Defective Endothelial Nitric Oxide Synthase Signaling Is Mediated by Rho-Kinase Activation in Rats with Secondary Biliary Cirrhosis

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In liver cirrhosis, down-regulation of endothelial nitric oxide synthase (eNOS) has been implicated as a cause of increased intrahepatic resistance. We investigated whether Rho-kinase activation is one of the molecular mechanisms involved in defective eNOS signaling in secondary biliary cirrhosis. Liver cirrhosis was induced by bile duct ligation (BDL). We measured mean arterial pressure (MAP), portal venous pressure (PVP), and hepatic tissue blood flow (HTBF) during intravenous infusion of saline (control), 0.3, 1, or 2 mg/kg/hour fasudil for 60 minutes. In BDL rats, 1 and 2 mg/kg/hour fasudil significantly reduced PVP by 20% compared with controls but had no effect on HTBF. MAP was significantly reduced in response to 2 mg/kg/hour fasudil. In the livers of BDL rats, 1 and 2 mg/kg/hour fasudil significantly suppressed Rho-kinase activity and significantly increased eNOS phosphorylation, compared with controls. Fasudil significantly reduced the binding of serine/threonine Akt/PKB (Akt) to Rho-kinase and increased the binding of Akt to eNOS. These results show in secondary biliary cirrhosis that (1) Rho-kinase activation with resultant eNOS down-regulation is substantially involved in the pathogenesis of portal hypertension and (2) Rho-kinase might interact with Akt and subsequently inhibit the binding of Akt to eNOS. (HEPATOLOGY **2008;47:966-977.**)

Portal hypertension is a common clinical syndrome associated with liver cirrhosis and characterized by increased intrahepatic resistance and elevated splanchnic blood flow, leading to a pathological increase in portal pressure and the development of portosystemic collaterals such as esophagogastric varices.[1-6] Pharmacological therapy for portal hypertension should be aimed at reducing intrahepatic vascular tone or elevated splanchnic blood flow. However, the only available treatment is a nonselective beta-blocker that reduces portal venous pressure (PVP) by decreasing cardiac output and splanchnic blood flow.[7] Several randomized trials indicate that a nonselective beta-blocker is the first choice for primary and secondary prophylaxis against esophageal variceal bleeding.[8-10] However, the mean decrease in PVP in response to beta-blockers is only approximately 15%. Moreover, 15% of patients with cirrhosis are beta-blocker intolerant, whereas another approximately 30% do not respond to beta-blockers despite adequate blockade.[11] Therefore, there is a pressing need for a more effective pharmacological therapy for portal hypertension.

Previous studies have shown that hepatic stellate cells (HSCs) through their contraction play a crucial role in regulating sinusoidal vascular tone.[12] The contractility of HSCs is regulated by the balance of vasoactive agents such as endothelin-1, and vasorelaxing agents such as nitric oxide (NO).[13] Recent studies have shown that the production of NO by endothelial nitric oxide synthase (eNOS) in hepatic sinusoidal endothelial cells (SECs) is

Abbreviations: α-SMA, alpha-smooth muscle actin; Akt, serine/threonine Akt/PKB; BDL, bile duct ligation; eNOS, endothelial nitric oxide synthase; ERM, ezrin-radixin-moesin; GRK2, G protein–coupled receptor kinase 2; HSC, hepatic stellate cell; HTBF, hepatic tissue blood flow; L-NAME, N-nitro-L-arginine methyl ester; MAP, mean arterial pressure; NO, nitric oxide; NOx, nitrogen oxide; PVP, portal venous pressure; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEC, sinusoidal endothelial cell; SEM, standard error of the mean; SO, sham-operated.

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decreased in cirrhotic livers, leading to increased intrahepatic resistance.[14] Although eNOS was first identified as a Ca^{2+}-calmodulin–dependent enzyme,[15] eNOS activity has been shown to be affected by binding to caveolin and phosphorylation at serine 1177 by serine-threonine kinase Akt in endothelial cells.[16-19] An increase in the binding of eNOS to caveolin with a concomitant decrease in the binding of eNOS to calmodulin has been found in livers of cirrhotic rats.[20] A more recent paper showed that phosphorylation of eNOS by Akt is impaired in secondary biliary cirrhosis and that this is mediated, at least in part, by G protein–coupled receptor kinase 2 (GRK2).[21] However, the molecular mechanisms underlying defective eNOS signaling in liver cirrhosis have not been fully elucidated.

Rho-kinase/ROK/ROCK is a downstream effector of small guanosine triphosphatase (GTPase) Rho that was initially identified as a signaling molecule regulating cytoskeletal rearrangement and was considered to be involved primarily in cellular functions and cell migration.[22-26]Rho-kinase is substantially involved in the contraction of activated HSCs, which play an important role in hepatic microcirculation.[13,27-29] It has also been shown that Rho-kinase negatively regulates eNOS phosphorylation through inhibition of Akt.[30-32] Therefore, we hypothesized that, even in hepatic SECs, Rho-kinase might be involved in the down-regulation of eNOS via inhibition of Akt, and that Rho-kinase might be a potential therapeutic target for the reduction of intrahepatic resistance in liver cirrhosis.

In the current study, we examine whether the Rho-kinase–mediated pathway is one of the molecular mechanisms involved in defective eNOS signaling in biliary cirrhotic rats *in vivo* and, if so, whether fasudil, a selective Rho-kinase inhibitor, could significantly ameliorate portal hypertension, particularly through the up-regulation of eNOS.

Materials and Methods

The study was approved by the Institutional Animal Care and Use Committee of the Kyushu University Graduate School of Medical Sciences.

Animal Model of Portal Hypertension due to Liver Cirrhosis. We induced portal hypertension by performing bile duct ligation (BDL) in adult male Sprague-Dawley rats (Charles River, Yokohama, Japan; 200-250 g body weight) following the method previously described by Kountouras et al.[33] Animals were housed with free access to food and drink and were maintained at 25 ± 1°C with a 12-hour light and dark cycle. Laparotomy was performed under sodium pentobarbital (40 mg/kg, intraperitoneally) anesthesia, and the common bile duct was isolated, ligated with 4–0 silk, and cut between the ligatures. Sham operations were performed in the same manner but without BDL. After 4 weeks, 5 BDL rats and 5 sham-operated (SO) rats were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally). Mean arterial pressure (MAP), portal venous pressure (PVP), and hepatic tissue blood flow (HTBF) were measured under baseline conditions in both groups. In the SO rats, the trunk of the portal vein was isolated and exposed. The probe was secured to the vessels using droplets of glue. The 6-mm-diameter probe on the trunk of the portal vein was connected to a transit time flow meter (T402, Transonic Systems Inc, Ithaca, NY). However, in BDL rats, the portal venous flow could not be measured because of the severe adhesion around the portal trunk. After hemodynamic measurement, the livers and abdominal aortas were excised and snap frozen in liquid nitrogen for baseline experiments.

