Carbon Monoxide-Mediated Activation of Large-Conductance Calcium-Activated Potassium Channels Contributes to Mesenteric Vasodilatation in Cirrhotic Rats

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ABSTRACT

Large-conductance calcium-activated potassium channels (BKCa) are important regulators of arterial tone and represent a mediator of the endogenous vasodilator carbon monoxide (CO). Because an up-regulation of the heme oxygenase (HO)/CO system has been associated with mesenteric vasodilation of cirrhosis, we analyzed the interactions of BKCa and of HO/CO in the endothelium-dependent dilatation of mesenteric arteries in ascitic cirrhotic rats. In pressurized mesenteric arteries (diameter, 170–350 μm) of ascitic cirrhotic rats, we evaluated the effect of inhibition of BKCa, HO, and guanylyl-cyclase on dilatation induced by acetylcholine and by exogenous CO; and HO-1 and BKCa subunit protein expression. Inhibition of HO and of BKCa reduced acetylcholine-induced vasodilatation more in cirrhotic rats than in control rats, whereas inhibition of guanylyl-cyclase had a similar effect in the two groups. CO was more effective in cirrhotic rats than in control rats, and the effect was hindered by BKCa inhibition. The expression of HO-1 and of BKCa α-subunit was higher in mesenteric arteries of cirrhotic rats compared with that of control animals, whereas the expression of the BKCa β1-subunit was lower. In conclusion, an overexpression of BKCa α-subunits, possibly due to HO up-regulation with increased CO production, participates in the endothelium-dependent alterations and mesenteric arterial vasodilatation of ascitic cirrhotic rats.

Mesenteric arterial vasodilation is a key mechanism in the pathophysiology of the hyperdynamic circulatory syndrome of cirrhosis. This syndrome is responsible for serious complications, such as ascites, heporenal syndrome, and gastrointestinal hemorrhage. The pathophysiological mechanism that supports the vasodilation of mesenteric arteries in cirrhosis is a decrease in the response of the arteries to vasoconstricting agents (Sieber et al., 1993), caused by an increase in vasodilating substances of endothelial origin, such as nitric oxide (NO), prostacyclin, and, as recently demonstrated, carbon monoxide (CO) (Wiest and Groszmann, 1999; Fernandez et al., 2001; Gonzales-Abraldes et al., 2002).

We have recently shown that an increased action of the heme oxygenase (HO)/CO system plays a role in the hyporesponsiveness of small resistance mesenteric arteries to phenylephrine (PE) only in the advanced stage of experimental cirrhosis (Bolognesi et al., 2005). Therefore, the increased activity of the HO/CO system may participate in the evolution of cirrhosis from compensated to decompensated.

HO is a microsomal enzyme with two main distinct isoforms: the inducible isoenzyme HO-1 and the constitutive one HO-2 (Zhang et al., 2001; Johnson et al., 2003a). It is the rate-limiting enzyme in the degradation of heme to biliverdin, CO, and free iron (Motterlini et al., 1998). CO, generated by HO in endothelial and smooth muscle layers of arterial vessels, modulates vascular tone by inducing relaxation of vascular smooth muscle cells through stimulation on soluble guanylyl cyclase (sGC) and opening of large-conductance calcium-activated potassium channels (BKCa) (Zhang et al., 2001).

