has been implicated in the pathogenesis of hypertension.4 Inhibition of Rho-kinase leads to the lowering of systemic blood pressure in a rat model of hypertension.5 Rho-kinase plays an important role in various other cellular functions such as actin cytoskeleton organization, as well as cell adhesion and motility.6 There is growing evidence that Rho-kinase may also be involved in regulating gene expression and other signaling pathways. For example, Rho-kinase might be involved in the increased expression of NAD(P)H oxidase and the development of myocardial hypertrophy after long-term treatment with angiotensin II.7 In addition, inhibition of Rho-kinase may contribute to some of the cholesterol-independent effects of statins by increasing the expression of endothelial nitric oxide synthase (eNOS).8,9 Specifically, reduction in geranylgeranylpyrophosphate after statin treatment reduces the activity of RhoA and Rho-kinase that results in the stabilization of eNOS mRNA and an increased bioavailability of nitric oxide (NO).8,10

Another important pathway of eNOS activation in endothelial cells is the post-translational phosphorylation of eNOS by protein kinase Akt.11,12 A recent study found that activation of RhoA leads to inhibition of Akt and eNOS activities.13 However, the physiological relevance of this pathway with regard to eNOS activation was not demonstrated. Because increased Rho-kinase activity is associated with cardiovascular risks such as hypertension,5,14 it is quite likely that eNOS regulation by Rho-kinase may play an important role in the pathogenesis of cardiovascular diseases. The purpose of this study, therefore, was to determine whether direct inhibition of
Rho-kinase could have cardiovascular protective effects in vivo, and, if so, to determine the mechanisms involved.

**Methods**

**Animal Care**

The animal studies were performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. The Animal Care and Use Committees at the Thomas Jefferson University and the University of Schleswig-Holstein approved all animal protocols.

**Cell Culture**

Human saphenous endothelial cells or bovine aortic endothelial cells (BAECs) at a passage <5 were cultivated as described previously15,16 in M199 cell medium and Dulbecco modified Eagle medium (Life Technologies Gibco BRL, Grand Island, NY), respectively, containing 100 μg/mL heparin sulfate, 10% fetal calf serum, and an antibiotic mixture of 100 U/mL penicillin and 100 μg/mL streptomycin until confluence was reached. Human saphenous endothelial cells and BAECs were then starved for 5 and 12 hours, respectively, in phenol red and serum-free medium before treatment was started.

**Western Blot Analysis**

Cells were harvested in cell lysis buffer (Cell Signaling Technology, Beverly, Mass.). Mice hearts and aortas were homogenized in the same buffer using a dounce homogenizer. Lysate was rotated for 30 minutes at 4°C and insoluble material was removed by centrifugation (12,000g for 15 minutes). Protein concentration was measured (BCA method, Pierce Biotechnology, Rockford, Ill) and 5 μg (cells) or 20 μg (tissue) of protein from each sample were separated by 10% SDS-polyacrylamide gels using standard methods. Membranes were incubated with polyclonal antibodies against phospho-Ser473 and total Akt (dilution 1:500; Cell Signaling Technology). In separate experiments, polyclonal-specific antibodies against pMB-S AS220L (1:200) or total MBS (1:500), which were developed in our separate experiments, polyclonal-specific antibodies against pMB-SDS-polyacrylamide gels using standard methods. Membranes were incubated with polyclonal antibodies against phospho-Ser473 and total Akt (dilution 1:500; Cell Signaling Technology). In separate experiments, polyclonal-specific antibodies against pMB-S AS220L (1:200) or total MBS (1:500), which were developed in our laboratory, were used to demonstrate the efficacy of Rho-kinase inhibition by fasudil in vivo.

**Akt Kinase Assay**

Cells were lysed as described and Akt kinase activity was determined in 100 μg of protein by detection of phosphorylated GSK-3 fusion protein using the Akt kinase assay kit (Cell Signaling Technology) according to the manufacturer’s guidelines.