Hemodynamic Effects of Intravenous Infusion of Fasudil on BDL Rats. Thirty-six BDL rats were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally) and polyethylene catheters inserted into the carotid artery and vein of each rat for MAP measurement and drug infusion. PVP was measured in the portal trunk via the ileocolic vein with 24-gauge catheters, which were connected to a polygraph system (AP-601G; Nihon Kohden, Tokyo, Japan). After cannulation of all catheters, animals were stabilized hemodynamically for 20 minutes. Thereafter, MAP and PVP were measured every 5 minutes for 60 minutes from the beginning of intravenous infusion of saline (control), 0.3 mg/kg/hour, 1.0 mg/kg/hour, or 2 mg/kg/hour fasudil (Eril; Asahi Kasei Corporation, Tokyo, Japan).[34,35] HTBF was also measured using a laser Doppler flow meter (Omega flow type II; Omega Flow Company Ltd., Tokyo, Japan) every 5 minutes for 60 minutes from the beginning of drug infusion.[36,37]

Sixty minutes after intravenous infusion of a graded dose of fasudil, the livers and abdominal aortas were isolated and snap-frozen in liquid nitrogen.

The role of NO in mediating the hepatic vascular response to fasudil was evaluated by using the NO synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME), at a dose of 15 mg/kg by intravenous bolus administration 20 minutes before the infusion of fasudil.[38]

These hemodynamic studies were carried out in all 36 BDL rats, which were randomized into 6 equal groups: saline-treated (1 mL/hour); fasudil-treated (0.3 mg/kg/hour, 1 mg/kg/hour, and 2 mg/kg/hour); and fasudil + L-NAME treated (fasudil: 1 mg/kg/hour; L-NAME: 15

mg/kg); and L-NAME treated (L-NAME: 15 mg/kg) groups.

Protein Extraction. For western blotting and immunoprecipitation, proteins were isolated from fresh-frozen tissues as previously described.[39] After homogenization of the tissues in lysis buffer (CelLytic MT; Sigma-Aldrich Inc., St. Louis, MO) containing a protease inhibitor cocktail (Sigma-Aldrich Inc.) composed of 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (104 mM), aprotinin (80 mM), leupeptin (2 mM), bestatin (4 mM), pepstatin A (1.5 mM), and E-64 (1.4 mM), the lysates were centrifuged for 15 minutes at 14,000 rpm and the supernatants retained. Protein concentrations were determined by the Bradford method using a protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA). Extracts were stored at −80°C until analysis.

Western Blot Analysis. Equal amounts of protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Millipore Co., Bedford, MA). After the blockade of nonspecific binding sites with Block Ace (Dainippon Sumitomo Pharmaceutical Co., Ltd., Osaka, Japan), membranes were incubated overnight at 4°C with specific antibodies against phosphorylated ezrin-radixin-moesin (ERM) (1:200 dilution),[40,41] phospho-eNOS (1:200 dilution; Cell Signaling Technology, Boston, MA), phospho-Akt-Ser-473 (1:200 dilution; Cell Signaling Technology Inc., Beverly, MA), Akt (1:500 dilution; Cell Signaling Technology Inc., Beverly, USA), alpha-smooth muscle actin (α-SMA) (1:500 dilution; Sigma-Aldrich Inc., St. Louis, MO), beta-actin (1:500 dilution; Sigma-Aldrich Inc., St. Louis, MO), ROCK2 (dilution 1:250; BD Biosciences, San Jose, CA), and glyceraldehyde 3-phosphate dehydrogenase (dilution 1:250; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by incubation with horseradish peroxidase–conjugated goat or rabbit secondary antibody (dilution 1:5000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 hour at room temperature. Chemiluminescence was developed using an electrogenerated enzymatic enhanced chemiluminescence detection kit (Amersham Biosciences Corporation, Piscataway, NJ), and detection and quantification were performed with an image analyzer (LAS 3000 mini; Fuji Film, Tokyo, Japan). After measurement of phosphorylated ERM or phosphorylated eNOS, the same membranes were stripped and reprobed with specific antibodies against ERM (dilution 1:500) or eNOS (dilution 1:500; BD Biosciences, San Jose, CA) to determine the amounts of total proteins. The phosphorylation level was normalized to the level of total ERM or eNOS protein expression. After measurement of α-SMA or ROCK2 (Rho-kinase), the same membranes were stripped and reprobed with specific antibodies against beta-actin or glyceraldehyde 3-phosphate dehydrogenase. The protein expression of α-SMA or ROCK2 (Rho-kinase) was normalized to the level of beta-actin or glyceraldehyde 3-phosphate dehydrogenase protein expression.

Rho-Kinase Activity. ERM is phosphorylated by Rho-kinase at T567 (ezrin), T5648 (radixin), and T558 (moesin). The extent of ERM phosphorylation was normalized to the level of total ERM protein expression detected by western blotting. We used the extent of ERM phosphorylation as a marker of Rho-kinase activity.[42-44]

Determination of Akt Binding to eNOS. Akt was immunoprecipitated from liver tissue lysates (800 mg) using 5 mL rabbit polyclonal anti-Akt antibody conjugated with protein G-Sepharose (30 mL; GE Healthcare, Uppsala, Sweden). Akt immunoprecipitates were then separated by SDS-PAGE and immunoblotted with the eNOS antibody (1:500 dilution). eNOS was immunoprecipitated from liver tissue lysates (800 mg) using 5 mL mouse monoclonal anti-eNOS antibody conjugated with protein G-Sepharose (30 mL). eNOS immunoprecipitates were then separated by SDS-PAGE and immunoblotted with the Akt antibody (1:500 dilution).

Determination of Akt Binding to Rho-kinase (ROCK2). Akt was immunoprecipitated from liver tissue lysates (800 mg) using 5 mL rabbit polyclonal anti-Akt antibody conjugated with protein G-Sepharose (30 mL). Akt immunoprecipitates were then separated by SDS-PAGE and immunoblotted with the ROCK2 antibody (1:500 dilution). Nonimmune rabbit serum was used as an immunoprecipitation control. ROCK2 was immunoprecipitated from liver tissue lysates (800 mg) using 5 mL mouse monoclonal anti-ROCK2 antibody conjugated with protein G-Sepharose (30 mL). ROCK2 immunoprecipitates were then separated by SDS-PAGE and immunoblotted with the Akt antibody (1:500 dilution). Nonimmune mouse serum was used as an immunoprecipitation control. Membranes were stripped and reprobed with the same antibody as that used for immunoprecipitation, as previously described.[45]

Measurement of Nitrogen Oxide. Under baseline conditions and at 60 minutes after the infusion of graded doses of fasudil, NO metabolites [nitrite and nitrate as nitrogen oxide (NOx)] in the liver were measured using an NOx-analyzing high-performance liquid chromatography system (ENO-20, Eicom Co., Kyoto, Japan) as previously described.[46,47] In brief, liver tissues were homogenized with the addition of twice the volume of methanol. Homogenates were centrifuged for 20 minutes at 15,000 rpm and supernatants extracted. Nitrite and nitrate were separated by a reverse-phase column. Nitrate was reduced to nitrite by a Cd reduction column. An azo

dye compound formed from nitrite by Griess reaction was measured using an ultraviolet detector at 540 nm.