ABBREVIATIONS: NO, nitric oxide; CO, carbon monoxide; HO, heme oxygenase; PE, phenylephrine; sGC, soluble guanylyl cyclase; BKCa, large-conductance calcium-activated K⁺ channel; ACh, acetylcholine; CrMP, chromium mesoporphyrin; PSS, physiological salt solution; SNP, sodium nitroprusside; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; Indo, indomethacin; L-NAME, N^6-nitro-L-arginine-methyl-ester; IbTx, iberiotoxin; CORM, CO-releasing molecule; pEC₅₀, log EC₅₀; ANOVA, analysis of variance; COX, cyclooxygenase; NOS, nitric-oxide synthase.
BKCa, alone and in combination with CrMP, on ACh-induced ACh. We then examined the effect of inhibition of sGC and of Ito, on the endothelium-dependent vasodilation induced by ACh. In cirrhotic rats with ascites, we preliminarily analyzed the effect of inhibition of HO, sGC, and BKCa, and the vasodilation. Finally, the hemodynamic effect of a CO donor, with diameters narrower than 500 μm in diameter, 1–2 mm in length) were isolated from surrounding perivascular tissue, removed from the mesenteric vascular bed, and mounted on glass micropipettes in a water-jacketed perfusion chamber (Living Systems Instrumentation, Burlington, VT) in warmed (37°C), oxygenated (95% O2 and 5% CO2) PSS. The vessels were mounted on a proximal micropipette connected to a pressure servo controller. Subsequently, the lumen of the vessel was flushed to remove residual blood, and the end of the vessel was mounted on a micropipette connected to a three-way stopcock. After the stopcock was closed, the intraluminal pressure was allowed to increase slowly until it reached 80 mm Hg. The vessel was superfused with PSS (4 ml/min) at 37°C gassed with 95% O2 and 5% CO2 for a 45-min period of equilibration (Bolognesi et al., 2005). Intraluminal pressure was maintained at 80 mm Hg throughout the experiment. After the equilibration period, the vessels were challenged with PE, an α1-adrenoceptor agonist (1 μM). An artery was considered unacceptable for experimentation if it demonstrated leaks or failed to constrict by more than 20% to PE. The presence of a functional endothelium was determined on the basis of a prompt relaxation to ACh (1 μM) in the vessel precontracted with PE (1 μM). To remove the endothelium, 2 ml of air was flushed through the lumen (Sun et al., 1994). In these arteries, the absence of a functional endothelium was confirmed after preconstriction with PE by the absence of response to ACh with a normal response to sodium nitroprusside (SNP), an endothelium-independent vasodilator. The effects of ACh and CO administration were evaluated as variations in the internal diameter of the vessels precontracted with 10 μM PE; all responses were reported as percent inhibition of the contraction induced by PE.

**Evaluation of the Response to ACh of Small Mesenteric Arteries Precontracted with Phenylephrine in CCl4 Cirrhotic Rats.** Responses to increasing doses of ACh (10−9 to 10−4 M) were determined in arteries superfused with PSS-containing vehicles for the inhibitors tested. Inhibitors were added to freshly prepared PSS, and 20- to 30-min drug-tissue contact time was allowed before retesting the response to ACh in the same vessel. ACh was added to the bath (extraluminal application), and cumulative dose-response curves were generated, with 2- to 3-min intervals between doses. After each dose-response test, the tissues were washed with fresh PSS for at least 20 min. Vascular diameters were measured 1 to 3 min after the addition of ACh with the use of a video system consisting of a microscope with a charge-coupled device television camera (Eclipse TS100-F; Nikon, Tokyo, Japan), a television monitor (Ultrak Inc., Lewisville, TX), and a video measuring system (Living Systems Instrumentation). In control rats and in ascitic cirrhotic rats, concentration-response curves to ACh were evaluated before and after 20-min superfusion with the HO inhibitor CrMP (15 μM); before and after 20-min superfusion with the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 μM) in arteries treated with indomethacin (Indo) (2.8 μM) and Nω-nitro-l-arginine-methyl-ester (l-NNAME) (1 mM) and then after a further 20 min of CrMP (15 μM) plus ODQ (10 μM) superfusion in arteries already evaluated after ODQ superfusion alone; and before and after 20-min superfusion with the BKCa inhibitor iberiotoxin (IbTx) (25 nM) in arteries treated with Indo (2.8 μM), l-NNAME (1 mM), and ODQ (10 μM) and then after a further 20 min of CrMP (15 μM) plus IbTx (25 nM) superfusion in arteries already evaluated after IbTx superfusion alone. Concentration-response curves to ACh were also evaluated in small mesenteric arteries of control rats before and after 20-min superfusion with three different amount of the BKCa inhibitor IbTx (25, 50, and 100 nM). Only one experiment was performed in each artery.

