Rho-Kinase Mediates Hyperglycemia-Induced Plasminogen Activator Inhibitor-1 Expression in Vascular Endothelial Cells

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Background—Elevated levels of plasminogen activator inhibitor-1 (PAI-1) are associated with myocardial infarction and stroke, especially in patients with diabetes. The induction of PAI-1 expression by hyperglycemia involves oxidative stress and protein kinase C (PKC). However, the mechanism by which hyperglycemia increases PAI-1 expression is unknown.

Methods and Results—Compared with normoglycemia, exposure of human endothelial cells to hyperglycemia, but not mannitol, increased Rho-kinase activity in a time- and concentration-dependent manner. This increase was inhibited by a PKC inhibitor, GF109203X, and antioxidants N-acetylcysteine (NAC) and reduced form of glutathione (GSH). This correlated with inhibition of hyperglycemia-induced PAI-1 expression by GF109203X, NAC, and GSH. Hyperglycemia-increased PAI-1 mRNA and protein levels were inhibited by Rho-kinase inhibitors hydroxyfasudil and Y27632 and by a dominant-negative mutant of Rho-kinase. The mechanism for this inhibition occurs at the level of gene transcription because Rho-kinase inhibitors repress hyperglycemia-stimulated PAI-1 promoter activity without affecting mRNA stability. Hyperglycemia failed to stimulate Rho-kinase activity and PAI-1 expression in heterozygous ROCK I–knockout murine endothelial cells.

Conclusions—Hyperglycemia stimulates Rho-kinase activity via PKC- and oxidative stress–dependent pathways, leading to increased PAI-1 gene transcription. These results suggest that inhibition of ROCK I may be a novel therapeutic target for preventing thromboembolic complications of diabetes and cardiovascular disease. (Circulation. 2005;111:3261-3268.)

Key Words: diabetes mellitus ■ glucose ■ molecular biology ■ plasminogen

Endothelium plays a critical role in the regulation of fibrinolysis, and impaired endothelial fibrinolytic potential contributes to development of thrombosis. Plasminogen activator inhibitor-1 (PAI-1), a member of the serine protease inhibitor family, is a key regulator of fibrinolysis by inhibiting plasminogen activators. Indeed, PAI-1 plays an important role in the pathogenesis of myocardial infarction and stroke. Elevated plasma PAI-1 levels are observed in subjects with metabolic syndrome and diabetes as well as in patients with ischemic heart disease. The endothelium contributes to the PAI-1 found in plasma, and elevated plasma PAI-1 levels are associated with endothelial dysfunction.

Recent studies, including the Insulin Resistance Atherosclerosis Study, have shown that high plasma PAI-1 levels correlate with the development of diabetes in patients with metabolic syndrome and future cardiovascular events. Thus, inhibition of PAI-1 may be beneficial not only in preventing cardiovascular complications of diabetes but the development of diabetes as well. Although hyperglycemia activates protein kinase C (PKC) and stimulates the production of reactive oxygen species (ROS) in endothelial cells, little is known about the downstream signaling mechanism by which hyperglycemia induces PAI-1 expression.

Rho-kinase (ROCK) is a serine/threonine kinase and includes 2 isoforms, ROCK I and ROCK II, which mediate the downstream signaling of the small GTP-binding protein, Rho. There is growing evidence that Rho-kinase plays an important role in pathophysiological conditions such as cerebral and coronary vasospasm, hypertension, vascular inflammation and remodeling, and atherosclerosis. Diabetes is one of the major risk factors for thromboembolic diseases such as stroke and acute coronary syndromes. However, the role, particularly the isoform-specific role, of Rho-kinase in mediating the vascular complications of diabetes is unknown. The aim of the present study was to test the hypothesis that Rho-kinase mediates the downstream effects of hyperglycemia-induced oxidative stress and PKC activation in terms of PAI-1 expression.

Methods

Materials

N-Acetylcysteine (NAC) and reduced form of glutathione (GSH) were obtained from Sigma. GF109203X and Y27632 were obtained...
from Calbiochem. Hydroxyfasudil was a gift from Asahi-Kasei Pharma Corp.