**Measurement of NO Release**

Nitrite, the stable breakdown product of NO in aqueous solutions, was measured as described.15 After 12 hours of starving, medium was aspirated from BAECs and replaced with 1 mL fresh serum-free DMEM. After 60 minutes, an aliquot was taken as background NO2 production and treatment with either vehicle or HF (30 μmol/L) was started for an additional 60 minutes in fresh medium before a second aliquot was taken. In separate experiments, cells were pretreated with the PI3-kinase inhibitor LY294002 (10 μmol/L) or the Akt inhibitor SH-5 (D-3-Deoxy-2-O-methyl-myo-inositol 1-(R)-2-methoxy-3-(octadecyloxy)propyl hydrogen phosphate; 10 μmol/L; Calbiochem) 2 hours before the NO2 measurement. SH-5 has previously been shown to selectively inhibit Akt without affecting other kinases like PKB-1 or MAPK.17 Bradykinin in a concentration of 100 μmol/L, 100 μmol/L, and the Ca2+ ionophore A23187 (1 μmol/L) served as positive controls. Nitrite levels were then measured using a Sievers NO analyzer as described in and compared with freshly made standards of NO2 in DMEM.

**Mesenteric Hemorrhagic Reinfusion Model**

Male 8- to 14-week-old mice C57Bl/6 mice (Jackson Laboratory, Bar Harbor, Me) were divided into the following treatment groups: (1) vehicle (NaCl 0.9%); (2) the Rho-kinase inhibitor fasudil (10 mg/kg); (3) the PI3-kinase inhibitor LY294002 (5 mg/kg); or (4) LY294002 plus fasudil. All drugs were administered intraperitoneally 1 hour before the onset of ischemia. The hemorrhage-induced I/R protocol was performed as described.16 Briefly, mice were subjected to ischemia by withdrawal of blood to allow mean arterial blood pressure (MAP) to be maintained at 40 mm Hg for 45 minutes. Reperfusion was accomplished by re-infusion of the shed blood and intravenous injection of 0.5 mL 0.9% NaCl. The effect of I/R injury on endothelial-leukocyte adhesion was assessed using intravital microscopy. Intravital microscopy was performed on mouse peritoneal venules after exteriorization of a loop of ileal tissue via a midline laparotomy as described previously.16 Observations of rolling and adherent leukocytes were made with a Microphot microscope and a 40× saltwater immersion lens.

**Myocardial Ischemia Model**

Male Wistar rats (270- to 310-g body weight; Charles River, Sulzfeld, Germany) were randomized into the following treatment groups: (1) vehicle (NaCl 0.9%); (2) the Rho-kinase inhibitor fasudil (10 mg/kg); (3) the PI3-kinase inhibitor wortmannin (15 μg/kg); (4) wortmannin + fasudil; (5) the NOS inhibitor L-NAME (Nω-nitro-L-arginine methyl ester; 15 mg/kg); or (6) L-NAME plus fasudil. Fasudil and wortmannin were administered intraperitoneally, 1 hour before left anterior descending artery occlusion. L-NAME was administered 15 minutes before fasudil injection. The myocardial infarction model was performed as described.16 Briefly, a lateral thoracotomy was performed in anesthetized and ventilated rats, and a 6-0 suture was looped under the left descending coronary artery for induction of coronary artery occlusion for 30 minutes, followed by 150 minutes of reperfusion. Hearts were then removed and the area at risk (AAR) and the infarct size (IS) were delineated using staining with Chinese ink and 2,3,5-triphenyltetrazolium chloride as described.20 The myocardial infarct size was expressed relatively to the AAR.

**Calculations and Statistics**

All quantitative data are given as means±SEM of 3 to 14 independent experiments. All data were compared with the corresponding treatment groups using a 1-way ANOVA with Dunn correction, or with a Student t test if only 2 groups were compared (MBS phosphorylation). Differences were considered as being statistically significant at an error level of P<0.05.