Measurement of Hepatic and Renal Function. After the intravenous infusion of fasudil for 60 minutes, blood samples were obtained from the inferior vena cava. These were used to measure the level of total bilirubin, direct bilirubin, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, and alkaline phosphatase as parameters indicative of hepatic function. They were also used to measure blood urea nitrogen and creatinine as parameters indicative of renal function.

Plasma Concentrations of Fasudil. Plasma concentrations of fasudil were measured after intravenous infusion of 1 mg/kg/hour or 2 mg/kg/hour fasudil for 60 minutes. Blood samples were obtained from the inferior vena cava in each rat. Plasma concentrations were measured by high-performance liquid chromatography.[48]

Chronic Administration of Fasudil in BDL Rats. Eighteen BDL rats received saline (control), 10 mg/kg/day, or 30 mg/kg/day fasudil by oral administration for 4 weeks from 3 weeks after the BDL, as previously described.[34,49] Rats were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally), and MAP, PVP, and HTBF were measured. After hemodynamic measurement for 15 minutes, blood samples were obtained from the inferior vena cava to assess hepatic and renal functions.

Statistical Analysis. Results are expressed as mean ± standard error of the mean (SEM). Comparisons of data between BDL rats and SO rats were evaluated by Student *t* test. Comparisons of data among multiple groups were evaluated with analysis of variance followed by Tukey-Kramer's post hoc test. Two-way repeated-measures analysis of variance was used to compare responses to the intravenous infusion of graded doses of fasudil. A value of *P* less than 0.05 was considered statistically significant.

Table 1. Hemodynamic Parameters at Baseline in SO and BDL Rats

Variable	SO Rats	BDL Rats	*P* Value
Mean arterial pressure (mm Hg)	147.7 ± 1.4	122.3 ± 6.0	<0.01
Portal venous pressure (mm Hg)	6.4 ± 0.6	15.8 ± 0.7	<0.0001
Hepatic tissue blood flow (mL/minute)	19.9 ± 0.4	16.8 ± 0.6	<0.01
Portal venous flow (mL/minute)	19.7 ± 0.5	–	–

SO, sham-operated; BDL, bile duct ligation.
Results are expressed as mean ± SEM (n = 5, each group).

Results

Hemodynamic Parameters at Baseline in SO Rats and BDL Rats. As expected, MAP was significantly lower and PVP was significantly higher in BDL compared with SO rats (Table 1). HTBF was also significantly lower in BDL than SO rats. Although in BDL rats portal venous flow could not be measured because of the severe adhesion around the portal vein trunk, the portal venous flow was similar to the HTBF in SO rats.

Activation of Rho-Kinase Signaling in the Livers of BDL Rats. To test whether Rho-kinase signaling was

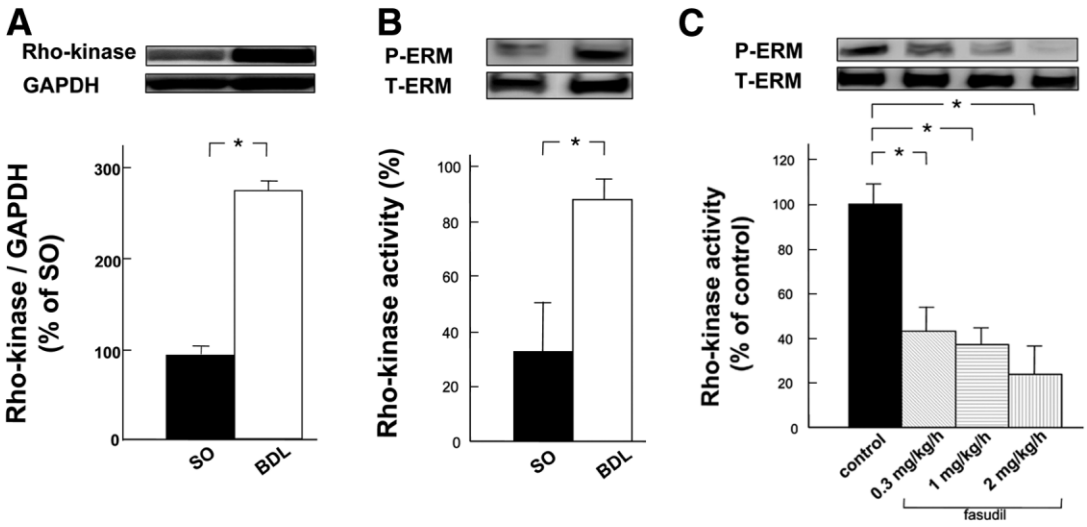


Fig. 1. Effects of fasudil on Rho-kinase activity in livers of BDL rats. (A) Rho-kinase protein expression was evaluated by western blotting in livers from BDL rats compared with SO rats. Results represent a percentage of the SO rats (100%) (mean ± SEM) (n = 5, each group). *$P < 0.05$ compared with SO rats. (B) Rho-kinase activity was evaluated by the extent of ERM phosphorylation in livers of SO and BDL rats under baseline conditions. Results represent the ratio of phosphorylation to total ERM (mean ± SEM) (n = 5). *$P < 0.05$ compared with SO rats. (C) Rho-kinase activity was evaluated in the livers at 60 minutes after intravenous infusions of graded doses of fasudil. Results represent a percentage of the controls (saline) (100%) (Mean ± SEM, n = 5). *$P < 0.05$.

implicated in the pathogenesis of portal hypertension in BDL rats, we examined the expression of Rho-kinase protein and Rho-kinase activities in whole livers of SO and BDL rats. Western blot analysis of whole liver homogenates showed a strong up-regulation of Rho-kinase protein levels in livers from BDL rats compared with sham operated rats (Fig. 1A). Rho-kinase activity is indicated by the extent of ERM phosphorylation normalized to the level of total ERM. Rho-kinase activity was found to be significantly increased in BDL compared with SO rats (Fig. 1B). We confirmed that fasudil, a specific Rho-kinase inhibitor, substantially inhibited Rho-kinase activity in a dose-dependent manner in the livers of BDL rats (Fig. 1C).

Hemodynamic Effects of Fasudil on BDL Rats. To investigate the role of Rho-kinase signaling in regulating portal and systemic hemodynamics, we studied the effects of the intravenous infusion of graded doses of fasudil on PVP, HTBF, and MAP in BDL rats. Both PVP and MAP reached a plateau at 50 minutes after the start of intravenous infusion of fasudil (Fig. 2A, D). Intravenous infusion of the 2 highest doses of fasudil (1 and 2 mg/kg/hour) significantly reduced PVP compared with controls; however, there was no significant difference in changes in PVP between these 2 doses (Fig. 2A). At 60 minutes after drug infusion, the reduction rate in PVP in response to fasudil at these 2 doses was as large as approximately 20% compared with controls, whereas 0.3 mg/kg/hour fasudil reduced PVP by only 10%, which was not statistically significant (Fig. 2B). No dose of fasudil changed HTBF as compared with controls (Fig. 2C).