**Evaluation of the Response to a CO Donor.** Small mesenteric arteries of control rats and of rats with cirrhosis and ascites were prepared and mounted on glass micropipettes in a water-jacketed perfusion chamber as already described. After the equilibration pe-
riod and after verifying their viability, the arteries were preconstricted with the addition of PE to the superfusion PSS. A CO donor (200 μM) [CO-releasing molecule (CORM-3)] (Clark et al., 2003; Foresti et al., 2004) was then added to the superfusion PSS for 15 min, and the change in lumen diameter was recorded. The chemical composition of CORM-3 is: tricarbonylchloro(glycinato)ruthenium(II) ([Ru(CO)3Cl(glycinato)]. The concentration of CORM-3 was chosen after testing the hemodynamic effect of four different CORM-3 concentrations (25, 50, 100, and 200 μM) (Foresti et al., 2004) in a preliminary experiment on small mesenteric arteries of control rats. Only with the higher dose (200 μM) did we obtain a clear and measurable effect. Therefore, 200 μM was the minimal efficacious dose in our experimental system. In this preliminary experiment, we also verified that the hemodynamic effect of CORM-3 on small mesenteric arteries is slow because it could only be detected a approximately 3 to 4 min after administration and could be fully evaluated only after an exposure of 10 to 15 min. For this reason, to evaluate CORM-3 effect in our experimental set, the changes in lumen diameter were recorded for 15 min during a continuous exposure to CORM-3. Taking all this into account, we decided to test a single dose of CORM-3 using the minimal dose that was efficacious in our control animals (200 μM).

The effect of CORM-3 was also verified in arteries superfused with CrMP (15 μM) for 30 min, in arteries superfused with CrMP (15 μM) plus ODQ (10 μM) for 30 min, and in arteries superfused with CrMP (15 μM) plus L-NAME for 30 min. In a second series of experiments, the effect of CORM-3 was verified in arteries preconstricted with PE after removal of the endothelium. In these arteries, after verifying CO effect, CORM-3 was removed by the superfusing PSS, and when a stable baseline was obtained, the NO donor SNP (100 nM to produce a detectible dilation) was added. When a stable baseline was observed, CORM-3 was added again to the superfusing PSS, and the dilating effect was measured again.

**Chemicals.** CrMP was obtained from Porphyrin Products (Logan, UT). CORM-3 was synthesized by Brian E. Mann (Clark et al., 2003), and stock solutions were prepared using deionized water. All other chemicals were obtained from Sigma Chemical (St. Louis, MO). PE and L-NAME were dissolved in deionized water and diluted with PSS. CrMP was dissolved in a solution of 50 mM NaCO3.

**Western Blot Analysis of HO-1 and of Subunits α and β1 of BKCa Protein Expression in Mesenteric Arteries of CCl4 Cirrhotic Rats.** Standard techniques were used to evaluate protein expression. After removal of veins and adipose tissue, small mesenteric arteries (30–40 arteries with diameter <500 μm) were collected from each rat, snap-frozen in liquid N2, and stored at −80°C until analyzed. The vessels were homogenized in urea lysis buffer. Protein extracts were assayed for protein content using the BCA protein assay kit (Pierce, Rockford, IL). SDS-polyacrylamide gel electrophoresis and immunoblotting were performed on 50 μg of total protein extracts. HO-1 protein expression was detected using a monoclonal murine antibody against HO-1 (StressGen Biotechnologies Corp., Victoria, BC, Canada). Only HO-1, and not HO-2, was tested in the present study because we had already demonstrated, in similar experimental condition, that among the two main HO isoforms (HO-1 and HO-2), only HO-1 is overexpressed in small mesenteric arteries of CCl4-induced cirrhotic rats (Bolognesi et al., 2005).

