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RESEARCH PAPER

Sorafenib targets dysregulated Rho kinase expression and portal hypertension in rats with secondary biliary cirrhosis

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Background and purpose: Extrahepatic vasodilation and increased intrahepatic vascular resistance represent attractive targets for the medical treatment of portal hypertension in liver cirrhosis. In both dysfunctions, dysregulation of the contraction-mediating Rho kinase plays an important role as it contributes to altered vasoconstrictor responsiveness. However, the mechanisms of vascular Rho kinase dysregulation in cirrhosis are insufficiently understood. They possibly involve mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK)-dependent mechanisms in extrahepatic vessels. As the multikinase inhibitor sorafenib inhibits ERK, we tested the effect of sorafenib on haemodynamics and dysregulated vascular Rho kinase in rats with secondary biliary cirrhosis.
Experimental approach: Secondary biliary cirrhosis was induced by bile duct ligation (BDL). Sorafenib was given orally for 1 week (60 $mg \cdot kg^{-1} \cdot d^{-1}$). Messenger RNA levels were determined by quantitative real time polymerase chain reaction, protein expressions and protein phosphorylation by Western blot analysis. Aortic contractility was studied by myographic measurements, and intrahepatic vasoregulation by using livers perfused *in situ*. *In vivo*, haemodynamic parameters were assessed invasively in combination with coloured microspheres.
Key results: In BDL rats, treatment with sorafenib decreased portal pressure, paralleled by decreases in hepatic Rho kinase expression and Rho kinase-mediated intrahepatic vascular resistance. In aortas from BDL rats, sorafenib caused up-regulation of Rho kinase and an improvement of aortic contractility. By contrast, mesenteric Rho kinase remained unaffected by sorafenib.
Conclusions and implications: Intrahepatic dysregulation of vascular Rho kinase expression is controlled by sorafenib-sensitive mechanisms in rats with secondary biliary cirrhosis. Thus, sorafenib reduced portal pressure without affecting systemic blood pressure.
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Keywords: portal hypertension; hepatic cirrhosis; chronic liver disease; cholestasis; vasocontraction; intrahepatic microcirculation; Rho kinase; hepatic stellate cells; sorafenib

Abbreviations: BDL, bile duct ligation; Ct, number of cycles required to exceed a threshold; ERK, extracellular signal-regulated kinase; HSC, hepatic stellate cell(s); L-NAME, Nω-nitro-L-arginine methyl ester; MEK, mitogen-activated protein kinase/ERK kinase; mRNA, messenger RNA; NOS, nitric oxide synthase; PDGF-R, platelet-derived growth factor receptor; RT-PCR, real time polymerase chain reaction; SEC, sinusoidal endothelial cell; SMA, smooth muscle actin; Y27632, (R)-(+)-*trans*-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide

Introduction

In advanced hepatic cirrhosis, increased intrahepatic vascular resistance and splanchnic hyperperfusion contribute to portal hypertension (Groszmann and Abraldes, 2005). The increase in splanchnic flow results from persistent mesenteric vasodilation together with angiogenesis (Tsai, 2007; Wiest, 2007; Hennenberg *et al.*, 2008), whereas the increased resistance of the intrahepatic vasculature is explained by hyperresponsiveness to vasoconstrictors and concomitant fixed structural changes of the organ (Rockey, 2003; Hernandez-Guerra *et al.*, 2005). In both phenomena, the alterations in vasoconstrictor responsiveness are associated with a dysregulated expression of the contraction-mediating vasoconstrictor effector Rho kinase (Hennenberg *et al.*, 2006; 2007; 2008; Zhou *et al.*, 2006; Trebicka *et al.*, 2007; 2008; Wiest, 2007).

In accordance with its considerable role in the regulation and maintenance of systemic and local vascular resistances

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under normal conditions (Somlyo and Somlyo, 2000; 2003; Loirand *et al.*, 2006), dysregulation of Rho kinase is highly involved in the haemodynamic and vascular abnormalities which lead to portal hypertension, at least in experimental cirrhosis (Hennenberg *et al.*, 2006; 2007; 2008; Zhou *et al.*, 2006; Trebicka *et al.*, 2007; 2008; Wiest, 2007; Anegawa *et al.*, 2008). Rho kinase is up-regulated in the intrahepatic vasculature in experimental and most likely also in human cirrhosis contributing to the increase in intrahepatic vascular resistance and vasoconstrictor hyperresponsiveness (Zhou *et al.*, 2006; Trebicka *et al.*, 2007; Anegawa *et al.*, 2008). In contrast, in aortas and mesenteric arteries from rats with secondary biliary cirrhosis, and in hepatic arteries from cirrhotic patients, down-regulation of Rho kinase results in vasoconstrictor hyporesponsiveness and hypocontractility (Hennenberg *et al.*, 2006; 2007; 2008; Wiest, 2007; Trebicka *et al.*, 2008). The mechanisms leading to the adverse Rho kinase up- and down-regulation in liver cirrhosis have not been identified to date.

We found recently that a Raf kinase/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK)-dependent mechanism might be responsible for post-transcriptional Rho kinase down-regulation in hypocontractile vessels from rats with bile duct ligation (BDL) (Hennenberg *et al.*, 2006; 2008) and patients with cirrhosis (Hennenberg *et al.*, 2007). We speculated that this might be targeted by the multikinase inhibitor sorafenib, as sorafenib inhibits ERK (Adnane *et al.*, 2006; Liu *et al.*, 2006; Wilhelm *et al.*, 2006). Therefore, we investigated the effects of a 1 week treatment with sorafenib on haemodynamic abnormalities and dysregulated vascular Rho kinase expression in rats with secondary biliary cirrhosis. The present study suggests that such treatment of BDL rats may indeed reduce portal pressure by correction of Rho kinase expression.

Methods

Animals

All animal care and protocols for the study were approved by the local committee for animal studies (Cologne District Government, 50.203-Bn 15, 23/03). Secondary biliary cirrhosis in male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) was induced by BDL as previously described (Hennenberg *et al.*, 2006). In brief, the common bile duct of rats, each with an initial body weight of approximately 200 g, was exposed after median laparotomy and ligated twice. The segment between the two ligations was resected and the abdomen was sutured. Four to 5 weeks after BDL, when cirrhosis with ascites was present, cirrhotic rats were randomly divided into two groups. Rats of one group were treated with the multikinase inhibitor sorafenib (60 $mg \cdot kg^{-1} \cdot d^{-1}$) for 7 days by gavage, while the remaining group was treated with solvent. Sham-operated rats served as controls and were treated equally. In these rats, the common bile duct was exposed, but no ligation or resection was performed.

Quantitative real time polymerase chain reaction (RT-PCR)

RNA was isolated from 30 mg shock frozen aortic, mesenteric arterial or liver tissue with the RNeasy-mini kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. RNA concentrations were measured spectrophotometrically at 260 nm. For each sample, 1 μg total RNA was used. Prior to reverse transcription, samples were DNA-digested by incubation with RQ1 RNase-free DNAse (Promega, Madison, WI, USA). Reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Karlsruhe, Germany) and random primers (250 ng, Microsynth, Balgach, Switzerland). Control reactions did not contain reverse transcriptase. For detection of Rho kinase, custom synthesized primers and probes were used as described recently (Hennenberg *et al.*, 2006). Primers and probes for β-arrestin 2, platelet-derived growth factor receptor (PDGF-R)β and the house-keeping gene (18SrRNA) were provided by Applied Biosystems as a ready-to-use mix and used according to the manufacturer's guidelines. RT-PCR was performed using the ABI 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). The PCR reaction was performed in a volume of 25 μL containing 12.5 μL 2× TaqMan PCR master mix (Roche Molecular Systems, Mannheim, Germany/Applied Biosystems, Foster City, CA, USA) and 2 μL (equivalent to 67 ng total RNA) cDNA. 18SrRNA served as endogenous control, the final concentrations for primers were 100 $nmol \cdot L^{-1}$, and for the probe 200 $nmol \cdot L^{-1}$. The results were expressed as the number of cycles required to exceed a threshold (Ct value) at which the fluorescence signal exceeded a defined threshold. The difference in Ct value of the target cDNA and the endogenous control are expressed as negative ΔCt values. Therefore, higher $-\Delta Ct$ values denote higher messenger RNA (mRNA) levels. For all of the target genes and for 18SrRNA, a validation experiment was performed according to the manufacturer's guidelines. In these experiments, it was demonstrated that the efficiencies of the RT-PCR for the target gene and the endogenous control were approximately equal. Therefore, the ΔCt method could be used for relative quantification.