Fasudil 2 mg/kg/hour was found to evoke a significant decrease in MAP compared with controls (Fig. 2D). However, 1 mg/kg/hour fasudil tended to decrease MAP compared with controls, but not significantly. In response to 2 mg/kg/hour fasudil, at 60 minutes after infusion, the reduction rate in MAP was approximately 20%, which was significantly larger than in controls (Fig. 2E). However, 1 mg/kg/hour fasudil reduced MAP by just 8%, which was not statistically significant in comparison with controls (Fig. 2E).

Hemodynamic Effects of L-NAME on BDL Rats Treated With or Without Fasudil. Fasudil potentially affects intrahepatic circulation through the inhibition of Rho-kinase in HSCs, NO production from SECs, or both. To determine whether the hemodynamic effects of fasudil in BDL rats are associated with the production of NO by SECs, we investigated the effects of L-NAME (15 mg/kg) on fasudil-induced responses of PVP and HTBF in BDL rats. Pretreatment with L-NAME tended to increase PVP at baseline in BDL rats, although L-NAME treatment did not change PVP for 60 minutes. Fasudil 1 mg/kg/hour significantly reduced PVP compared with controls in BDL rats, whereas it did not significantly reduce PVP in BDL rats pretreated with L-NAME (Fig. 3A). The reduction rate of PVP by fasudil (1 mg/kg/hour)

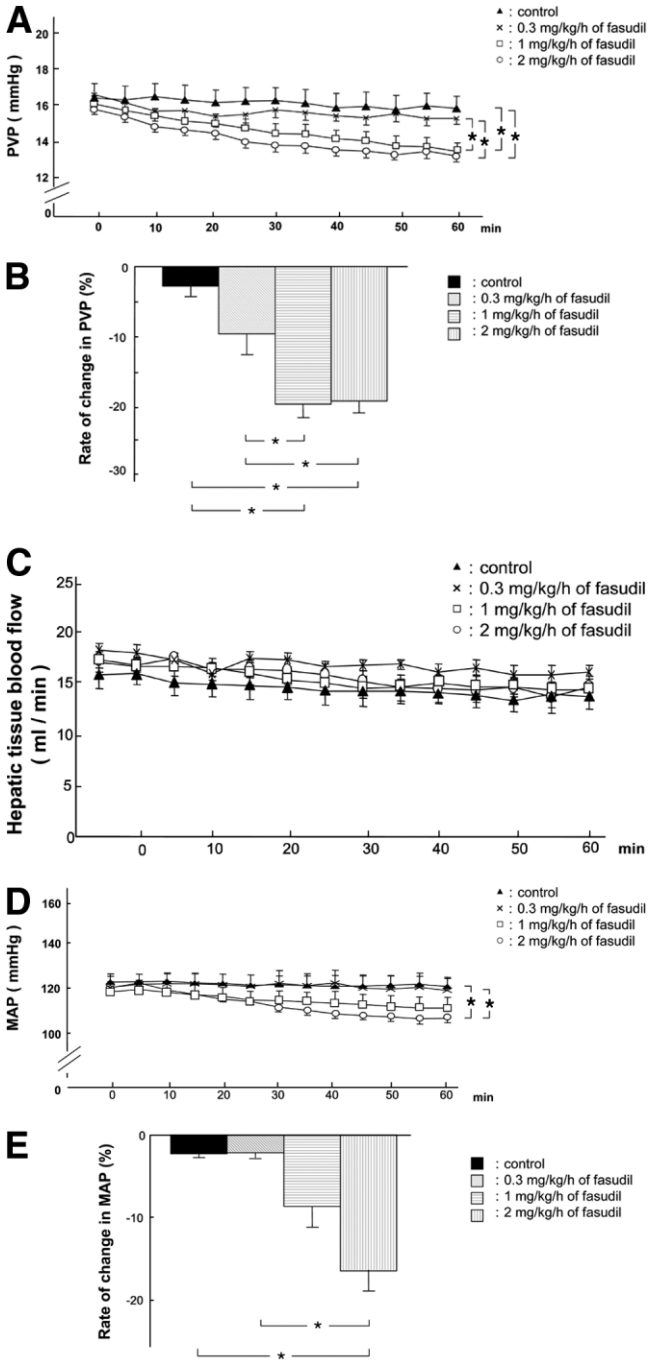
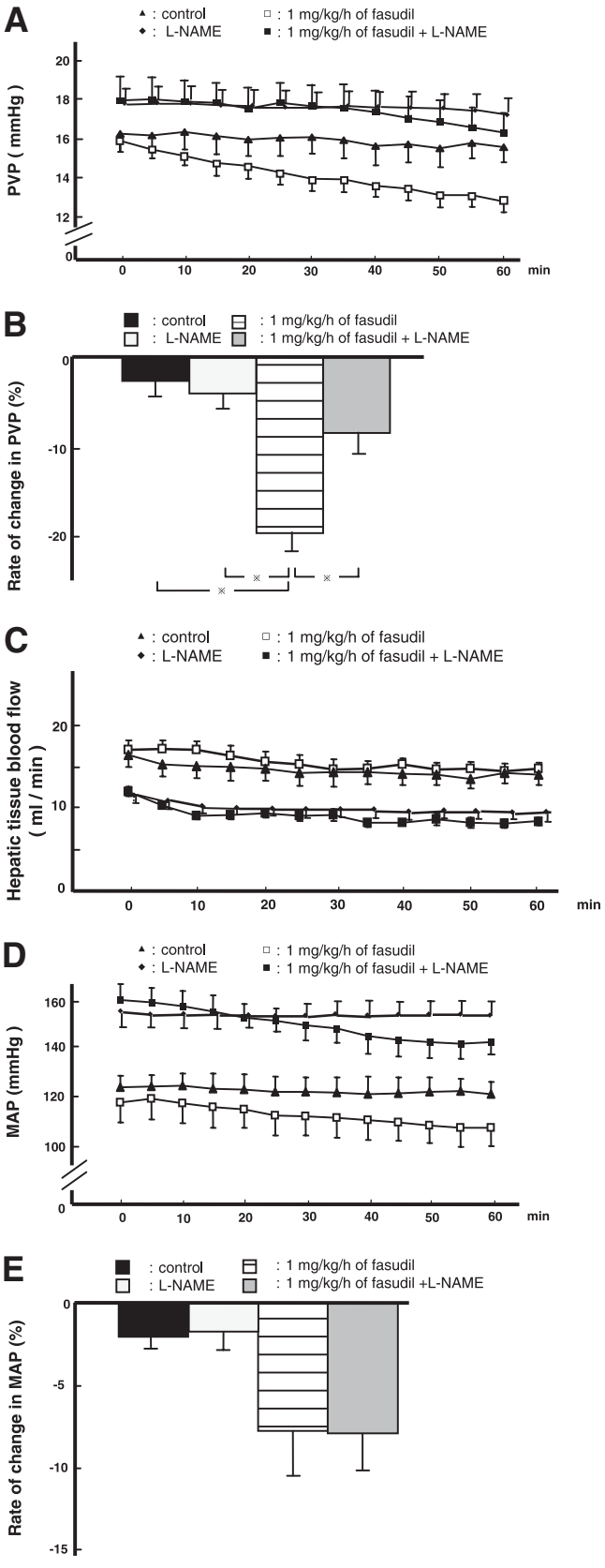