The protein expression of the two subunits of BKCa, β1- and α-subunits, were detected using polyclonal rabbit anti-slo β1 (KC-NMBI) and anti-κα 1,1 (α subunit 1) (KCNaM1) antibody (Alomone Labs Ltd., Jerusalem, Israel). The secondary antibodies, anti-rabbit conjugated to horseradish peroxidase, were diluted 1:1000 in phosphate-buffered saline containing 2% nonfat dry milk. Antibiotic detection was visualized by standard ECL-enhanced chemiluminescence (Amersham, Arlington Heights, IL) with exposure to X-ray film. Control antigens (Alomone Labs Ltd.) were used as positive controls. Protein expression was determined by densitometric analysis using the VersaDoc Imaging System (Bio-Rad Laboratories, Hercules, CA). After stripping, the blots were assayed for β-actin content as standardization of sample loading. The quantitative densitometric values of each protein of interest were normalized to β-actin and displayed in histograms.

Protein expression of HO-1 was evaluated in four control rats, in six cirrhotic rats with asites, and also in four cirrhotic rats without asites. Protein expression of BKCaα subunits was evaluated in five control rats, in four cirrhotic rats with asites, and also in four cirrhotic rats without asites.

**Data Analysis.** Data were expressed as mean ± S.E. Vasorelaxant responses were expressed as percent inhibition of the contraction induced by PE. Concentration-response data derived from each vessel were fitted separately to a logistic function by nonlinear regression and EC50 (molar concentration of ACh causing 50% of the maximal vasorelaxant effect) was calculated and expressed as log (molar) (pEC50). From the same regression, the maximal relaxation of the artery was also calculated. Two-way ANOVA was used to compare dose-response curves from controls and treated groups. Other data were analyzed by one-way ANOVA or Student’s t test for paired or unpaired observations when appropriate. The α values quoted indicate the number of experiments and animals used. The null hypothesis was rejected at p < 0.05.

**Results.** All rats treated with CCl4 included in the study had macronodular or micronodular cirrhosis. In 27 of 35 cirrhotic rats, the presence of ascites was confirmed by visual examination at laparotomy. The presence of ascites was dubious in two cirrhotic rats. These “intermediate” rats were classified as nonascitic, but they were not used for any experiment. Control rats had no appreciable alteration in liver appearance. At the time of the study, no difference in body weight between cirrhotic (nonascitic rats, 570 ± 24 g; ascitic rats, 548 ± 11 g) and control rats (575 ± 20 g) was observed.

**Evaluation of the Response to ACh.** Comparing dose-response curves with ACh in baseline condition, ascitic cirrhotic rats (n = 6) showed a higher sensitivity to ACh with respect to control rats (p = 0.036) (Fig. 1).

**Effect of HO Inhibition.** Inhibition of HO with CrMP caused a slight shift of the concentration-response curve to ACh in control rats (F = 8.61, p < 0.001, two-way ANOVA), without a change in EC50 (Fig. 1). On the contrary, a marked rightward shift of the concentration-response curve to ACh was detected after CrMP in cirrhotic rats with asites (F = 4.38, p = 0.0027, two-way ANOVA), with a significant increase in EC50 (Fig. 1). The increase in pEC50 after CrMP was significantly higher in cirrhotic rats with asites (p = 0.03) (Fig. 1). Following CrMP, sensitivities of control and cirrhotic rats with asites to ACh were the same (Fig. 1).

**Effect of sGC Inhibition and of HO Inhibition.** After treatment with Indo and L-NAME, mesenteric arteries of cirrhotic rats with asites maintained a higher sensitivity to ACh with respect to control rats (p = 0.05) (Fig. 2). In arteries treated with Indo and L-NAME, the inhibition of sGC with ODQ provoked a shift of the dose-response curve to ACh, both in control rats (F = 3.58, p = 0.01, two-way ANOVA) and in cirrhotic rats with asites (F = 8.09, p = 0.0005, two-way ANOVA) (Fig. 2). The addition of CrMP to ODQ provoked a further decrease in the response to ACh, both in control rats (F = 9.77, p < 0.001, two-way ANOVA) and in cirrhotic rats with asites (F = 15.64, p < 0.001, two-way ANOVA) (Fig. 2). The effect of ODQ and of CrMP + ODQ on maximal relaxation of the artery was slightly but not significantly greater in control rats than in cirrhotic rats with asites (Fig. 2).
Rats (difference in the change of pEC50, effect of ACh more in cirrhotic rats with ascites with respect to controls with ascites with respect to control rats, \( p \). IbTx did not provoke a further decrease in the response to between the two groups, §, significantly different (\( p < 0.01 \)) from baseline (two-way ANOVA). #, significantly different with respect to control rats; $, \( p = 0.05 \) with respect to control rats; §, significantly different (\( p < 0.05 \)) from Indo + L-NAME; **, significantly different (\( p < 0.05 \)) from Indo + L-NAME + ODQ.