Western blotting

Western blot analysis was performed as previously described (Hennenberg *et al.*, 2006; 2007). In detail, the following primary antibodies were used: mouse anti-β-arrestin-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse anti-G-protein-coupled receptor kinase 2 (Biomol, Hamburg, Germany); rabbit anti-phospho-ERK Tyr202-Tyr204 (Cell Signaling Technologies, Danvers, MA, USA); rabbit anti-phosphomoesin Thr558 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit anti-phospho-PDGF-Rβ phospho-Tyr751 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-phospho-PDGF-Rβ Tyr857 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit anti-Rho kinase (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with appropriate peroxidase-coupled secondary antibodies, detection was performed with enhanced chemiluminescence, ECL (Amersham, Amersham, UK). Films were developed using a Kodak X-omat. Densitometric quantification was performed using a FLA-300 phosphoimager (Fuji Film, Dusseldorf, Germany).

Assessment of kinase activities

To evaluate Rho kinase activity, we compared the phosphorylation state of the Rho kinase substrate moesin between

different samples. In different cells and tissues, moesin is phosphorylated by Rho kinase at Thr558 (Hennenberg *et al.*, 2006; 2007; Zhou *et al.*, 2006; Trebicka *et al.*, 2007; 2008). To evaluate the activity of ERK1 and 2, its phosphorylation state at Tyr202 and Tyr204 was detected. ERK is activated by this phosphorylation (Abou-Alfa *et al.*, 2006; Liu *et al.*, 2006). Phospho-moesin and phospho-ERK were detected by Western blot analysis with site- and phospho-specific antibodies. Activation of the PDGF-Rβ was assessed as the phosphorylation state at its autophosphorylation sites Tyr751 and Tyr857.

Analysis of aortic ring contraction

For measurement of contractility of rat isolated aortic rings, freshly excised aortas were used. Aortas were cut into 3–4 mm wide rings and mounted in organ bath chambers containing carbogen-bubbled Krebs-Henseleit solution (37°C) as previously described (Hennenberg *et al.*, 2006; 2007). After an equilibration period of 45 min, a stable baseline was achieved and cumulative concentration response curves for the α_1 -adrenoceptor agonist, methoxamine, were constructed.

Haemodynamic measurements

Haemodynamic studies were performed under ketamine anesthesia (60 mg·kg^{-1} i.m.). This condition has been shown to approximate most closely the conscious state in terms of cardiac output and regional blood flow (Seyde and Longnecker, 1984) and it has been used extensively to investigate haemodynamic effects of drugs lowering portal pressure in animal models of portal hypertension (van de Casteele *et al.*, 2001; Hennenberg *et al.*, 2006; Zhou *et al.*, 2006; Trebicka *et al.*, 2007; 2008). Median laparotomy was performed, a PE-50 catheter was introduced into a small ileocoecal vein and advanced to the portal vein for the measurement of portal pressure. The left femoral artery and vein were cannulated with PE-50 catheters for measurement of arterial pressure and blood withdrawal. Another PE-50 catheter was advanced via the right carotid artery into the left ventricle under pulse curve control. This catheter was used for microsphere application. The catheter in the femoral artery and the portal vein were connected to a pressure transducer (Hugo Sachs Elektronik, March-Hugstetten, Germany) for blood pressure measurement. The zero point was 1 cm above the operating table. After insertion of all catheters, rats were allowed to stabilize hemodynamically for 30 min.

Cardiac index was measured using the coloured microsphere technique as previously described (Hakkinen *et al.*, 1995; Hennenberg *et al.*, 2006). The coloured microsphere technique was validated by the more frequently used radioactive microsphere method (Hakkinen *et al.*, 1995). It has the advantage of lacking radioactivity. A reference sample was obtained for 1 min at a rate of 0.65 mL·min^{-1}, using a continuous withdrawal pump (Hugo Sachs Elektronik, March-Hugstetten, Germany). A total of 300 000 yellow microspheres (15 µm diameter, Triton Technologies, San Diego, CA, USA) were suspended in 0.3 mL saline containing 0.05% Tween and injected into the left ventricle 10 s after the withdrawal pump had been started. Porto-systemic shunting was estimated after injection of 150 000 blue microspheres in 0.3 mL saline containing 0.05% Tween in an ileocoecal vein within 30 s.

Tissue samples were digested by addition of 3.8 mL of 5.3 mol·L^{-1} potassium hydroxide and 0.5 mL of Tween 80 and subsequent boiling for 1 h. The digested tissues and digested blood reference sample were vortexed and filtered using Whatman Nucleopore filters (Whatman International Limited, Madison, UK). The colour of the filtered microspheres was dissolved in 0.2 mL DMF, and the absorption was measured by spectrophotometry. Thereafter, cardiac index and porto-systemic shunting was calculated using software obtained by Triton Technologies. Cardiac index was expressed per 100 g body weight. Splanchnic perfusion pressure was defined as the difference between mean arterial pressure and portal pressure. Splanchnic vascular resistance was calculated from the ratio between splanchnic perfusion pressure and splanchnic blood flow. Hepatic vascular resistance was calculated as the portal pressure divided by sum of gastrointestinal and splenic perfusion minus shunt flow. Systemic vascular resistance was estimated as the ratio between mean arterial pressure and cardiac index.

In situ *liver perfusion*

In situ liver perfusion was performed in a recirculating system as previously described (Zhou *et al.*, 2006; Trebicka *et al.*, 2007). Briefly, rats were fasted overnight but allowed free access to water. After being anaesthetized with ketamine hydrochloride (60 mg·kg^{-1} body weight), the abdomen was opened and the bile duct was cannulated with a polyethylene tube to monitor bile flow in sham-operated rats. Loose ligatures were placed around portal vein, common hepatic artery, spleen vein and inferior vena cava just cranially to the confluence of the right renal vein. A 500 U dose of heparin was injected into the inferior vena cava. The portal vein was cannulated with a 14-gauge Teflon catheter, initiating liver exsanguinations by infusion (30 mL·min^{-1}) of Krebs-Henseleit solution containing heparin (2 U·mL^{-1}) and oxygenated with carbogen. The posterior vena cava was immediately cut caudally to the loose ligature, allowing the perfusate to escape. Thereafter, the thorax was opened and the right atrium was cut. Another catheter was introduced in the right atrium and pushed forward to the inferior vena cava. Next, all ligatures were pulled tight. At a constant flow (30·mL·min^{-1}), perfusion pressure was continuously monitored and recorded digitally online. The temperature of Krebs-Henseleit solution was 37°C and controlled continuously during the entire experiment. The preparation was allowed to stabilize for 10 min without any procedure.

Viability and stability of liver perfusion. The criteria for liver viability included gross appearance of the liver, stable perfusion, stable buffer pH (7.4 ± 0.1), and in sham-operated rats, bile production >0.4 µL (min·g)$^{-1}$, and during the initial 10-minute stabilization period. If one of the viability criteria was not met, the experiment was discarded.