Fig. 2. Hemodynamic effects of fasudil on BDL rats. (A, C, D) In BDL rats, PVP (A), HTBF (C), and MAP (D) were measured during intravenous infusion of saline (control), 0.3, 1, or 2 mg/kg/hour fasudil. Results are expressed as mean ± SEM (n = 6, each group). *$P < 0.05$. (B, E) Rates of change in PVP (B) and MAP (D) were measured at 60 minutes after the intravenous infusion of saline (control) or graded doses of fasudil in comparison with baseline. Results are expressed as mean ± SEM (n = 6, each group). *$P < 0.05$.



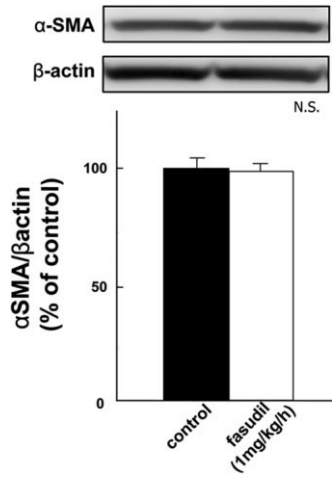


Fig. 4. Effects of fasudil on α-SMA expression in livers of BDL rats Alpha-smooth muscle actin was evaluated by western blotting in livers at 60 minutes after intravenous infusions of fasudil (1 mg/kg/hour) and normal saline (control). Results represent a percentage of the controls (saline) (100%) (Mean $\pm$ SEM, n = 4). $^*P < 0.05$.

was as small as 8% in BDL rats pretreated with L-NAME, compared with 20% in BDL rats without L-NAME (Fig. 3B). The rate of the reduction of PVP by fasudil in BDL rats pretreated with L-NAME was only 8%, reasonably similar to the 4% found in BDL rats pretreated with L-NAME without fasudil. Pretreatment with L-NAME significantly reduced HTBF at baseline, whereas even subsequent treatment with fasudil could not recover HTBF (Fig. 3C).

To determine whether the relaxing effect of fasudil on MAP may be associated with either the contraction of vascular smooth muscle cells, the production of NO by endothelial cells, or both, we investigated the effect of L-NAME on fasudil-induced response of MAP. L-NAME significantly increased MAP in BDL rats at baseline. Fasudil treatment tended to decrease MAP both in BDL rats and in L-NAME–treated BDL rats (Fig. 3D). The reduction rate of MAP by fasudil (1 mg/kg/hour) was approximately 8% in BDL rats, which was similar to that in BDL rats pretreated with L-NAME (Fig. 3E).

Fig. 3. Effects of L-NAME on fasudil-induced responses in PVP, HTBF, and MAP. (A, C, D) PVP (A), HTBF (C), and MAP (D) were measured in BDL rats during infusion of saline (control) or fasudil (1 mg/kg/hour), in BDL rats pretreated with L-NAME (15 mg/kg) during the infusion of fasudil (1 mg/kg/hour) and in BDL rats pretreated with L-NAME (15 mg/kg/hour). (B,E): Rates of change in PVP (B) and MAP (E) were measured at 60 minutes after the infusion of saline (control) or fasudil (1 mg/kg/hour) in BDL rats, in BDL rats pretreated with L-NAME (15 mg/kg) at 60 minutes after the infusion of fasudil, and in BDL rats pretreated with L-NAME. Results are expressed as mean $\pm$ SEM (n = 6, each group). $^*P < 0.05$.