**Effect of BKCa Inhibition.** Even after treatment with Indo, L-NAME, and ODQ, mesenteric arteries of cirrhotic rats with ascites maintained a higher sensitivity to ACh with respect to control rats (\( p = 0.024 \)) (Fig. 3). The addition of IbTx provoked a decrease in the response to ACh both in control and in cirrhotic rats with ascites (\( F = 11.59, p = 0.001 \), two-way ANOVA) (Fig. 3). The effect of IbTx was more evident in ascitic cirrhotic rats than in controls (the increase in pEC50 after IbTx was significantly higher in cirrhotic rats with ascites with respect to control rats, \( p = 0.010 \), whereas the decrease in maximal relaxation was not different between the two groups, \( p = N.S.) \). The addition of CrMP to IbTx did not provoke a further decrease in the response to ACh both in control rats and in cirrhotic rats with ascites (\( p = N.S., \) two-way ANOVA) (Fig. 3). Final sensitivity and maximal relaxation to ACh were similar in controls and in ascitic cirrhotic rats (Fig. 3). In control rats, 25 nM IbTx did not change the response of mesenteric arteries to ACh, whereas a significant rightward shift of the concentration-response curve to ACh was evident after incubating the arteries with the higher concentrations of IbTx (50 and 100 nM) (\( F = 6.77, p < 0.001 \), two-way ANOVA) (Fig. 4).

**Evaluation of the Response to CORM-3 of Small Mesenteric Arteries Preconstricted with Phenylophrine in CCl4 Cirrhotic Rats.** The addition of CORM-3 to the superfusion caused a slight but not significant dilatation (5 ± 3%) in mesenteric arteries of control rats (\( p = N.S. \)), whereas it caused a significant vasorelaxation (25 ± 6%) in cirrhotic rats with ascites (\( p = 0.05 \)) (Fig. 5). Vasorelaxation was more evident in ascitic cirrhotic rats than in controls (\( p = 0.012 \)) (Fig. 5). In arteries treated with CrMP and preconstricted with PE, CORM-3 provoked a significantly greater vasorelaxation both in controls and in ascitic cirrhotic rats (\( p = 0.016 \) and \( p = 0.022 \), respectively), but in the latter group, the vasorelaxation was more evident (\( p = 0.036 \)) (Fig. 5). In arteries pretreated with CrMP and ODQ and preconstricted with PE, CORM-3 did not provoke any significant dilatation, both in control and in cirrhotic rats with ascites (Fig. 5). In arteries pretreated with CrMP and IbTx and preconstricted
Fig. 3. Concentration-response curves to Ach obtained in small resistance mesenteric arteries incubated with Indo (2.8 μM), L-NAME (1 mM), and ODQ (10 μM), to inhibit COX, NOS, and sGC, respectively. In the presence of vehicle (open circle), the effect of Ach was increased in cirrhotic rats with ascites with respect to control rats (difference in pEC50, p = 0.024). With respect to vehicle (open circle), incubation with the BKCa inhibitor IbTx (25 nM) (closed square) decreased the effect of Ach both in control rats and in rats with cirrhosis and ascites. The inhibition of BKCa was more effective on the endothelium-dependent vasodilation induced by Ach in cirrhotic rats with ascites with respect to control rats (difference in p(EC50), p = 0.010). The addition of CrMP (15 μM) to IbTx (25 nM) (closed triangle) did not further modify the effect of Ach in both groups. $\#$, significantly different (p < 0.01) from Indo + L-NAME + ODQ (two-way ANOVA); $\*$, p = 0.024 with respect to control rats. $\*$, significantly different (p < 0.05) from Indo + L-NAME + ODQ.