**Results**

**Rapid Activation of PI3-Kinase/Akt Pathway by Rho-Kinase Inhibition**

To determine whether Rho-kinase can regulate Akt, we treated serum-starved human endothelial cells with the Rho-kinase inhibitor, HF. In a concentration-dependent manner, treatment of endothelial cells with HF (0.01 to 100 μmol/L, 30 minutes) increased Akt serine-473 phosphorylation (Figure 1A). At a concentration of 30 μmol/L, HF increased Akt serine-473 phosphorylation by 3.4-fold (P<0.05 versus baseline). Similar results were obtained using BAECs (data not shown). In a time-dependent manner, HF (30 μmol/L) produced a 1.8-fold increase in Akt serine-473 phosphorylation as early as 5 minutes, with a maximum 4.6-fold increase after 15 minutes (Figure 1B). To determine the specificity of HF on Rho-kinase inhibition, we used another structurally different Rho-kinase inhibitor, Y-27632, in our studies. Y-27632 (3 to 30 μmol/L) produced a similar increase in Akt phosphorylation with a maximum after 30 minutes of incubation (1.4-, 1.9-, and 4.0-fold for 3, 10, or 30 μmol/L, respectively), suggesting that Rho-kinase inversely regulates Akt phosphorylation (data not shown).
To determine whether serine-473 phosphorylation of Akt is associated with an increase in Akt kinase activity, we measured phosphorylation of GSK-3, the downstream target of Akt. Treatment with HF (30 μmol/L, 30 minutes) produced a 2.2-fold increase in GSK-3 phosphorylation (P<0.05, n=4) (Figure 2). Cotreatment with the PI3-kinase inhibitor, LY294002 (10 μmol/L), decreased basal and HF-induced Akt kinase activity. Similar results were obtained with another PI3-kinase inhibitor, wortmannin (100 nmol/L; n=2; data not shown). These findings suggest that Rho-kinase tonically inhibits the PI3-kinase/Akt pathway in endothelial cells under serum-starved culture conditions.

**Activation of eNOS by Rho-Kinase Inhibition**

Because eNOS is an important downstream target of Akt in vascular endothelial cells, and because its product, NO, has been shown to induce a variety of cardiovascular protective effects, we investigated whether HF can stimulate NO production in endothelial cells. Treatment of endothelial cells with HF (30 μmol/L for 60 minutes) led to a 4-fold increase in NO2 production as compared with untreated or control cells (60±18% of baseline NO2 production in HF-treated cells versus 15±7% in untreated or control cells, P<0.05, n=14) (Figure 3). The increase in NO2 production by HF was comparable to the one obtained by 100 nmol/L of bradykinin (46±5%; n=7) but less potent than 1 μmol/L of the strong NO stimulator A23187 (121±14%; n=5; data not shown). However, the effect of HF was completely blocked by pretreatment with LY294002 or the Akt inhibitor, SH-5 (n=4 to 6).

**Inhibition of Rho-Kinase and Activation of PI3-Kinase/Akt Pathway by Fasudil**

To determine whether the activation of PI3-kinase/Akt and eNOS by Rho-kinase inhibition is of physiological significance, we studied the effects of the Rho-kinase inhibitor, fasudil, in vivo. Fasudil is rapidly and completely converted to HF in vivo. Accordingly, fasudil (10 mg/kg) was injected intraperitoneally in mice or rats 1 hour before initiation of ischemia. Approximately 1 hour after fasudil administration, mouse aortas showed a 42% decrease in MBS phosphorylation (P<0.05, n=6). These findings indicate that fasudil is effective in inhibiting vascular Rho-kinase activity in vivo (Figure 4A).

To determine whether fasudil can activate PI3-kinase/Akt pathway in vivo, we measured Akt phosphorylation in the presence and absence of PI3-kinase inhibitor. Treatment with fasudil (10 mg/kg; intraperitoneal) increased Akt serine-473 phosphorylation in mouse hearts (P<0.05 versus control), which was blocked by cotreatment with LY294002 (Figure 4B). These findings suggest that inhibition of Rho-kinase leads to the activation of PI3-kinase/Akt.