Cell Culture and Treatment Conditions
Human saphenous vein endothelial cells (HSVECs) were cultured as described.20,21 The protocol to isolate and culture HSVECs was approved by the Committee on Human Studies at Harvard Medical School. Confluent HSVECs were cultured in a 0.1% gelatin-coated P-60 dish in Medium 199 supplemented with 5% fetal bovine serum (FBS), endothelial cell growth factor, and antibiotics in the indicated concentrations of d-glucose. In some experiments, media were changed after treatment for 48 hours and further incubated for 24 hours in the same media with various inhibitors as indicated. Adenovirus vectors carrying a dominant-negative mutant of Rho-kinase (Ad.DN.Rho-K) or β-galactosidase (Ad.LacZ) were infected as described.22

Development of Anti-Phospho-Specific Thr853-MBS Polyclonal Antibody
Rho-kinase phosphorylates the myosin-binding subunit (MBS) of myosin light chain phosphatase at Thr853.23–25 A rabbit polyclonal antibody was raised against MBS phosphorylated at Thr853 with the use of the following synthetic peptide: Cys-Pro-Arg-Glu-Lys-Arg-Arg-Ser-phospho-Thr853-Gly-Val-Ser-Phe853 (BioSource International). The antisera was affinity purified with a column containing immobilized phosphopeptide.

Assay for Rho-Kinase Activity
Cells were fixed and harvested in 10% trichloroacetic acid and 10 mmol/L of dichlorodiphenyltrichloroethane on ice. After centrifugation, pellets were dissolved in 10 μL of 1 mol/L Tris base and then mixed with 100 μL of extraction buffer (8 mol/L urea, 2% SDS, 5% sucrose, and 2% mercaptoethanol). Equal amounts of cell extracts were subjected to 7.5% SDS-PAGE and transferred onto PVDF membrane (Immobilon-P, Millipore). Membranes were incubated with rabbit anti-phospho-specific Thr853-MBS polyclonal antibody or rabbit anti-MBS polyclonal antibody (Covance). Band intensities from Western and Northern blots were quantified by the NIH Image Program. Results are expressed as mean ± SEM. All statistical analyses were determined by dual-luciferase reporter assay system (Promega) with the use of a Berthold L9501 luminometer.

Western Blotting
Proteins extracted from cells were separated on SDS-PAGE, transferred to PVDF transfer membrane, and probed with anti-PAI-1 monoclonal antibody (Santa Cruz Biotechnology), anti-ROCK I monoclonal antibody, anti-ROCK II monoclonal antibody (BD Bioscience), or mouse anti-α-tubulin antibody (Sigma).

Generation of ROCK I–Knockout Mice
The conditional targeting vector was constructed to delete a genomic fragment containing exon 1b of the ROCK I gene by homologous recombination. TwoloxP sites were introduced so that exon 1b of ROCK I was flanked by theseloxP sites. The neomycin resistance gene (Neo) fused to the phosphoglycerokinase (pGK) promoter (pGK-Neo) was inserted between exon 1b and 3′loxP site. The 5′ homology arm and the 3′ homology arm were inserted upstream of 5′loxP site and downstream of 3′loxP site, respectively. The linearized targeting vector was introduced to embryonic stem cells derived from C57Bl/6 mice. Neomycin-resistant clones were screened for homologous recombination by polymerase chain reaction after identification by genomic Southern blot. TheloxP-flanked exon 1b and pGK-Neo gene in correctly targeted clones were deleted by transfection with Cre. The deletion of exon 1b introduced a frame shift creating a stop codon in exon 1c, preventing translation of the functional protein. The correctly targeted embryonic stem cell clones were injected into C57Bl/6 blastocysts. Male heterozygous ROCK I–knockout mice (ROCK I+/−) were bred to C57Bl/6 females to obtain heterozygous pups. ROCK I−/− mice are viable and fertile, show no developmental abnormalities, and display no growth, anaatomic defects. The mice were maintained in the Harvard Medical School animal facilities. All animal experimentation protocols were approved by the Standing Committee on Animals at Harvard Medical School.