with PE, CORM-3 caused a significant dilatation in controls rats (14 ± 3%, p = 0.004), which was slightly but not significantly lower than the dilatation obtained in the arteries pretreated only with CrMP (14 ± 3 versus 20 ± 3%, respectively, p = N.S.) (Fig. 5); on the contrary, in cirrhotic rats with ascites, pretreatment with CrMP and IbTx markedly reduced the dilator effect of CORM-3 with respect to the effect obtained after pretreatment with only CrMP (41 ± 1 versus 46 ± 11%, respectively, p = 0.003) (Fig. 5).

After endothelium removal, CORM-3 provoked a significant but slight dilatation both in controls (p = 0.002) and in cirrhotic rats (p = 0.056) but more evident in controls (p = 0.015) (Fig. 6). After the addition of a low concentration of SNP, which caused per se a modest dilation (25 ± 8% versus 25 ± 5%, p = N.S., in controls and cirrhotic rats, respectively), the vasodilating effect of CORM-3 was more evident, both in controls (p = 0.0003) and in cirrhotic rats (p = 0.037), but remained more evident in control rats (p = 0.0008) (Fig. 6).

Western Blot Analysis. In small mesenteric resistance arteries, HO-1 and BKCa α-subunit protein expression was increased in rats with cirrhosis, particularly in those with ascites (Figs. 7 and 8). On the contrary, BKCa β1-subunit protein expression was significantly decreased both in cirrhotic rats with and without ascites (Fig. 8).
Discussion

This study demonstrates that in small mesenteric arteries of rats with CCl4-induced cirrhosis, the response to ACh is increased, and it is normalized by the inhibition of HO and BKCa. CO was more effective in cirrhotic than in control rats, and the effect was hindered by BKCa inhibition. In mesenteric arteries of cirrhotic rats, there is an overexpression of the α-subunit of BKCa, which together with the increased expression of HO-1 and an increased production of CO may cause the increased response to ACh.

BKCa channels are composed of the pore-forming α-subunit and of the auxiliary regulatory β-subunits that modulate channel gating (Tanaka et al., 2004). A change in the expression of BKCa subunits has been reported in experimental arterial hypertension. Indeed, a decrease in β1-subunit expression has been reported in genetic (Amberg and Santana, 2003a) and in acquired angiotensin II-induced hypertension (Amberg et al., 2003b), suggesting that it may contribute to the development of hypertension. Bratz et al. (2005) reported a decrease in the expression of BKCa α-subunit in superior mesenteric arteries from rats made hypertensive with N(ω)-nitro-L-arginine. Liu et al. (1997, 1998) reported an increase in the expression of the pore-forming α-subunit in aortas (Liu et al., 1997) and in cerebral arteries (Liu et al., 1998) of spontaneously hypertensive rats, and they suggested that such an increase may be a compensatory vasodilatory reaction in systemic hypertension (Liu et al., 1998). Our data demonstrate that an altered expression of BKCa may participate in the exaggerated mesenteric vasodilatation of cirrhosis. BKCa is stimulated by the CO locally produced by endothelial HO (Naik et al., 2003a), which is also overexpressed in experimental cirrhosis (Bolognesi et al., 2005).

We showed that inhibition of HO was more effective on the endothelium-dependent vasodilatation in cirrhotic rats with ascites with respect to controls rats (Fig. 1). To analyze the mechanisms that could link HO to the ACh-induced mesenteric vasorelaxation, we studied the relationship between HO and the effectors of CO on smooth muscle cells, i.e., sGC and BKCa (Zhang et al., 2001; Jaggar et al., 2002; Wu et al., 2002).