**Vascular-Protective Effects of Rho-Kinase Inhibition**

There was a tendency for reduced baseline MAP in mice at 1 hour after intraperitoneal administration of fasudil (77±5 versus 85±6 mm Hg in controls, P>0.05, n=6 to 7). However, this effect did not reach statistical significance and there was no detectable difference in MAP after resuscitation from...
hemorrhagic shock (77±4 in fasudil versus 80±5 mm Hg in controls, \( P > 0.05 \)). To determine whether the activation of PI3-kinase/Akt pathway by fasudil confers vascular protection, we subjected mice to hemorrhage-induced I/R injury. In the mesenteric postcapillary venules, there were no differences in leukocyte rolling or adhesion after fasudil injection as compared with the vehicle-treated group (Figure 5). During mesenteric ischemia achieved by hemorrhagic shock, leukocyte rolling was significantly reduced as a result of loss in shear forces in the microcirculation. However, compared with vehicle, fasudil attenuated the increase in leukocyte rolling by 55% during reperfusion. This effect was maintained after 30 and 45 minutes of reperfusion (reduction by 48% and 57%, respectively; \( P < 0.05 \) versus vehicle, \( n = 7 \)). Similar results were obtained for leukocyte adhesion to postcapillary venules in the mouse mesentery (reduction by 68% after 15 minutes of reperfusion, \( P < 0.05 \) versus vehicle, \( n = 7 \)). Cotreatment with the PI3-kinase inhibitor, LY294002 (5 mg/kg), completely abolished this vascular-protective effect of fasudil, whereas treatment with LY294002 alone did not statistically differ from the control group. These findings suggest that the interaction between leukocytes and the endothelium is not regulated by basal PI3-kinase activity unless PI3-kinase activity is increased by Rho-kinase inhibition.

**Cardioprotective Effects of Rho-Kinase Inhibition**

To determine whether inhibition of Rho-kinase can lead to tissue protection, we tested whether fasudil could reduce infarct size after myocardial ischemia. Similar to the effects in mice, administration of fasudil to rats did not produce a significant reduction in MAP compared with baseline levels of control group (78±10 versus 88±9 mm Hg in the control group, \( P > 0.05, n = 5 \)). In addition, during the time course of the experiments, there were no differences in MAP between fasudil and the control groups, with both groups showing a comparable decrease in blood pressure (73±10 versus 73±8 mm Hg in the control group) followed by a readaptation phase after ischemia and reperfusion (97±14 versus 90±10 mm Hg in the control group). Pretreatment with l-NAME caused an increase in MAP compared with vehicle or fasudil group (92±14 mm Hg at baseline). Treatment with wortmannin alone decreased MAP (71±9 mm Hg at baseline), which reached statistical significance at the end of the reperfusion period (57±7 versus 85±7 mm Hg in the control group, \( P < 0.05, n = 5 \)). However, cotreatment with fasudil enhanced the reduction in MAP with a tendency to lower MAP during the entire experimental period (66±9 mm Hg at baseline, \( P > 0.05 \) versus wortmannin alone; 49±2 mm Hg at the end of reperfusion, \( P > 0.05 \) versus wortmannin alone).

To determine whether activation of PI3-kinase/Akt by Rho-kinase inhibition could mediate tissue protection, we tested whether fasudil could decrease myocardial infarct size
in rats subjected to coronary artery occlusion. The ratio between AAR and left ventricular area did not differ between the treatment groups, indicating a constant placement of the coronary ligature (Figure 6). Pretreatment with fasudil (10 mg/kg; intraperitoneal) 60 minutes before induction of myocardial ischemia reduced myocardial IS by 40% (IS/AAR 50% to 31 ± 1%, P < 0.05, n = 5). This cardioprotective effect of fasudil was completely blocked after pretreatment with the NOS inhibitor, L-NAME (IS/AAR 50 ± 4%) or the PI3-kinase inhibitor, wortmannin (IS/AAR 49 ± 10%) (P > 0.05 for both, n = 5). Consistent with previously published data from our group, L-NAME alone, at the concentration given, did not affect myocardial infarct size.19,22 Similarly, wortmannin alone did not affect myocardial infarct size.

**Discussion**

Previously, we have reported that activation of Rho-kinase by hypoxia leads to the downregulation of eNOS expression and activity.8 Because activation of eNOS is fundamental to the maintenance of normal vascular function, these data suggest that inhibition of Rho-kinase might exert beneficial effects in diseases in which endothelial function is impaired. A recent study found that long-term treatment with fasudil was effective in preventing the development of atherosclerosis.23