Isolation and Culture of Murine Lung Endothelial Cells
Isolation of murine lung endothelial cells (MLECs) was performed with the use of Dynal beads coated with anti–platelet–endothelial cell adhesion molecule-1 or anti–intercellular adhesion molecule-2 monoclonal antibody, anti–CD31 monoclonal antibody, or anti–intercellular adhesion molecule-2 monoclonal antibody. MLECs isolated from wild-type or ROCK I−/− mice were cultured with DMEM supplemented with 20% FBS, heparin, endothelial cell growth factor, nonessential amino acids, and antibiotics. Confluent MLECs were cultured in a 0.1% gelatin-coated P-60 dish in Medium 199 supplemented with 5% FBS, heparin, endothelial cell growth factor, and antibiotics in the indicated concentrations of d-glucose for 72 hours and then processed for protein extraction.

Statistical Analyses
Band intensities from Western and Northern blots were quantified by the NIH Image Program. Results are expressed as mean ± SEM. All data were analyzed by means of 1-way ANOVA followed by Fisher exact test for post hoc analyses. A value of P<0.05 was considered statistically significant.

Results
Cell Culture
Relatively pure (>98%) HSVECs and MLECs were confirmed by phase contrast microscopy and staining for CD31 and von Willebrand factor (data not shown). There were no observable adverse effects of reagents, including hydroxyfasudil and Y27632, on cellular viability at all of the concentrations used in this study.
Hyperglycemia Stimulates Rho-Kinase Activity

In a time-dependent manner, treatment of HSVECs with high glucose (25 mmol/L) significantly increased Rho-kinase activity as measured by phosphorylation of MBS, one of the downstream targets of Rho-kinase (Figure 1A). Compared with normoglycemia (5.5 mmol/L of glucose), exposure to hyperglycemia (12 to 25 mmol/L) significantly increased Rho-kinase activity in a concentration-dependent manner (Figure 1B). Similar concentrations of mannitol did not affect Rho-kinase activity, suggesting that changes in osmolarity were not responsible for hyperglycemia-induced Rho-kinase activation.

Because hyperglycemia induces PKC activation and stimulates ROS production in a variety of cells, including endothelial cells, we investigated whether PKC and ROS are involved in hyperglycemia-stimulated Rho-kinase activation. Interestingly, activation of Rho-kinase (ie, increased phosphorylation of MBS) by hyperglycemia was completely blocked by a nonselective PKC inhibitor, GF109203X (5 μmol/L), but not by dimethyl sulfoxide (P<0.01) (Figure 1C). The antioxidants NAC and GSH, which scavenge ROS, also inhibited hyperglycemia-induced Rho-kinase activation (ie, MBS phosphorylation; P<0.01) (Figure 1D). These results suggest that PKC and ROS are upstream mediators of hyperglycemia-induced Rho-kinase activation.

Rho-Kinase Mediates Hyperglycemia-Induced PAI-1 mRNA Expression

Northern blot analysis detected 2 species (3.2 and 2.4 kb) of PAI-1 mRNA, in which the length of 3′ untranslated region differs. Indeed, treatment with phorbol ester phorbol myristate acetate or tumor necrosis factor-α increased both PAI-1 mRNA transcripts to a similar extent (data not shown). Total PAI-1 mRNA expression (combination of 3.2 and 2.4 transcripts) was increased by hyperglycemia in a time-dependent manner (Figure 2A). The effects of hyperglycemia were concentration dependent, and, similar to Rho-kinase activity, mannitol also did not affect total PAI-1 mRNA expression (Figure 2B). The Rho-kinase inhibitors hydroxyfasudil and Y27632 inhibited hyperglycemia-induced PAI-1 mRNA expression in a concentration-dependent manner (Figure 3A), suggesting that Rho-kinase is involved in high glucose–mediated PAI-1 upregulation. Indeed, transfection of HSVECs with an adenovirus carrying a dominant-negative mutant of Rho-kinase (Ad.DN.Rho-K) attenuated hyperglycemia-induced PAI-1 mRNA expression (Figure 3B). In agreement with previous studies, a PKC inhibitor, GF109203X, and antioxidants NAC and GSH blocked hyperglycemia-induced PAI-1 mRNA expression (Figure 3C and 3D).