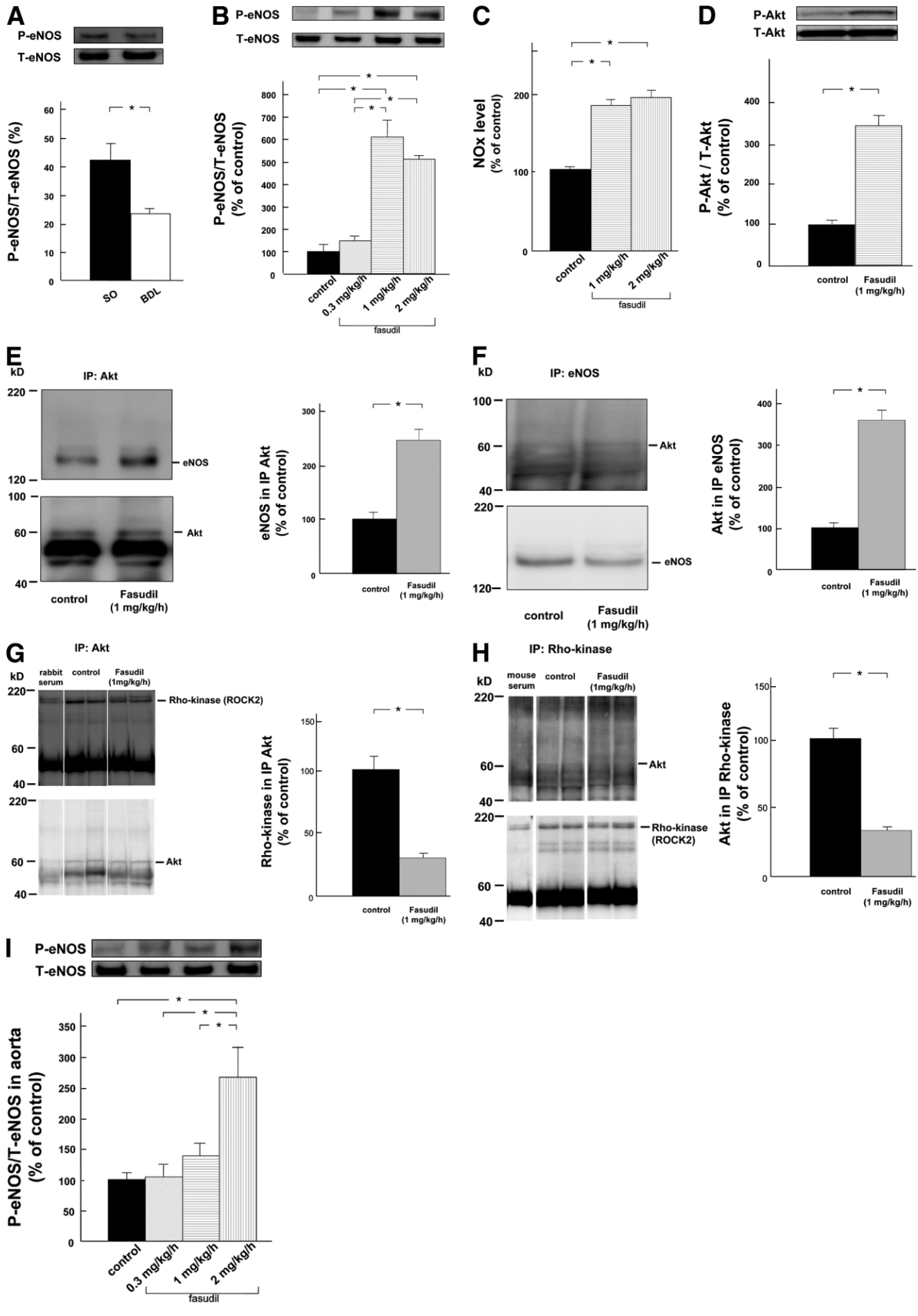

A
P-eNOS
T-eNOS
P-eNOS/T-eNOS (%)
SO
BDL
B
P-eNOS
T-eNOS
P-eNOS/T-eNOS (% of control)
control
0.3 mg/kg/h
1 mg/kg/h
2 mg/kg/h
fasudil
C
NOx level (% of control)
control
1 mg/kg/h
2 mg/kg/h
fasudil
D
P-Akt
T-Akt
P-Akt / T-Akt (% of control)
control
Fasudil (1 mg/kg/h)
E
kD
IP: Akt
eNOS
Akt
control
Fasudil (1 mg/kg/h)
eNOS in IP Akt (% of control)
F
kD
IP: eNOS
Akt
eNOS
control
Fasudil (1 mg/kg/h)
Akt in IP eNOS (% of control)
G
IP: Akt
kD
rabbit serum
control
Fasudil (1mg/kg/h)
Rho-kinase (ROCK2)
Akt
Rho-kinase in IP Akt (% of control)
H
IP: Rho-kinase
kD
mouse serum
control
Fasudil (1mg/kg/h)
Akt
Rho-kinase (ROCK2)
Akt in IP Rho-kinase (% of control)
I
P-eNOS
T-eNOS
P-eNOS/T-eNOS in aorta (% of control)
control
0.3 mg/kg/h
1 mg/kg/h
2 mg/kg/h
fasudil

Fig. 5.

Effects of Fasudil on α-SMA Expression in Livers of BDL Rats. To determine the effect of fasudil on HSC activation related to HSC contraction, we examined expressions of α-SMA by western blotting in livers of BDL rats. We found that 1 mg/kg/hour fasudil did not change the expression of α-SMA in livers of BDL rats (Fig. 4).

The Role of Rho-Kinase in eNOS Signaling in Secondary Biliary Cirrhosis. eNOS has been shown to be regulated by the phosphorylation of eNOS by Akt, and the down-regulation of eNOS has been implicated in liver cirrhosis.[14,21] The current study with L-NAME suggests that intravenous infusion of fasudil in the livers of BDL rats may affect NO production in SECs rather than the inhibition of Rho-kinase in HSCs. Therefore, to determine whether Rho-kinase might negatively regulate eNOS signaling in livers of BDL rats, we studied the effect of fasudil on Akt-eNOS signaling. Consistent with a previous study,[21] the eNOS phosphorylation level in the liver was significantly decreased in BDL rats compared with SO rats (Fig. 5A). Fasudil 1 and 2 mg/kg/hour significantly increased the eNOS phosphorylation level compared with controls (Fig. 5B). Fasudil at these 2 doses also significantly increased the NOx level in livers compared with controls (Fig. 5C).

Fasudil treatment with 1 mg/kg/hour significantly increased the phosphorylation of Akt in BDL rats compared with controls (Fig. 5D).

In BDL rats, with or without fasudil treatment, eNOS was detected in immunoprecipitates of Akt, whereas Akt was also detected in the immunoprecipitates of eNOS. The Akt-eNOS binding was significantly enhanced in livers of BDL rats by fasudil treatment (Fig. 5E,F). Rho-kinase was detected in the immunoprecipitates of Akt (Fig. 5G,H), and Akt was detected in the immunoprecipitates of Rho-kinase in BDL rats with or without fasudil treatment. This clearly indicated a direct association between these 2 enzymes in livers of BDL rats. Binding of Rho-kinase to Akt was greatly decreased in BDL rats by fasudil treatment.

Effect of Fasudil on eNOS Phosphorylation in the Aortas of BDL Rats. Fasudil 2 mg/kg/h significantly increased the level of eNOS phosphorylation compared with controls, but fasudil treatment with lower doses did not significantly increase eNOS phosphorylation in the aortas of BDL rats (Fig. 5I).

Effect of the Intravenous Infusion of Fasudil on Hepatic and Renal Function. We measured blood chemistry after intravenous infusion of fasudil for 60 minutes (Table 2) and found that intravenous infusion for 60 minutes of any dose of fasudil did not exacerbate hepatic or renal functions.

Plasma Concentrations of Fasudil. The plasma concentrations of fasudil in the BDL rats that received intravenous infusions of fasudil are shown in Table 3. The plasma concentration of fasudil in rats that received 1 mg/kg/hour fasudil was within the range of plasma concentrations described in a previous report with healthy human subjects treated with 1 mg/kg/hour fasudil (299.2 ng/mL).[50]

Effect of Chronic Administration of Fasudil in BLD Rats. To assess the safety of the chronic administration of fasudil in BDL rats, we investigated hemodynamic changes and the alterations in hepatic and renal functions in BDL rats treated with graded doses of fasudil