Effect of the α_1-adrenoceptor agonist methoxamine on portal perfusion pressure. After the stabilization period of 10 min, cumulative concentration-response curves with the α_1-adrenoceptor agonist methoxamine (100 nmol·L^{-1}–1 mmol·L^{-1}) were obtained by addition of the agonist to the

perfusate. The effect of methoxamine was expressed as change in perfusion pressure elicited by the given concentration of methoxamine in the perfusate (i.e. perfusion pressure after methoxamine – basal pressure).

Effect of the Rho kinase inhibitor Y27632 on portal perfusion pressure. (R)-(+)-*trans*-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y27632) was added to the perfusate (final concentration 10 $\mu mol \cdot L^{-1}$) after stabilization for 10 min. The effect was expressed as the decrease in perfusion pressure (i.e. basal perfusion pressure – perfusion pressure after Y27632).

Data analysis and statistical procedures

Data are presented as means ± standard error of the mean with the indicated number (*n*) of experiments. Different animal groups were investigated with knowledge of the treatments. *P*-values < 0.05 were considered statistically significant. For the analysis of *in vitro* contractility studies, concentration response curves were fitted by non-linear regression, using the computer software Prism® (Graph Pad Software Inc., San Diego, CA, USA). E_{max} (maximum contraction) and pEC_{50} values (negative logarithm of the concentration producing a half-maximum effect) were calculated from the fitted curves.

Materials and nomenclature

Sorafenib (Nexavar® 200 mg) was obtained from Bayer Healthcare (Germany). After removal of the outer coat, pills were ground with a tissue mill and the resulting powder was mixed with tap water, and applied by gavage to rats. Y27632 was obtained from Biomol (Cologne, Germany), and added as aqueous stock solution to the perfusate in liver perfusion experiments. Methoxamine and Nω-nitro-L-arginine methyl ester were obtained from Sigma (Schnelldorf, Germany) and added as aqueous stock solution to the perfusate or organ bath solution in liver perfusion or contractility experiments respectively. Nomenclature of receptors and enzymes conforms to the 'Guide to Receptors and Channels' (Alexander *et al.*, 2008).

Results

General characteristics

Mean body weight was decreased by treatment with sorafenib for 1 week only in sham-operated rats (Table 1). Liver and spleen weights were increased in untreated BDL rats compared with non-cirrhotic controls (Table 1). In BDL rats, treatment with sorafenib resulted in a decrease of spleen weight and induced a trend to a reduction in liver weight (Table 1). Mortality was less than 15% and did not differ between both groups.

Effects of sorafenib on intracellular signalling

Sorafenib inhibits Raf kinase, which specifically activates ERK via MEK. To assess whether sorafenib was present in the liver and the extrahepatic vessels in our BDL rats, we tested the effect of 1 week's treatment with sorafenib on ERK activity. The treatment of BDL rats with sorafenib caused profound inhibition of ERK1 and ERK2 in aortas and mesenteric arteries (Figure 1A). In the liver, ERK inhibition by sorafenib was less pronounced and confined to ERK2 (Figure 1A).

Besides Raf kinase sorafenib targets different receptor tyrosine kinases including the PDGF-Rβ. PDGF-Rβ activation is associated with autophosphorylation at Tyr751 and Tyr857. In livers of BDL rats, PDGF-Rβ phosphorylation at these sites was clearly diminished in response to chronic sorafenib (Figure 1B). However, we did not find any experimental conditions which allowed the detection of phosphorylated PDGF-Rβ in extrahepatic vessels of BDL rats or sorafenib-treated BDL rats.

Effects of sorafenib on vascular and hepatic Rho kinase

In BDL rats, the treatment with sorafenib resulted in posttranscriptional up-regulation of Rho kinase expression in aortas. While Rho kinase mRNA levels remained unaltered by sorafenib (Figure 2A), the protein expression was raised (Figure 2B). The up-regulation of Rho kinase expression by sorafenib was paralleled by an increased activity of the enzyme in aortas from BDL rats, as assessed by the phosphorylation of the Rho kinase substrate moesin at Thr558 (Figure 2B). By contrast, in aortas from sham-operated rats, sorafenib only induced a trend towards an up-regulated Rho kinase protein expression or activity (data not shown).

In mesenteric arteries of BDL rats, treatment with sorafenib was without effect on Rho kinase expression or activity (Figure 2B). In livers of BDL rats, mRNA and protein expression of Rho kinase was decreased by sorafenib (Figure 2). This change in expression level was accompanied by a corresponding change in hepatic Rho kinase activity (Figure 2B).

Effects of sorafenib on aortic contractility

Next, we tested whether the up-regulation of aortic Rho kinase after sorafenib treatment in BDL rats results in an

Table 1 General characteristics of sorafenib-treated and -untreated rats (means ± SEM, five sham-operated rats, seven BDL rats)

	Body weight (g)	*Liver weight (g)*	*Spleen weight (g)*	*Gastrointestinal tract weight (g)*
Sham	332 ± 6.6	10.2 ± 0.48	0.57 ± 0.02	11.3 ± 1.12
Sham + sorafenib	295 ± 3.7#	11.5 ± 0.21	0.57 ± 0.04	12.8 ± 0.475
BDL	333 ± 19.2	19.4 ± 2.73#	1.84 ± 0.19#	13.4 ± 1.07
BDL + sorafenib	289 ± 10.4	15.5 ± 1.34	1.34 ± 0.10†	11.6 ± 1.05

#*P* < 0.05 versus sham, †*P* < 0.05 versus BDL.
BDL, bile duct ligation.

