Defective RhoA/Rho-Kinase Signaling Contributes to Vascular Hypocontractility and Vasodilation in Cirrhotic Rats

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Background & Aims: **Portal hypertension is associated with arterial hypotension and vascular hypocontractility, which persists despite elevated plasma levels of vasoconstrictors. We investigated the role of the RhoA/Rho-kinase pathway in vascular smooth muscle hypocontractility of rats with secondary biliary cirrhosis.** ***Methods:*** **Aortic expressions of RhoA and Rho-kinase were analyzed in sham-operated and BDL rats by reverse-transcription polymerase chain reaction (RT-PCR) and immunoblots. Activation of aortic RhoA was examined by pull down of guanosine triphosphate (GTP)-RhoA and membrane translocation of RhoA. Rho-kinase activity was assessed as phosphorylation of its substrate, moesin. Contractility of isolated aortic rings was determined myographically. The hemodynamic effect of the Rho-kinase inhibitor (R)-(+)-*trans*-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y-27632) was determined in vivo by measuring changes in mean arterial pressure and systemic vascular resistance (SVR) (microspheres).** ***Results:*** **Contraction of aortic rings from BDL rats was impaired in response to the α_1-adrenergic receptor agonist methoxamine but not to high molar KCl. Aortic expression of RhoA was unchanged in cirrhotic rats, whereas Rho-kinase was down-regulated posttranscriptionally. Methoxamine-induced activation of RhoA as well as basal and methoxamine-induced phosphorylation of moesin were strongly reduced in aortas from cirrhotic rats. Aortic rings from cirrhotic rats precontracted with methoxamine showed an increased sensitivity to relaxation with Y-27632. The drop in SVR induced by Y-27632 was larger in cirrhotic rats than in sham-operated rats.** ***Conclusions:*** **An impaired vascular activation of RhoA and a down-regulation of Rho-kinase might contribute to vasodilation and vascular hypocontractility in BDL-induced cirrhosis.**

Cirrhosis is associated with hyperdynamic circulation despite increased plasma levels of vasoconstrictors such as norepinephrine.[1–3] One explanation for this vascular hypocontractility is the formation of counteracting vasodilators such as endothelial production of nitric oxide (NO).[4–8] However, elevated levels of NO or other vasodilators can only partly explain hypocontractility, and several studies postulated the existence of additional, NO- and endothelium-independent mechanisms contributing to vascular hypocontractility in cirrhosis.[9–12]

Contraction of vascular smooth muscle is dependent on phosphorylation of myosin light chains (MLC), which is tightly regulated by the activities of both MLC kinase and MLC phosphatase.[13] The initial signal causing contraction is the binding of contractile agonists to G-protein-coupled receptors. The resulting activation of heterotrimeric G-proteins containing α-subunits of the $G\alpha_{q/11}$ and the $G\alpha_{12/13}$ families then leads to activation of 2 different signaling pathways, both resulting in an increased MLC phosphorylation. In the well-known phospholipase C/inositol 1,4,5-trisphosphate (PLC/IP_3) cascade, contraction occurs due to an IP_3-induced elevation of the cytosolic calcium concentration activating the calcium/calmodulin-dependent MLC kinase. In the last decade, the G-protein-dependent activation of a second pathway, the RhoA/Rho-kinase pathway has been the focus of many investigations. Its role as one of the main mechanisms leading to vascular smooth muscle contraction is now well established[14–20] (Figure 1). The main players in this pathway are the small monomeric GTPase RhoA and its direct downstream effector, Rho-kinase. Like PLC, RhoA is activated in a receptor-dependent fashion, a process stimulating the exchange of guanosine diphosphate (GDP) by guanosine triphosphate (GTP)

Abbreviations used in this paper: BDL, bile duct ligation; EC_{50}, concentration producing a half-maximum effect; GST, glutathione S-transferase; GTP, guanosine triphosphate; IP_3, 1,4,5-triphosphate; L-NAME, N^G-nitro-L-arginine methyl ester; MAP, mean arterial pressure; MLC, myosin light chain; MLC-P, phospho-MLC; NO, nitric oxide; NOS, nitric oxide synthase; pEC_{50}, negative logarithm of EC_{50}; PLC, phospholipase C; P-moesin, moesin phosphorylated at Thr-558; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SVR, systemic vascular resistance; Y-27632, (R)-(+)-*trans*-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide.