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Fig. 5. Effects of fasudil on eNOS phosphorylation in BDL rats. (A) Phosphorylated eNOS was determined by western blotting using a specific antibody of phosphorylated eNOS in livers of BDL rats. The same membrane was stripped and total eNOS protein expression was determined by reprobing with anti-eNOS antibody. Phosphorylation levels of eNOS were determined after normalization to total eNOS expression. Results are expressed as mean ± SEM (n = 5). *$P < 0.05$ versus SO rats. (B) Phosphorylation levels of eNOS were determined in the livers of BDL rats at 60 minutes after intravenous infusion of saline (control), 0.3, 1, or 2 mg/kg/hour fasudil. Results are expressed as a percentage of the control (100%) (mean ± SEM) (n = 5). *$P < 0.05$. (C) NOx (nitrite and nitrate) were measured using an NOx-analyzing high-performance liquid chromatography system (ENO-20) in livers of BDL rats at 60 minutes after intravenous infusion of saline (control), or 1 or 2 mg/kg/hour fasudil. Results represent a percentage of the control (100%) and are expressed as mean ± SEM. *$P < 0.05$. (D) Phosphorylation levels of Akt were determined in livers of BDL rats at 60 minutes after intravenous infusion of control and 1 mg/kg/hour fasudil. Results are expressed as a percentage of the control (100 %) (mean ± SEM) (n = 4). *$P < 0.05$. (E, F) Liver tissues were obtained from BDL rats treated with saline (controls) or 1 mg/kg/hour fasudil. eNOS was immunoprecipitated from liver tissue lysates with anti-eNOS antibody conjugated with protein G-Sepharose. Akt was immunoprecipitated from the liver tissue lysates with anti-Akt antibody conjugated with protein G-sepharose. The immunoprecipitates of either eNOS or Akt were immunoblotted with reciprocal antibodies. Membranes were then stripped and reprobed with the same antibody as used for immunoprecipitation. Results represent a percentage of controls (100%) and are expressed as mean ± SEM. *$P < 0.05$ (n = 4 to 5 per group). (G, H) Liver tissues were obtained from BDL rats treated either with saline (controls) or with fasudil (1 mg/kg/hour). Rho-kinase was immunoprecipitated from liver tissue lysates with anti-Rho-kinase (ROCK2) antibody conjugated with protein G-sepharose. Nonimmune mouse serum was used as an immunoprecipitation control. Akt was immunoprecipitated from liver tissue lysates with anti-Akt antibody conjugated with protein G-sepharose. Nonimmune rabbit serum was used as an immunoprecipitation control. The immunoprecipitates of either Rho-kinase (ROCK2) or Akt were immunoblotted with reciprocal antibodies. Membranes were then stripped and reprobed with the same antibody as used for immunoprecipitation. Results represent a percentage of controls (100%) and are expressed as mean ± SEM. *$P < 0.05$ (n = 4 per group). (I) Phosphorylation levels of eNOS were determined in the aortas of BDL rats at 60 minutes after intravenous infusion of saline (control) and 0.3, 1, or 2 mg/kg/hour fasudil. Results are expressed as a percentage of the control (100%) (mean ± SEM) (n = 5). *$P < 0.05$.

Table 2. Effect of Fasudil on Hepatic and Renal Function

Factor	SO Rats (n = 5)	BDL Rats (n = 5)			
		Saline	0.3 mg/kg/hour	1 mg/kg/hour	2 mg/kg/hour
Total bilirubin (mg/dL)	0.03 ± 0.007	6.6 ± 0.6	6.7 ± 0.4	6.4 ± 0.7	5.6 ± 0.6
Direct bilirubin (mg/dL)	0.02 ± 0.002	4.9 ± 0.5	5.1 ± 0.3	4.8 ± 0.6	4.3 ± 0.5
AST (IU/L)	96 ± 5	717 ± 103	537 ± 112	517 ± 57	543 ± 47
ALT (IU/L)	29 ± 2	104 ± 24	97 ± 9	103 ± 22	88 ± 4
LDH (IU/L)	729 ± 174	782 ± 108	419 ± 69	430 ± 188	426 ± 148
ALP (IU/L)	794 ± 142	1056 ± 51	1166 ± 220	1110 ± 70	1120 ± 72
BUN (mg/dL)	14.5 ± 0.2	23.7 ± 0.6	20.7 ± 1.3	22.3 ± 1.7	25.5 ± 2.4
Creatinine (mg/dL)	0.17 ± 0.01	0.21 ± 0.01	0.26 ± 0.01	0.26 ± 0.03	0.25 ± 0.01

SO, sham-operated; BDL, bile duct ligation; AST, aspartate aminotransferase; ALT, alamine aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; BUN, blood urea nitrogen.

Results are expressed as mean ± SEM (n = 5, each group).

(0, 10, 30 mg/kg/day) by oral administration for 4 weeks from 3 weeks after BDL (Fig. 6). Chronic administration of fasudil at 10 and 30 mg/kg/day significantly reduced PVP by 18% and 19%, respectively, compared with BDL rats treated with saline (controls). However, no dose of fasudil significantly changed MAP. Both 10 and 30 mg/kg/day fasudil significantly increased HTBF. No dose of chronically administered fasudil exacerbated hepatic or renal functions in BDL rats (Table 4).

Discussion

The current study demonstrates that in secondary biliary cirrhosis (1) Rho-kinase activation is substantially involved in the defective eNOS signaling; (2) Rho-kinase may directly interact with Akt and subsequently inhibit Akt-eNOS signaling; and (3) the intravenous infusion of a regular dose of fasudil (1 mg/kg/hour), a specific inhibitor of Rho-kinase, significantly decreases PVP without reducing HTBF through the up-regulation of eNOS.

Liver cirrhosis is associated with increased intrahepatic vascular resistance leading to portal hypertension. HSC contractility, which is regulated by the balance between vasoconstricting agents such as endothelin-1 and vasorelaxing agents such as NO, contributes to increased intrahepatic vascular resistance. HSCs are also a major source of extracellular matrix in liver cirrhosis, and their activation increases hepatic fibrosis, resulting in increased intrahepatic vascular resistance. Rho-kinase, a downstream effector of GTPase Rho, has been shown to be involved in the molecular mechanisms of arteriosclerosis in general, particularly vascular smooth muscle hypercontraction/hypertrophy and the down-regulation of eNOS.[22-26,30,51] Rho-kinase also has been shown to contribute to increased intrahepatic resistance in liver cirrhosis. Rho-kinase mediates the contraction of HSCs induced by endothelin-1 and activates HSCs, leading to the progression of liver fibrosis.[27-29] Recent studies have shown that in human umbilical vein endothelial cells, Rho-kinase inhibits eNOS phosphorylation through the inhibition of Akt.[30] Therefore, it is possible that Rho-kinase also may be associated with the down-regulation of eNOS in SECs, contributing to increased intrahepatic resistance. In the current study, we focused on the association between Rho-kinase and eNOS signaling in the livers of rats with secondary biliary cirrhosis.

Table 3. Plasma Concentration of Fasudil in BDL Rats

Fasudil	1 mg/kg/hour	2 mg/kg/hour
Plasma concentration	207.5	394.9

Results are expressed as mean ± SEM (n = 4, each group).

In the current study, protein expression of Rho-kinase in the liver of BDL rats significantly increased, by 200% compared with SO rats. Rho-kinase activity in the liver of BDL rats also significantly increased, by 200%, compared with SO rats (Fig. 1B). In BDL rats, the increase in Rho-kinase activity might have resulted from increased protein expression, and the transcriptional regulation of Rho-kinase might influence the inhibitory interaction of Rho-kinase and Akt in BDL rats. Inhibition of Rho-kinase by fasudil can potentially affect Rho-kinase in HSCs, where it mediates the contraction of HSCs and liver fibrosis, or NO production from SECs, or both. The fact that fasudil acutely ameliorated portal hypertension indicates that the acute hemodynamic effect of fasudil was not associated with an improvement in liver fibrosis. In the current study, the intravenous infusion of fasudil at both 1 and 2 mg/kg/hour significantly decreased PVP without causing a reduction in HTBF, indicating that fasudil may be able to reduce intrahepatic vascular resistance in secondary biliary cirrhosis. Furthermore, the inhibition of NO production by L-NAME greatly reduced the fasudil-induced effects on PVP and HTBF. Acute administration of fasudil did not affect the expression of α-SMA, which is