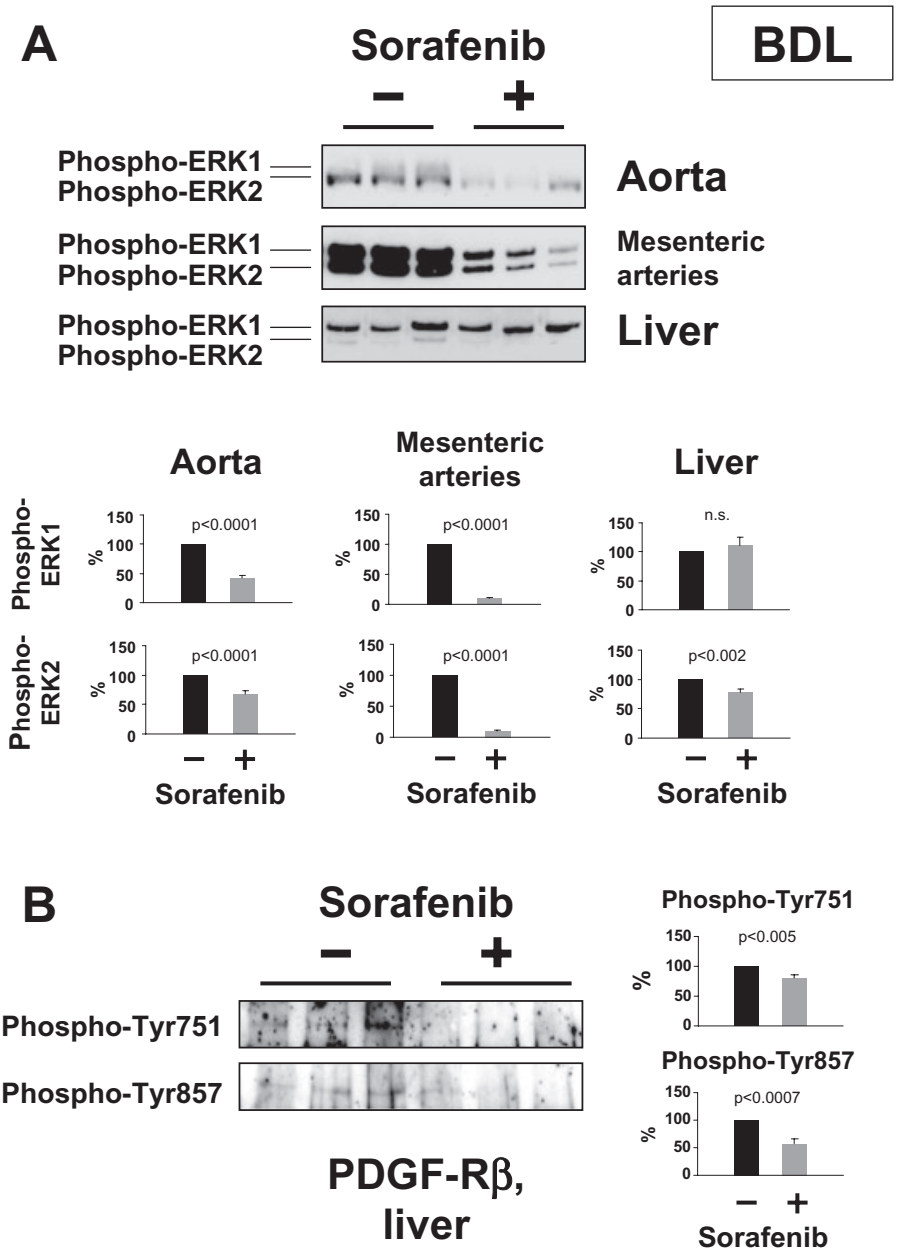

Figure 1 Vascular and intrahepatic targets of sorafenib in BDL rats: intracellular effects of sorafenib in the liver and different vessels. (A) Activity of ERK1 and 2 was monitored as Tyr202-Tyr204 phosphorylation. Shown are representative Western blots and densitometric quantification of all experiments (means ± SEM, $n = 9$ for each group). (B) The activity of the PDGF-Rβ was assessed as its phosphorylation state at the indicated autophosphorylation sites (Tyr751, Tyr857). Representative Western blots and densitometric quantification of all experiments (means ± SEM, $n = 9$ for each group) are shown. BDL, bile duct ligation; ERK, extracellular signal-regulated kinase; PDGF-R, platelet-derived growth factor receptor.

improvement of contractility. Indeed, the treatment with sorafenib increased the contractility to the α_1-adrenoceptor agonist methoxamine in aortic rings from BDL rats in terms of the Emax, which was increased by sorafenib (Figure 3A). However, the treatment of BDL rats with sorafenib did not change the reduced EC_{50} of aortic rings to methoxamine (Figure 3A).

Effects of sorafenib on aortic β-arrestin 2 expression

In BDL rats, up-regulated β-arrestin 2 expression represents an important determinant of aortic vasoconstrictor responsiveness and Rho kinase activation (Hennenberg *et al.*, 2006; 2008). In contrast to its effect on Rho kinase, treatment with sorafenib was without effects on mRNA or protein expression of β-arrestin 2 in aortas from BDL rats (Figure 3B).

Haemodynamic effects of sorafenib

Our BDL rats showed the characteristic haemodynamic abnormalities including portal hypertension, splanchnic and systemic vasodilation, an increased intrahepatic vascular resistance, and excessive collateralization (Figure 4). Treatment with sorafenib reduced portal pressure in BDL rats

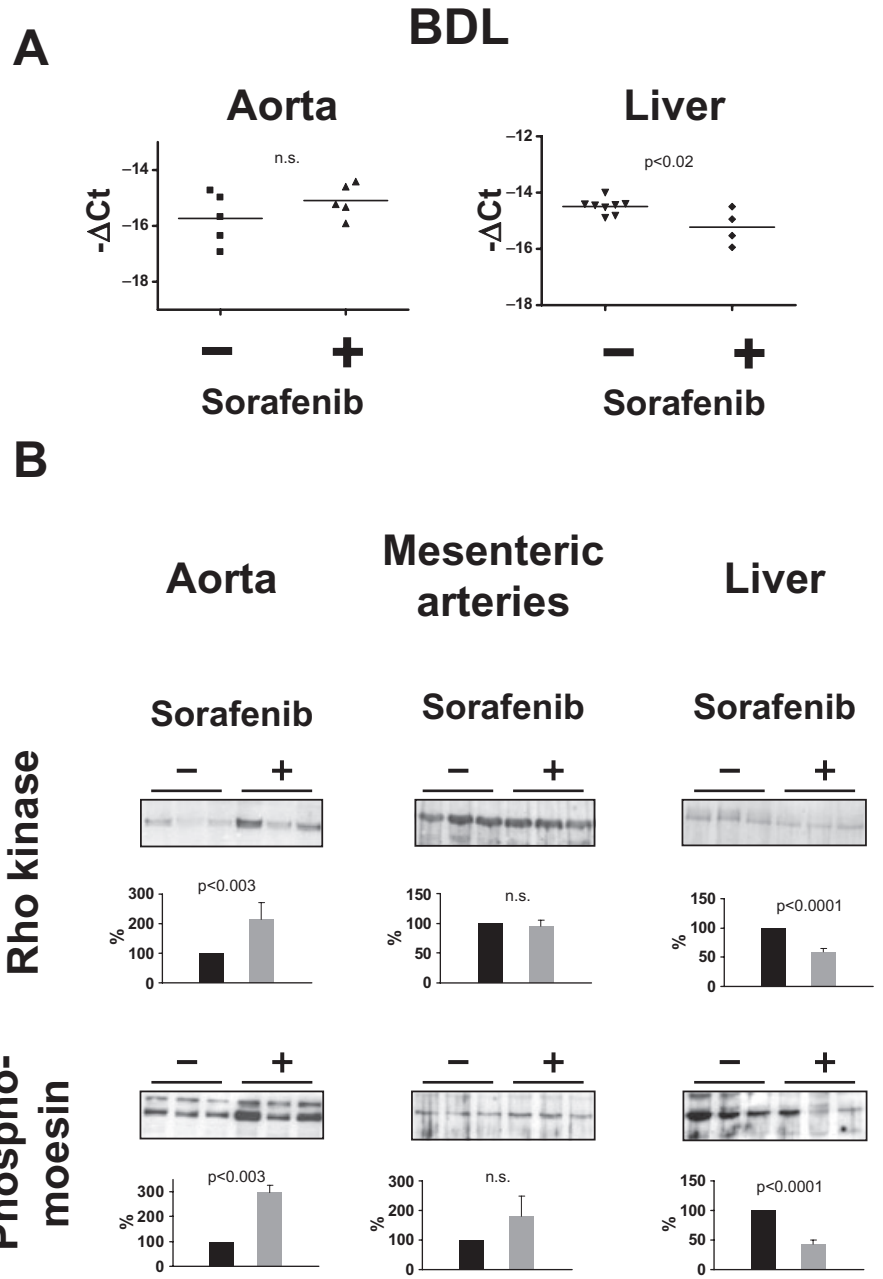

Figure 2 Vascular and intrahepatic effects of sorafenib on Rho kinase in BDL rats. (A) Aortic and hepatic Rho kinase mRNA levels. The higher the $-\Delta$Ct, the higher the mRNA concentration. Results from all experiments are shown. (B) Western blot analysis for Rho kinase protein expression and activity. The latter was assessed as the phosphorylation state of the Rho kinase substrate moesin at Thr558. Representative Western blots and densitometric quantification of all experiments (means $\pm$ SEM, $n = 9$ for each group for livers and mesenteric arteries, $n = 12$ for each group for aortas) are shown. BDL, bile duct ligation; Ct, number of cycles required to exceed a threshold; mRNA, messenger RNA.

(Figure 4A). This was associated with a decrease in intrahepatic vascular resistance of approximately 50% (Figure 4B). Splanchnic and systemic vascular resistance as well as mean arterial pressure and cardiac index remained unaffected by sorafenib in BDL rats (Figure 4C–F).