Rho-Kinase Inhibitors Attenuate High Glucose–Stimulated PAI-1 Promoter Activity

To determine whether Rho-kinase mediates hyperglycemia-induced PAI-1 gene transcription, we measured PAI-1 promoter activity in endothelial cells treated with normal or high glucose in the presence or absence of Rho-kinase inhibitors hydroxyfasudil and Y27632. Under normal glucose conditions (ie, 5.5 mmol/L), cotreatment with hydroxyfasudil and...
Y27632 decreased PAI-1 promoter activity by 22±2% (P<0.01), suggesting that basal Rho-kinase activity contributes to basal PAI-1 gene transcription (Figure 4A). High glucose (ie, 25 mmol/L) increased PAI-1 promoter activity by 1.5-fold compared with normal glucose conditions (P<0.01). Cotreatment with Rho-kinase inhibitors completely blocked the glucose-induced PAI-1 promoter activity, indicating that glucose-induced PAI-1 gene transcription is mediated in part by Rho-kinase.

To determine whether Rho-kinase also regulates the stability of PAI-1 mRNA, we studied the half-life of PAI-1 mRNA in the presence of an RNA synthetase inhibitor, DRB. Neither glucose nor the Rho-kinase inhibitor hydroxyfasudil affected PAI-1 mRNA half-life (Figure 4B). These findings suggest that Rho-kinase mediates glucose-induced PAI-1 expression, primarily at the level of gene transcription.

Rho-Kinase Mediates Hyperglycemia-Induced PAI-1 Protein Expression

Exposure of endothelial cells to high glucose (25 mmol/L) but not mannitol increased PAI-1 protein levels to a greater extent at 72 hours than at 24 hours (Figure 5A). This increase in PAI-1 protein levels under high-glucose conditions was completely blocked by cotreatment with Rho-kinase inhibitors hydroxyfasudil and Y27632 (Figure 5B). Furthermore, in agreement with PAI-1 mRNA levels, the hyperglycemia-induced PAI-1 protein levels were similarly inhibited by the nonselective PKC inhibitor GF109203X and antioxidants NAC and GSH. These results suggest that Rho-kinase is the final common mediator of PKC- and oxidative stress–induced PAI-1 expression under high-glucose conditions.

ROCK I Mediates Hyperglycemia-Induced PAI-1 Protein Expression

MLECs were isolated from wild-type (WT) and heterozygous ROCK I–knockout (ROCK I+) mice. Compared with WT
MLECs, ROCK I protein expression was significantly lower in ROCK I−/− MLECs (40.5 ± 4.5%; n = 5; P < 0.01; Figure 6A). However, ROCK II expression was similar between them (104.7 ± 6.4%; n = 5; P = NS). In WT MLECs, Rho-kinase activity was increased after exposure to high glucose (25 mmol/L) for 72 hours (167.3 ± 24.4%; n = 5; P < 0.05), whereas Rho-kinase activity was unchanged in ROCK I−/− MLECs (77.9 ± 16.9% versus 99.3 ± 33.0%, normal glucose versus high glucose, respectively; n = 5; P = NS; Figure 6B). Exposure of WT MLECs to high glucose increased PAI-1 protein levels (Figure 6C). This increase in PAI-1 protein levels under high-glucose conditions was completely absent in ROCK I−/− MLECs. These results suggest that ROCK I plays a predominant role in hyperglycemia-induced increases in Rho-kinase activity and PAI-1 expression.