In this study, we found that inhibition of Rho-kinase by HF rapidly increased Akt phosphorylation and activation (EC50 = 7.3 μmol/L). At the concentration used, HF is relatively selective for Rho-kinase and had minimal effects on other signaling pathways such as myosin light chain kinase and protein kinase C.24 Furthermore, another chemically distinct Rho-kinase inhibitor, Y-27632, had similar effects on Akt serine-473 phosphorylation as that of HF, suggesting relative selectivity of the observed effects. In contrast to the rapid stimulatory effect of Rho-kinase inhibition on Akt, a recent study could not demonstrate Akt activation using dominant-negative mutants of Rho and Rho-kinase.13 This discrepancy might be explained by the different experimental approaches used between these studies. For example, Akt activation was only examined 18 hours after inhibition of Rho-kinase using adenoviral gene transfer of dominant-negative mutants of Rho and Rho-kinase. At such a late time point, it is possible that Akt is no longer activated. In contrast, we observed activation of Akt within 15 minutes after inhibition of Rho-kinase by HF.

The lipid class I PI3-kinases generate 3′ phosphorylated phosphatidylinositols (eg, phosphatidylinositol 3,4,5-trisphosphate [PIP3]),25 which recruit Akt to the cell membrane, where it is phosphorylated.26 PI3-kinase inhibitors block the activation of Akt by HF, indicating that Rho-kinase tonically inhibits Akt through PI3-kinase. Therefore, our findings support our hypothesis that Rho-kinase negatively regulates Akt via inhibitory effects on PI3-kinase. Interestingly, a potential interaction between Rho-kinase and PI3-kinase/Akt pathway has been reported earlier by Farah et al.27 In this study, using yeast 2-hybrid analyses, a Xenopus homolog of Rho-associated kinase (ROK)-α was found to bind to the pleckstrin-homology (PH)-phosphotyrosine binding domain of the insulin receptor substrate-1. Microinjection of mRNA corresponding to a Rho-kinase mutant inhibited insulin-mediated signal transduction. Thus, Rho-kinase may inhibit PI3-kinase through an inactivating serine phosphorylation of insulin receptor substrate-1.27 In addition, the phosphorylation motif of Rho-kinase (RXRX or RXRT) can be found in the catalytic p110 subunit of PI3-kinases as well as in the PH-phosphotyrosine binding domain of the regulatory p85 subunit. However, it is not known whether Rho-kinase can directly phosphorylate PI3-kinase subunits and alter its activity.

A major downstream target of Akt in endothelial cells is eNOS, which is activated by phosphorylation at serine-1179.11,12 We found that HF also stimulated NO production, which was blocked by PI3-kinase and Akt inhibitors. Surprisingly, the NO2 release achieved by HF was comparable to 100 nmol/L of bradykinin. However, because NO2 represents cumulative NO release after 1 hour of incubation, the kinetics of NO release after HF and bradykinin may differ.

NO not only is a potent vasodilator but also inhibits platelet aggregation and leukocyte recruitment to the vascular wall.28 We found that induction of NO by fasudil leads to decreased leukocyte rolling and adhesion after hemorrhage-induced I/R injury in mice. These anti-inflammatory effects of fasudil were completely reversed by the PI3-kinase inhibitors, indicating the involvement of the PI3-kinase/Akt pathway. Similarly, the anti-ischemic effects of HF were mediated by PI3-kinase and NO because the cardioprotective effects of HF were blocked by inhibitors of PI3-kinase and eNOS. Interestingly, we could not observe an effect on myocardial infarct size in animals treated with L-NAME alone, although MAP was significantly increased after L-NAME administration. The lack of effect of L-NAME is in accordance with a recent study from our group, in which L-NAME itself had no effect on infarct size but abolished cardioprotection achieved by statin treatment.19 In addition, these findings agree with those of Ockaili et al,29 who reported no difference in rate pressure product or infarct size in L-NAME–treated versus control rabbits. Although fasudil was given systemically and therefore may inhibit Rho-kinase in the vascular wall as well as inflammatory cells, our findings with L-NAME suggest that
the beneficial effects of Rho-kinase inhibition must occur downstream of NO.

In summary, our findings indicate that acute inhibition of Rho-kinase leads to cardiovascular protection mediated by the rapid activation of eNOS. It remains to be determined, however, whether targeting Rho-kinase in cardiovascular disease will yield therapeutic benefits in clinical trials.

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References


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