In sham-operated rats, sorafenib caused slight and non-significant increases in mean arterial pressure and systemic vascular resistance (Figure 4C and D). No changes in portal pressure or intrahepatic vascular resistance were observed in sham-operated rats in response to sorafenib (Figure 4A and B). Collateral blood flow and shunting in BDL rats remained unaffected by the sorafenib treatment (Figure 4G).

Effects of sorafenib on intrahepatic vasoregulation

To confirm our findings of the effects of sorafenib on intrahepatic Rho kinase and intrahepatic vascular resistance *in vivo*, we performed further experiments using livers perfused *in situ*. Isolated livers of BDL rats, perfused *in situ*, showed the typically increased resistance to portal outflow under resting

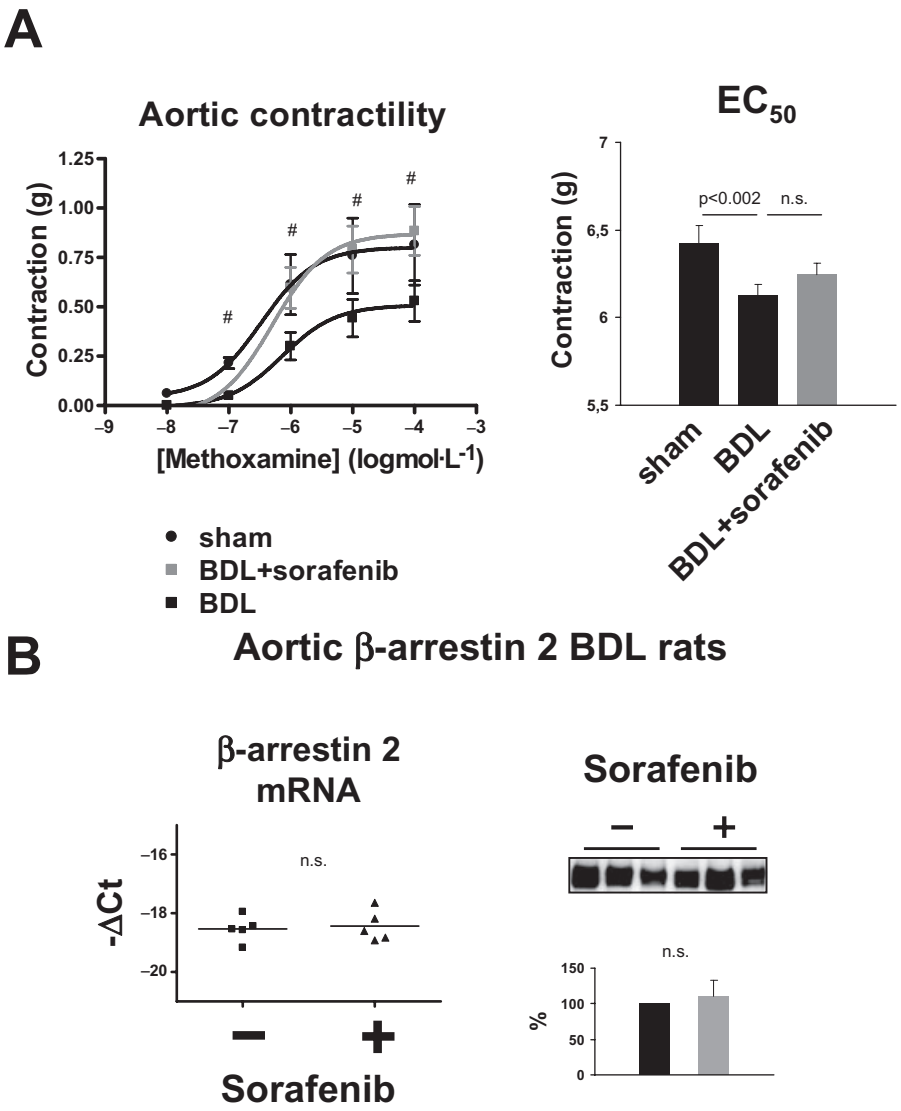

Figure 3 Effects of sorafenib on aortic contractility and regulators of Rho kinase in BDL rats. (A) Contractility of isolated aortic rings to the α_1-adrenoceptor agonist methoxamine. All vessels were incubated with the NOS inhibitor L-NAME 30 min before and during the entire measurements. Concentration response curves are shown in the right panel and the EC_{50} values in the left panel. Data are means ± SEM from experiments with eight sham-operated rats, seven BDL rats and eight sorafenib-treated BDL rats. #$P < 0.05$ for BDL versus BDL + sorafenib. (B) Effect of sorafenib on aortic mRNA and protein expression of β-arrestin 2 in BDL rats. Results from all experiments for mRNA expression ($n = 5$ for each group), and representative Western blots and densitometric quantification of all experiments for protein expression ($n = 8$ for each group) are shown. BDL, bile duct ligation; L-NAME, Nω-nitro-L-arginine methyl ester; mRNA, messenger RNA; NOS, nitric oxide synthase.

conditions, and an enhanced responsiveness to vasoconstrictors (Figure 5). In BDL rats, the chronic treatment with sorafenib led to a reduction in resting perfusion pressure of *in situ*-perfused livers, which was of a similar degree as already observed for intrahepatic vascular resistance *in vivo* (Figure 5A). In sham-operated rats, sorafenib was without effect on hepatic perfusion pressure (Figure 5B). Although treatment with sorafenib reduced the responsiveness of perfused livers to methoxamine, this was not significant in the present setting (Figure 5B). Finally, livers from sorafenib-treated BDL rats were less susceptible to the Rho kinase inhibitor Y27632. While Y27632 (10 μmol·L^{-1}) strongly decreased hepatic perfusion pressure in untreated BDL rats, this was much less pronounced in livers from sorafenib-treated BDL rats (Figure 5C).

Effects of sorafenib on markers of hepatic stellate cells (HSC) activation

To assess the effects of 1 week's treatment with sorafenib on hepatic fibrosis of BDL rats, we studied different markers of HSC activation. As shown in Figure 6, the treatment with sorafenib induced a decrease in hepatic α-smooth muscle actin protein expression as well as PDGF-Rβ mRNA expression.

Discussion

A main finding of the present study is the ability of the multikinase inhibitor sorafenib to reduce portal hypertension in rats with secondary biliary cirrhosis. In liver cirrhosis,