**Discussion**

We have shown that exposure of endothelial cells to high-glucose conditions increases Rho-kinase activity and PAI-1 expression. These findings are consistent with previous studies showing that high glucose activates the Rho/Rho-kinase pathway in mesangial cells28 and vascular smooth muscle cells.29 The association between diabetes mellitus and activation of PKC has been well established. Activation of the diacylglycerol-PKC pathway occurs predominantly in vascular tissue. Indeed, incubation of vascular endothelial and smooth muscle cells with high glucose increases intracellular diacylglycerol levels, leading to PKC activation.12 Activation of PKCα has been implicated in the pathogenesis of the vascular complications of diabetes. Indeed, it was reported that high glucose stimulates PKCα in human aortic endothelial cells.30 PKCα induces the phosphorylation of RhoGDI, which leads to the membrane translocation and activation of RhoA.31 Furthermore, PKCα, but not PKCδ, mediates sphingosine-1 phosphate-induced RhoA activation in C2C12 myoblasts.32 Thus, it remains to be determined which PKC

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**Figure 4.** A, Bovine aortic endothelial cells were transfected with p800LUC and then cultured in media containing 5.5 mmol/L of glucose (NG) or 25 mmol/L of glucose (HG) for 48 hours. Cells were treated with or without 10 μmol/L of hydroxyfasudil (HFD) or Y-27632 (Y) for the last 24 hours. PAI-1 promoter activity was determined by luciferase activity. n = 3; †P < 0.01 compared with NG; ‡P < 0.01 compared with control. B, HUVECs were cultured in media containing 5.5 mmol/L of glucose (NG) or 25 mmol/L of glucose (HG) for 72 hours. Cells were treated with hydroxyfasudil (HG+HFD) for the last 24 hours and then incubated with DRB (50 μmol/L). Northern blotting of PAI-1 and GAPDH mRNA was performed. The relative intensity corresponds to time 0, and the time represents time after DRB treatment. n = 2–3.

**Figure 5.** Rho-kinase mediates high glucose-stimulated PAI-1 protein expression. A, HUVECs were cultured in media containing indicated concentrations of glucose or mannitol for 24 or 72 hours. PAI-1 levels were determined by immunoblotting with PAI-1-specific antibody and standardized to immunoblotting for α-tubulin as internal control. Three separate experiments yielded similar results. B, HUVECs were cultured in media containing 5.5 mmol/L of glucose (NG) or 25 mmol/L of glucose (HG) for 72 hours. Cells were treated with vehicle (Veh), GF109203X (GFX), GSH, NAC, Y-27632 (Y), or hydroxyfasudil (HFD) for the last 24 hours. n = 3; †P < 0.05 compared with NG; ‡P < 0.01 compared with HG.
isolated from WT and heterozygous ROCK I- knockout (ROCK I−/−) mice were cultured in media containing 5.5 mmol/L of glucose (NG) or 25 mmol/L of glucose (HG) for 72 hours. Rho-kinase activity was determined. n=5; *P<0.05 compared with NG. C, MLECs isolated from WT and heterozygous ROCK I−/− mice were cultured in media containing 5.5 mmol/L of glucose (NG) or 25 mmol/L of glucose (HG) for 72 hours. PAI-1 expression was determined. n=5; *P<0.01 compared with NG.

isoform(s) activates Rho-kinase in endothelial cells under high-glucose conditions.

Although high glucose induces intracellular ROS in various cell types, the mechanism by which ROS induces gene expression in vascular cells is not known. It is possible that PKC lies downstream of ROS. Indeed, incubation of cultured cells with high glucose increases mitochondrial ROS production, leading to subsequent PKC activation.33,34 However, other studies have shown that PKC inhibitors block high glucose–induced ROS production in vascular endothelial and smooth muscle cells.13,35 Thus, it is unclear whether PKC lies upstream of ROS production or vice versa. Nevertheless, in the present study we showed that a common downstream effect of PKC and ROS, which mediates high glucose–induced PAI-1 expression, is Rho-kinase. These findings agree with our earlier observation that Rho-kinase is activated in endothelial cells under hypoxic conditions in which ROS production and oxidant stress are increased.22 Recently, DEF6, a novel PH-DH–like domain protein, was identified as an upstream activator of RhoA, Rac1, and Cdc42.36 Increased ROS was found to stimulate DEF6, leading to the activation of Rho GTPases in fibroblasts. Thus, it is quite likely that Rho-kinase lies downstream of ROS in mediating hyperglycemia–induced PAI-1 gene transcription.