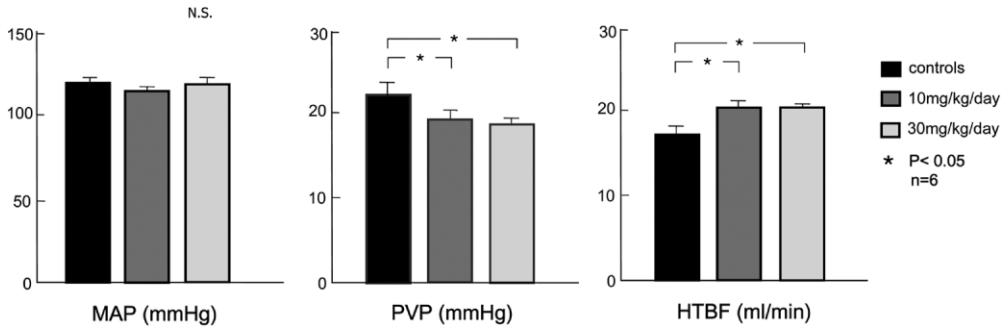

Fig. 6. Effects of chronic treatment of fasudil in BDL rats. PVP, MAP, and HTBF were measured in BDL rats that received saline, 10 mg/kg/day, or 30 mg/kg/day fasudil by oral administration for 4 weeks from 3 weeks after the BDL. Results are expressed as mean ± SEM (n = 6, each group). *$P < 0.05$.

related to HSC activation in the livers of BDL rats. These results indicated that the improvement in intrahepatic hemodynamics by acute administrations of fasudil might be mediated by an enhancement of NO production by SECs, rather than by direct inhibition of Rho-kinase in HSCs.

Next, we examined the effects of fasudil on eNOS signaling in secondary biliary cirrhosis to clarify the relationship between Rho-kinase and defective eNOS signaling. Previous studies have shown that decreased NO production by SECs leads to increased intrahepatic vascular resistance[14] and that this abnormality might be related to defective Akt-eNOS signaling in SECs.[21] However, the molecular mechanisms involved in this defective Akt-eNOS signaling are not fully understood. We showed that the inhibition of Rho-kinase by fasudil significantly increases eNOS phosphorylation levels with up-regulation of Akt phosphorylation in secondary biliary cirrhosis. We also showed that fasudil significantly enhanced the direct binding of Akt and eNOS. These results indicated that Rho-kinase activation is likely to result in the down-regulation of eNOS phosphorylation via the attenuation of Akt-eNOS binding in secondary biliary cirrhosis. To the best of our knowledge, this is the first demonstration of a role for Rho-kinase in defective eNOS signaling in cirrhotic liver. Recently, it was shown that GRK2 negatively regulates eNOS phosphorylation and NO production in SECs through its direct binding to Akt.[21] GRK2 and Rho-kinase have similar characteristics that allow them to phosphorylate ezrin at T567.[52] In the current study, to determine how Rho-kinase could inhibit Akt, we studied the direct association between Rho-kinase and Akt by co-immunoprecipitation. Although the current study has some limitations of the data collected from whole liver, Rho-kinase directly interacted with Akt and led to the inhibition of Akt-eNOS binding, such as the GRK2–Akt interaction (Fig. 5G, H). The interaction between GRK2 and Rho-kinase in Akt-eNOS signaling in liver cirrhosis remains unknown and will require further study.

The fasudil-induced hypotensive response was more sensitive in the intrahepatic vascular system than in aortas of BDL rats (Fig. 2A,B, D,E). This is consistent with the result that the significant increase in eNOS phosphorylation was achieved by a lower dose of fasudil in livers of BDL rats than in their aortas (Fig. 5B, I). This might be attributable to the preexisting NO overproduction by increased shear stress and elevated levels of tumor necrosis

Table 4. Effect of Chronic Administration of Fasudil on Hepatic and Renal Function

Factor	SO Rats (n = 5)	BDL Rats (n = 5)		
		Saline	10 mg/kg/day	30 mg/kg/day
Total bilirubin (mg/dL)	0.03 ± 0.007	5.4 ± 0.9	5.5 ± 0.3	4.7 ± 1.5
Direct bilirubin (mg/dL)	0.02 ± 0.002	4.0 ± 0.6	4.2 ± 0.2	3.6 ± 1.1
AST (IU/L)	96 ± 5	778 ± 115	567 ± 61	385 ± 70
ALT (IU/L)	29 ± 2	159 ± 28	125 ± 14	80 ± 8
LDH (IU/L)	729 ± 174	1089 ± 350	640 ± 85	683 ± 179
ALP (IU/L)	794 ± 142	1016 ± 113	1296 ± 52	1032 ± 147
BUN (mg/dL)	14.5 ± 0.2	25.0 ± 2.4	24.9 ± 1.5	27.0 ± 1.7
Creatinine (mg/dL)	0.17 ± 0.01	0.32 ± 0.05	0.26 ± 0.01	0.29 ± 0.01

SO, sham-operated; BDL, bile duct ligation; AST, aspartate aminotransferase; ALT, alamine aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; BUN, blood urea nitrogen.

Results are expressed as mean ± SEM (n = 5, each group).

factor alpha (TNF-α) in the aortas of BDL rats.[53,54] As well, in spite of the inhibition of NO by L-NAME, fasudil tended to decrease MAP in BDL rats (Fig. 3D,E). Although 1 mg/kg/hour fasudil did not increase eNOS phosphorylation in the aortas of BDL rats, it tended to decrease MAP. These results indicated that fasudil may have affected the relaxation of vascular smooth muscle cells, rather than NO production in endothelial cells in the aortas of BDL rats.

In Japan, intravenous infusion of fasudil (1 mg/kg/hour) is used clinically in patients with subarachnoid hemorrhage as prophylaxis against spasms of the brain vessels. The mean value of the plasma concentrations of fasudil in BDL rats that received intravenous infusion of 1 mg/kg/hour fasudil for 60 minutes was within the clinical therapeutic range as shown in a previous study (fasudil: 299.2 ng/mL).[50]

A regular dose of fasudil (1 mg/kg/hour) did not affect hepatic and renal functions in BDL rats. Moreover, in BLD rats, chronic treatment of fasudil significantly reduced PVP and significantly increased HTBF without any reduction of MAP. No dose of chronically administered fasudil affected hepatic and renal functions in BDL rats. These results indicated that acute and chronic administrations of fasudil would be useful in clinical settings involving liver cirrhosis.

These findings show a novel molecular and mechanistic basis for the increased intrahepatic resistance in liver cirrhosis and provide a novel therapeutic strategy for the treatment of portal hypertension.

Limitations of the Current Study. There are several limitations to the current study that need to be considered. First, the BDL-induced portal hypertension model may not fully represent portal hypertension in humans, and thus the usefulness of Rho-kinase inhibitors should be examined in other portal hypertension models with different causes. Second, we did not directly measure portal venous flow because of the severe adhesion around the portal venous trunk. Thus, liver tissue blood flow was substituted for portal venous flow.

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