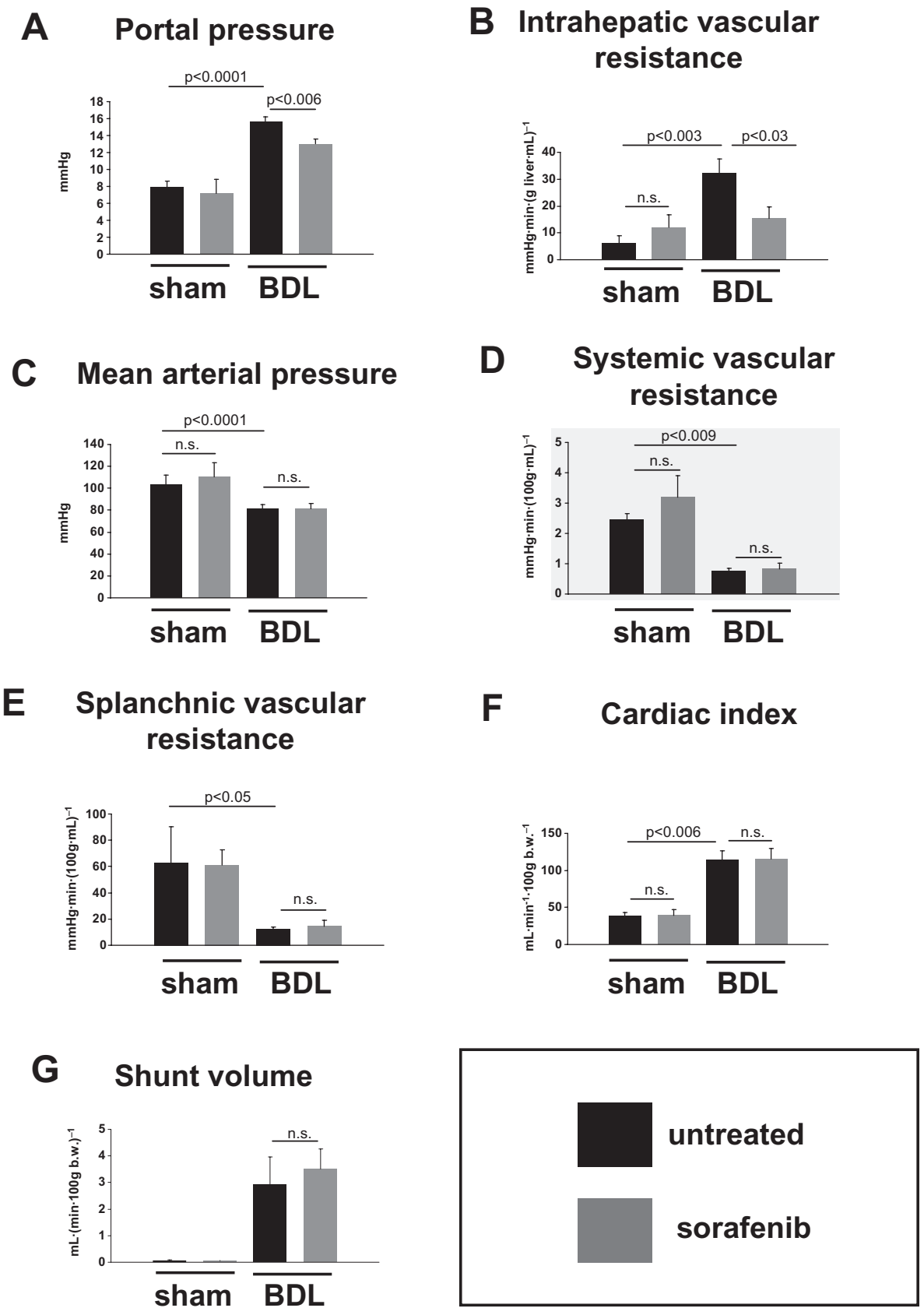

Figure 4 Haemodynamic effects of sorafenib in sham-operated and BDL rats. Results from experiments with five sham-operated rats and seven BDL rats (means ± SEM) are shown. BDL, bile duct ligation.

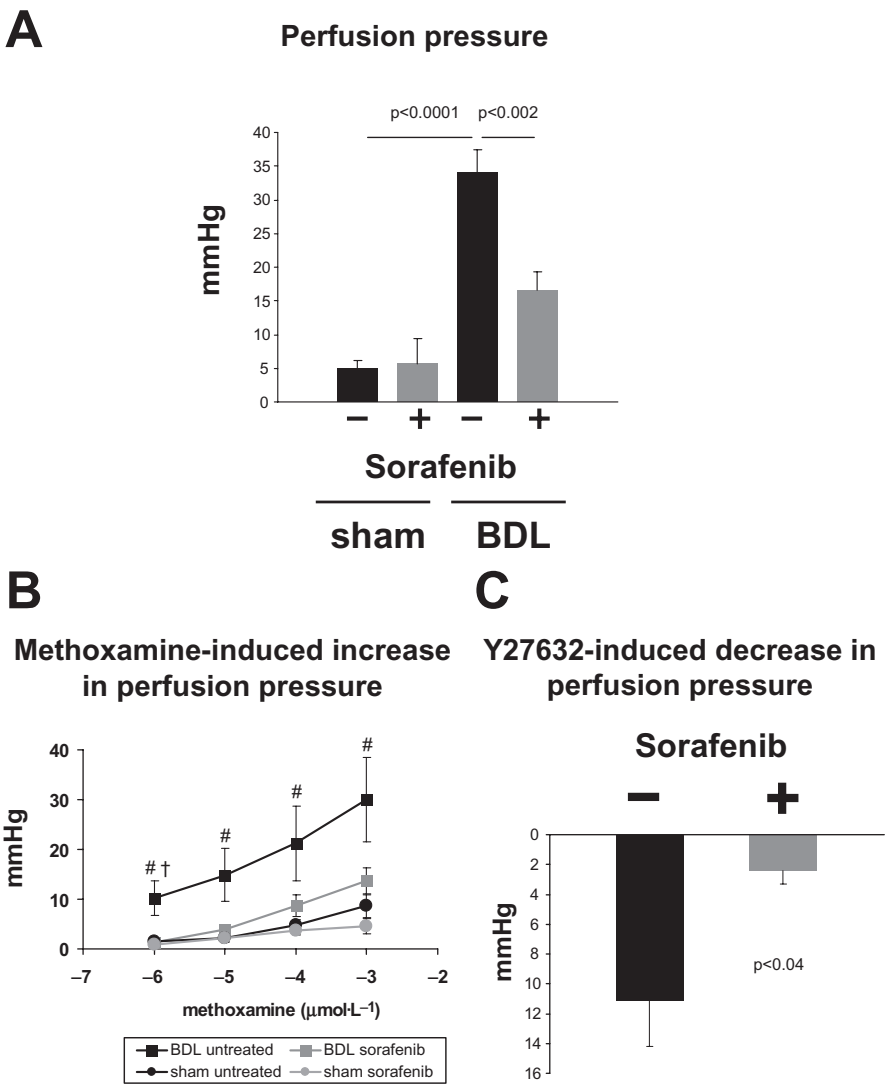

Figure 5 Effects of sorafenib on the perfusion pressure of livers perfused *in situ*. (A) Basal perfusion pressure of livers perfused *in situ* of sorafenib-treated and -untreated BDL rats. Shown are data from all experiments (means ± SEM, nine sham-operated rats, five sorafenib-treated sham-operated rats, eight BDL rats, seven sorafenib-treated BDL rats). (B) Methoxamine-induced increase in perfusion pressure (methoxamine-induced perfusion pressure – basal perfusion pressure) in sorafenib-treated and -untreated sham-operated and BDL rats. #$P < 0.05$ versus untreated sham-operated rats; †$P < 0.05$ versus sorafenib-treated BDL. Data from all experiments (means ± SEM, eight sham-operated rats, seven sorafenib-treated sham-operated rats, six BDL rats, six sorafenib-treated BDL rats) are shown. (C) Y27632-induced decrease in perfusion pressure in sorafenib-treated and -untreated BDL rats. Data from all experiments (means ± SEM, six BDL rats, six sorafenib-treated BDL rats) are shown. BDL, bile duct ligation; Y27632, (R)-(+)-*trans*-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide.

increased intrahepatic vascular resistance and decreased splanchnic vascular resistance are attractive targets for the treatment of portal hypertension (Rockey, 2003; Groszmann and Abraldes, 2005; Hernandez-Guerra *et al.*, 2005; Tsai, 2007; Wiest, 2007; Hennenberg *et al.*, 2008). According to our current data, the beneficial effect of sorafenib on portal pressure of BDL rats is explained by a reduction of the elevated intrahepatic vascular resistance of these animals. This involves a reduction in hepatic expression and function of Rho kinase, which represents a critical mediator of vasoconstrictor hyperresponsiveness (Zhou *et al.*, 2006; Laleman *et al.*, 2007; Trebicka *et al.*, 2007; Anegawa *et al.*, 2008) and fibrosis (Iwamoto *et al.*, 2000; Tada *et al.*, 2001; Murata *et al.*, 2001; Ikeda *et al.*, 2003; Fukushima *et al.*, 2005; Kitamura *et al.*, 2007) in the cirrhotic liver.