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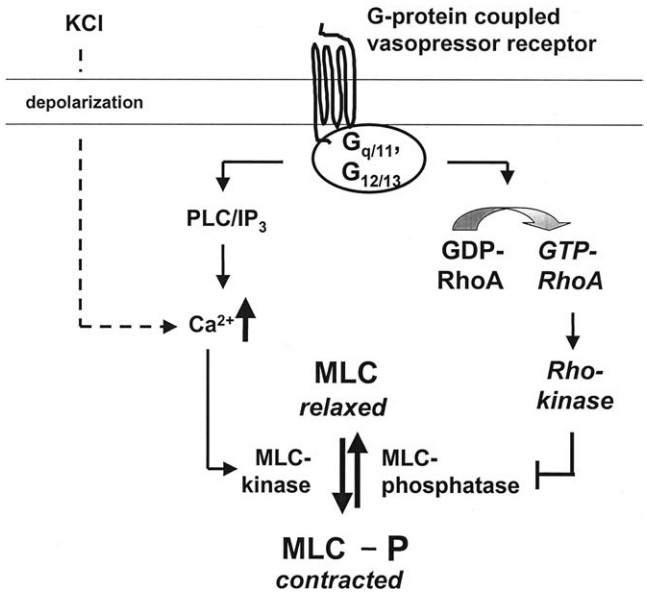

Figure 1. Contractile pathways in vascular smooth muscle. Agonist activation of vasopressor receptors coupled to $G\alpha_{q/11}$ and $G\alpha_{12/13}$-types of G-proteins stimulates the PLC/IP_3 and the RhoA/Rho-kinase pathways, both resulting in increased MLC phosphorylation and thus contraction of the vascular smooth muscle cell. Contraction induced by high molar KCl occurs exclusively via activation of Ca^{2+}/calmodulin-dependent MLC kinase after depolarization of the vascular smooth muscle cell.

bound to RhoA, and the translocation of RhoA to the membrane, causing its activation. Activated RhoA in turn activates Rho-kinase, which then phosphorylates and thereby inactivates MLC phosphatase, leading to increased MLC phosphorylation and contraction.

The aim of the present study was to examine possible changes in RhoA/Rho-kinase-mediated contraction in the aortic smooth muscle from rats with secondary biliary cirrhosis because a defect in RhoA/Rho-kinase signaling might explain the NO-independent vascular hypocontractility.

Materials and Methods

Animals

For our experiments, male Sprague–Dawley rats with an initial body weight of 180 to 200 g were used and divided into 2 groups. One group (n = 109) underwent bile duct ligation (BDL) as previously described.[21] The rats of the other group (n = 118) were sham operated and served as controls. In these rats, the common bile duct was exposed by median laparotomy, but no ligation or resection was performed. In both groups of rats, BDL and sham-operated rats, experiments were carried out after 5 weeks. The study was approved by the local committee for animal studies (Bezirksregierung Köln, 50.203-Bn 15, 23/03).

In 1 group of sham-operated (n = 6) and BDL rats (n = 5, 4 weeks after ligation), N^G-nitro-L-arginine methyl ester (L-NAME, 30 mg/kg/day), an inhibitor of NO synthases (NOS), was dissolved in 25 mL tap water and given overnight for 1 week.

Analysis of Aortic Ring Contraction

For in vitro contractility studies, aortas were excised, cleaned of adherent and connective tissue without stripping, and cut in 4-mm-wide rings. Rings were mounted in organ bath chambers filled with carbogen-bubbled Krebs–Henseleit solution maintained at 37°C with an outer water jacket. During an equilibration period of 45 minutes, a baseline tension of 1 g was adjusted. While experiments were performed, the tension of the aortic rings was continuously registered with an isometric force transducer (Fort 10; World Precision Instruments, Berlin, Germany), amplified (TBM4; World Precision Instruments), and recorded on a multichannel polygraph (Rikadenki R10; Tokyo, Japan). After the equilibration period, rings were contracted with 10 μmol/L methoxamine or 80 mmol/L KCl. For contraction with KCl, a Krebs–Henseleit solution was used in which a corresponding concentration of NaCl was replaced by 80 mmol/L KCl. After maximum contraction, cumulative concentration-response curves for (R)-(+)-*trans*-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y-27632) were evaluated. After maximum Y-27632-induced relaxation, rings were washed several times, contracted with Krebs–Henseleit solution containing 80 mmol/L KCl, and relaxed with 100 μmol/L acetylcholine to assess integrity or successful removal of endothelium. In 1 set of experiments, 1 or 2 of 3 rings were preincubated for 20 minutes with the NOS inhibitor L-NAME (200 μmol/L). In another set of experiments, endothelium-denuded aortic rings were used. Removal of endothelium was performed mechanically by gently rubbing the interior of the vessel with forceps. In further experiments, aortic rings from chronic L-NAME-treated rats (30 mg/kg per day for 7 days) were used. Here, the equilibration period was shortened to 15 minutes. Thereafter, all rings were directly contracted with methoxamine (10 μmol/L). With 2 of these rings, dose-response curves for Y-27632 were assessed, whereas another was used in parallel to confirm the lack of relaxation in response to acetylcholine (100 μmol/L).

Quantitative RT-PCR

RNA was isolated from 30 mg shock frozen aortic 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). Reverse transcription was performed using moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, Karlsruhe, Germany), and random primers (250 ng; Microsynth, Balgach, Switzerland). Control reactions did not contain reverse transcriptase. Primers and probes for RT-PCR were designed using the Primer Express Software (Applied Biosystems, Foster City,

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