The elevation of glucose significantly increased PAI-1 expression, an effect that was antagonized by 2 structurally different Rho-kinase inhibitors, hydroxyfasudil and Y27632, and by the overexpression of a dominant-negative mutant of Rho-kinase. Similarly, previous studies have demonstrated an important role of Rho-kinase in angiotensin II–induced PAI-1 expression in vascular smooth muscle cells.37 However, it was not known from that study whether the effect of Rho-kinase occurred at the level of PAI-1 gene transcription. Because PAI-1 promoter activity, but not mRNA stability, was affected by glucose and Rho-kinase inhibitors, the primary mechanism by which Rho-kinase regulates PAI-1 expression appears to occur at the transcriptional levels. Indeed, Rho/Rho-kinase regulates the activities of several transcription factors, such as serum response factor18,30 and activator protein-1.40,41 Because PAI-1 promoter contains consensus sequences for both serum response factor and activator protein-1, it is highly likely that these Rho-kinase–dependent transcription factors contribute to high glucose–induced PAI-1 expression. Further studies, however, are required to determine which transcription factors and which cis-acting elements are responsible for Rho-kinase–mediated PAI-1 gene transcription.

There are 2 isoforms of Rho-kinase, ROCK I and ROCK II, whose kinase domains share 92% identity. Rho-kinase inhibitors such as hydroxyfasudil and Y27632 have similar potency to inhibit both ROCK I and ROCK II. It is therefore impossible to clarify the isoform–specific role of Rho-kinase by application of Rho-kinase inhibitors. We generated ROCK I−/− mice and isolated ROCK I−/− MLECs. In ROCK I−/− MLECs, ROCK I protein expression was approximately half of that in WT MLECs, whereas there was no difference in ROCK II expression. Surprisingly, partial deficiency of ROCK I resulted in the complete lack of increases in PAI-1 protein levels after exposure to high glucose in association with the absence of elevation of Rho-kinase activity (ie, phosphorylation of MBS). These results suggest that ROCK I plays a predominant role in hyperglycemia–induced increases in Rho-kinase activity and PAI-1 expression despite the presence of the highly homologous ROCK II.

PAI-1 is associated with vascular complications in diabetics. Clinical studies reveal a strong correlation between plasma PAI-1 levels and cardiovascular events and mortality. Thus, therapeutic strategies that can decrease PAI-1 levels may be beneficial in patients with diabetes and cardiovascular risks. Current management of elevated PAI-1 levels and diabetic complications includes weight loss and thiazolidinediones.42 Thiazolidinediones decrease plasma PAI-1 levels in humans.53,44 Indeed, thiazolidinediones decrease PAI-1 expression in cultured vascular endothelial cells and adipocytes.45,46 Our results suggest that inhibition of Rho-kinase may be a novel therapeutic target for diabetic patients at risk for cardiovascular events. In addition to the currently available therapy with thiazolidinediones, Rho-kinase inhibitors may provide additional benefits for lowering PAI-1 levels. The clinical consequences of this, however, remain to be determined.

Acknowledgments

This work was supported by grants from the National Institutes of Health (HL52233) and the American Heart Association (Bugher Foundation Award). Dr Rikitake is a recipient of an American Heart Foundation Award. Dr Rikitake is a recipient of an American Heart Foundation Award.
Hydroxyfasudil is a gift from Asahi Kasei Pharma Corporation the Japan Heart Foundation/Bayer-Yakuhin Research Grant Abroad.

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Circulation. 2005;111:3261-3268; originally published online June 13, 2005;
doi: 10.1161/CIRCULATIONAHA.105.534024
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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