Vasoconstrictor hyperresponsiveness of the intrahepatic microcirculation and vasoconstrictor hyporesponsiveness of extrahepatic vessels are associated with altered expressions and activities of the contraction-mediating Rho kinase in the corresponding vascular beds of BDL rats and in patients suffering from cirrhosis (Hennenberg *et al.*, 2006; 2007; 2008; Zhou *et al.*, 2006; Trebicka *et al.*, 2007; 2008; Wiest, 2007; Anegawa *et al.*, 2008). Rho kinase is coupled to vasoconstrictor receptors and mediates the contraction of vascular smooth muscle and HSC (Somlyo and Somlyo, 2000; 2003; Loirand *et al.*, 2006; Laleman *et al.*, 2007; Trebicka *et al.*, 2007). Due to its central role in HSC activation, proliferation and survival, Rho kinase also critically participates in extracellular matrix production during hepatic fibrogenesis (Iwamoto *et al.*, 2000; Murata *et al.*, 2001; Tada *et al.*, 2001; Ikeda *et al.*, 2003;

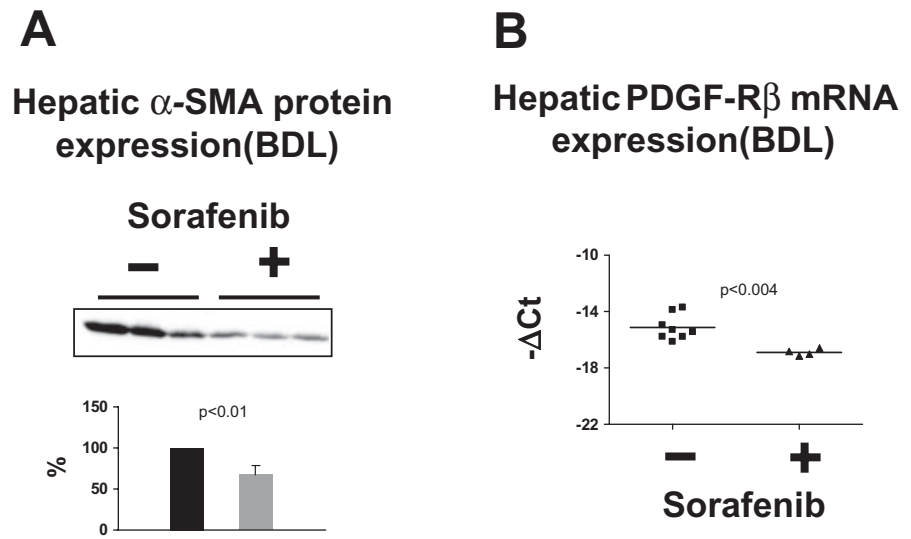

Figure 6 Effects of sorafenib on HSC markers in BDL rats. (A) α-SMA protein expression in livers from BDL and sorafenib-treated BDL rats. Representative Western blot and densitometric quantification of all experiments (means ± SEM, $n = 9$ for each group) are shown. (B) Hepatic mRNA levels of PDGF-Rβ in BDL and sorafenib-treated BDL rats. Data from all experiments are shown. BDL, bile duct ligation; Ct, number of cycles required to exceed a threshold; HSC, hepatic stellate cell(s); mRNA, messenger RNA; PDGF-R, platelet-derived growth factor receptor.

Fukushima *et al.*, 2005; Kitamura *et al.*, 2007). Our present study shows that the multikinase inhibitor sorafenib partially corrects intrahepatic and extrahepatic dysregulation of Rho kinase expression, and reduces portal pressure in rats with secondary biliary cirrhosis.

Sorafenib was developed for anti-tumour treatment and designed to inhibit cell cycle and angiogenesis (Abou-Alfa *et al.*, 2006; Liu *et al.*, 2006; Strumberg *et al.*, 2007; Furuse *et al.*, 2008; Takimoto and Awada, 2008). Molecular targets of sorafenib are Raf kinase, which represents the specific upstream activator of the MAPK ERK1 and 2, and different receptor tyrosine kinases including those associated with the vascular endothelial growth factor receptor and PDGF-R, also c-Kit, and Tie-2 (Abou-Alfa *et al.*, 2006; Adnane *et al.*, 2006; Liu *et al.*, 2006; Wilhelm *et al.*, 2006).

As revealed by our haemodynamic measurements, the decrease in portal pressure induced by treatment with sorafenib in BDL rats was due to a diminished intrahepatic vascular resistance. This reduction in intrahepatic vascular resistance was also observed using livers perfused *in situ*. As sorafenib was without effect on splanchnic or systemic vascular resistance of BDL rats, an extrahepatic portal pressure lowering mechanism of sorafenib appears unlikely. As shown recently, the increased intrahepatic vascular resistance of BDL rats depends substantially on up-regulation of Rho kinase expression and activity in HSC and sinusoidal endothelial cells (SEC) (Zhou *et al.*, 2006; Trebicka *et al.*, 2007; Anegawa *et al.*, 2008). Up-regulation of Rho kinase was also reported from livers of patients with alcohol-induced cirrhosis (Zhou *et al.*, 2006), suggesting that similar mechanisms play a role in patients with portal hypertension. As the reduction of intrahepatic vascular resistance by sorafenib in BDL rats was associated with down-regulation of hepatic Rho kinase expression, it appears likely that this contributed to the beneficial effect of sorafenib on intrahepatic perfusion and portal pressure. This was supported by our experiments applying the Rho kinase inhibitor Y27632 to livers perfused *in situ*. However, we cannot exclude that further, for example, nitric oxide-dependent, mechanisms are involved as well. Recent studies suggested that the contribution of Rho kinase up-regulation to intrahepatic vasoconstrictor hyperresponsivess includes its function as a vasoconstrictor effector in contractile cells such as HSC (Zhou *et al.*, 2006; Laleman *et al.*, 2007; Trebicka *et al.*, 2007), and the down-regulation of endothelial nitric oxide synthase activity in SEC in cirrhosis (Anegawa *et al.*, 2008).

The decrease in hepatic Rho kinase expression and intrahepatic vascular resistance in response to sorafenib may be explained by reduced activity or number of HSC. Activated HSC express high levels of Rho kinase, which is required to achieve its contractile properties, and contributes to portal hypertension in BDL rats (Iwamoto *et al.*, 2000; Murata *et al.*, 2001; Tada *et al.*, 2001; Ikeda *et al.*, 2003; Fukushima *et al.*, 2005; Zhou *et al.*, 2006; Kitamura *et al.*, 2007; Laleman *et al.*, 2007; Trebicka *et al.*, 2007). In a recent study, the multikinase inhibitor sunitinib reduced fibrosis and HSC activation in rats given CCl_4, together with HSC viability (Tugues *et al.*, 2007). This was attributed to inhibition of PDGF-Rs, which play a crucial role in HSC activation. A similar effect of sorafenib in our study might well be responsible for the decreases of intrahepatic vascular resistance and hepatic Rho kinase expression, as sorafenib also caused inhibition and down-regulation of hepatic PDGF-Rβ in our BDL rats (Figure 1B). PDGF-Rβ expression is highly increased in activated HSC, whereas other hepatic cells of the cirrhotic liver preferentially express the α-isoform with only low levels of the β-receptor (Heldin *et al.*, 1991; Pinzani and Marra, 2001; Tugues *et al.*, 2007; Borkham-Kamphorst *et al.*, 2008). We assume that the intrahepatic effects of sorafenib may be partially determined by increases in hepatic PDGF-Rβ expression, most likely on HSC (Pinzani and Marra, 2001; Tugues *et al.*, 2007; Borkham-Kamphorst *et al.*, 2008). Targeting this receptor might explain

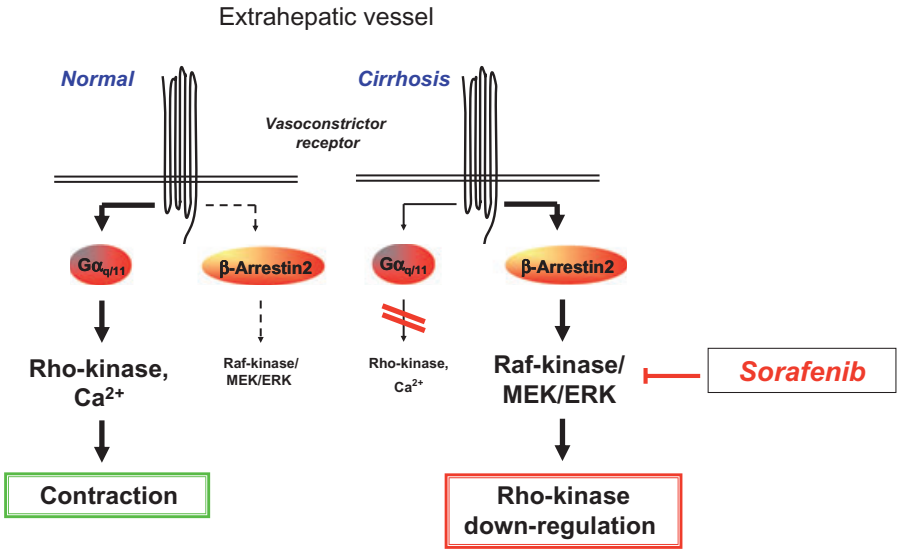

Figure 7 Assumed mechanisms of post-transcriptional Rho kinase down-regulation and its correction by sorafenib in hypocontractile vessels. Given an β-arrestin 2-dependent activation mode – which is likely to occur in cirrhosis due to up-regulation of β-arrestin 2 expression – ERK may cause post-transcriptional Rho kinase down-regulation in cultured fibroblasts and *in vivo* in the internal anal sphincter. We assume that aberrant signalling by usually G-protein-coupled vasoconstrictor receptors cause the Rho kinase down-regulation of hypocontractile vessels at least from BDL rats: changing the coupling from G-proteins to the overexpressed β-arrestin 2, these receptors may lead to down-regulation of Rho kinase which can be targeted by ERK inhibition through sorafenib. BDL, bile duct ligation; ERK, extracellular signal-regulated kinase.

the predominantly hepatic effect of sorafenib in our BDL rats. Indeed, sorafenib treatment of BDL rats was associated with a reduction of markers for HSC activation (Figure 6), suggesting that we targeted HSC among other contractile cells in our study. Thus, we assume that the reduction in intrahepatic vascular resistance after 1 week of sorafenib treatment was due to decreased HSC-mediated contractility of the intrahepatic vascular bed.

In contrast to its fate in the liver, Rho kinase is post-transcriptionally down-regulated in aortas and mesenteric arteries from BDL rats (Hennenberg *et al.*, 2006; 2008; Trebicka *et al.*, 2008). Down-regulation of Rho kinase protein expression was also found in hepatic arteries from cirrhotic patients (Hennenberg *et al.*, 2007). Contrary to the liver, no transcriptional mechanisms were involved in the sorafenib-induced up-regulation of aortic Rho kinase expression (Figure 2). We speculate that sorafenib corrects ERK-mediated Rho kinase down-regulation in these vessels via inhibition of ERK. We recently found that a MEK- and ERK-dependent mechanism might be responsible for post-transcriptional Rho kinase down-regulation in the vascular smooth muscle cells of extrahepatic vessels from cirrhotic species (Hennenberg *et al.*, 2006; 2008) (Figure 7). This is underlined by studies showing post-transcriptional regulation of Rho kinase expression via MEK-ERK in cultured fibroblasts (Pawlak and Helfman, 2002a,b). Similarly, Rho kinase expression is also post-transcriptionally regulated *in vivo* in the internal anal sphincter (de Godoy *et al.*, 2007). Such a phenomenon might be caused by ERK activation via β-arrestin 2 – which is highly up-regulated in extrahepatic vessels of cirrhotic rats (Hennenberg *et al.*, 2007; 2008) – instead of activation via G-proteins (Pawlak and Helfman, 2002a,b; Thomas *et al.*, 2004; Lefkowitz *et al.*, 2006; Smith and Luttrell, 2006; de Godoy *et al.*, 2007; Mehta and Griendling, 2007; Defea, 2008; Hennenberg *et al.*, 2008). Therefore, we speculate that the MEK-ERK system in BDL rats becomes amenable to sorafenib by β-arrestin 2 (Figure 7). Thus, our data support the idea of a participation of a switched ERK regulation in post-transcriptional Rho kinase down-regulation in aortas from BDL rats (Figure 7) (Hennenberg *et al.*, 2008). Of course, we cannot exclude the contribution of further mechanisms.

Although we found improvement of the decreased aortic contractility after sorafenib application *in vitro* (Figure 3A), there was no effect on systemic or splanchnic haemodynamics *in vivo*. The unchanged splanchnic vascular resistance may be explained by the unchanged Rho kinase expression of mesenteric vessels, while aortic Rho kinase was up-regulated by sorafenib. This may indicate that either different or additional mechanisms participate in the regulation of Rho kinase expression in these two vascular compartments.

Furthermore, the EC_{50} for methoxamine was still lower at aortic rings of sorafenib-treated BDL rats. This may be explained by the still increased β-arrestin 2 expression in vessels. This latter finding might be of special relevance in the *in vivo* situation with high systemic vasoconstrictor levels (Moller and Henriksen, 2008). In this context, it is interesting that also in patients with hepatocellular carcinoma who most often have liver cirrhosis, the otherwise well-known hypertensive effect of sorafenib was not observed (Abou-Alfa *et al.*, 2006; Veronese *et al.*, 2006; Furuse *et al.*, 2008; Wu *et al.*, 2008). In our study, we observed a trend towards a hypertensive effect in non-cirrhotic but not in BDL rats. This suggests that sorafenib-induced hypertension does occur less often in hepatic cirrhosis. We assume that a hypertensive effect in cirrhotic rats or patients may be prevented by high vascular β-arrestin 2 expression. In cirrhosis, negative regulation of G-protein-dependent signalling by β-arrestin 2 may be enhanced by the increased binding of β-arrestin 2 to vasocon-

strictor receptors (Hennenberg *et al.*, 2007; 2008). Thus, hypertensive effects of sorafenib in cirrhosis may be prevented by the unaltered, increased β-arrestin 2 expression of hypocontractile vessels.

The dosage used in our study (60 $mg \cdot kg^{-1} \cdot d^{-1}$) was based on dosing in studies performed previously in different mouse tumour models. In these studies, dosages between 20 and 100 $mg \cdot kg^{-1} \cdot d^{-1}$ were used (Liu *et al.*, 2006; Kim *et al.*, 2007). Sorafenib showed dose-dependent anti-tumour effects within a range of 20–100 $mg \cdot kg^{-1} \cdot d^{-1}$ in experiments with treatment periods of more than 40 days (Liu *et al.*, 2006; Kim *et al.*, 2007). In the SHARP trial, sorafenib was presumably safe for several months in those patients who tolerated the drug (Llovet *et al.*, 2008).

In conclusion, extrahepatic and intrahepatic dysregulation of Rho kinase expression in BDL rats can be targeted by the multikinase inhibitor sorafenib. This caused a sorafenib-induced reduction of intrahepatic vascular resistance, which leads to a decrease in portal hypertension.

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Conflict of interest

None.

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