In arteries treated with Indo and L-NAME, to inhibit any possible interfering action from cyclooxygenase (COX) and nitric oxide synthase (NOS), respectively, both the inhibition of sGC and, afterward, of HO had a similar effect on ACh-induced vasorelaxation in control and in ascitic cirrhotic rats. Hence, the action of HO on smooth muscle cells is not exclusively due to the activation of sGC; moreover, the action on sGC is not what differentiates the HO action in ascitic cirrhotic rats.

We evaluated the effect of inhibition of BKCa in mesenteric arteries pretreated with the inhibitors of COX, NOS, and sGC. Sensitivity to ACh was increased in ascitic cirrhotic rats also after the inhibition of these three systems. The inhibition of BKCa caused a decrease in the maximal relaxation to ACh, particularly in control rats, but it provoked a marked decrease in the sensitivity to ACh only in cirrhotic rats with ascites, suggesting a role of BKCa in the altered vasoactive response that occurs in this pathological condition. Further inhibition of HO did not lead to any modification in the response to ACh, both in controls and in cirrhotic rats with ascites, excluding other mediators of HO.

Because CO is the vasoactive molecule produced by HO (Zhang et al., 2001), we investigated the response to exogenous CO in small mesenteric arteries of cirrhotic rats with ascites. We used CORM-3, a CO-releasing water-soluble molecule (Clark et al., 2003; Foresti et al., 2004). In mesenteric
arteries of control rats, the vasodilating response to CORM-3 was insignificant, whereas it was evident after elimination of endogenous CO by pretreatment with the HO inhibitor (Sacredoti et al., 2006) (Fig. 5). A similar trivial effect of exogenous CO in baseline conditions has been reported by Kozma et al. (1999), who reported that in first order gracilis muscle arterioles, CO does not produce arteriolar dilatation unless the preparation is exposed previously to CrMP. One possible explanation for the ineffectiveness of exogenous CO as a vasodilator in preparations not exposed to an inhibitor to HO is that, in such a setting, the vasodilatory mechanism mediated by endogenous CO is maximally active (Kozma et al., 1999; Zhang et al., 2001). Another explanation may be that inhibition of endogenous CO results in increased NOS activity (Johnson and Johnson, 2003b), which, in turn, permits the exogenous CO to cause dilatation (Barkoudah et al., 2004). At any rate, in control rats, the vasodilatory effect of exogenous CO after HO inhibition was completely abolished after inhibition of sGC but only partially and not significantly reduced after inhibition of BKCa (the final response was significantly lower with respect to control rats, \( p < 0.015 \)), underlining the pivotal role of these channels for the action of CO in this condition. The increased vasodilating effect of CO in ascitic cirrhotic rats was reversed in mesenteric arteries without endothelium, a condition in which the CO effect was lower with respect to control rats, even after SNP pretreatment. These results, taken together, not only support the hypothesis that the HO/CO system plays a role in the vasodilatation of mesenteric arteries in ascitic cirrhosis but also suggest that in ascitic cirrhotic rats, the effect of CO is mainly through BKCa. The lack of CO effect after sGC inhibition, reported also by other authors (Villamor et al., 2000; Naik and Walker, 2003b), could be explained by the finding of Barkoudah et al. (2004), who hypothesized a permissive role of cGMP for effect of CO on BKCa. This interpretation is confirmed by the scarce effect of CO detected in arteries after removal of the endothelium (Barkoudah et al., 2004; Foresti et al., 2004), an effect that was restored after the administration of NO, which probably produces the minimal background level of cGMP necessary for CO to cause vasodilatation by activating BKCa (Barkoudah et al., 2004).

The administration of CORM-3 to mesenteric arteries of cirrhotic rats with ascites caused a vasodilation evident even in baseline condition, which was amplified by pretreatment with CrMP. The dilator effect of CO was abolished not only after sCG inhibition but also after the inhibition of BKCa (the final response was significantly lower with respect to control rats, \( p < 0.015 \)), underlining the pivotal role of these channels for the action of CO in this condition. The increased vasodilating effect of CO in ascitic cirrhotic rats was reversed in mesenteric arteries without endothelium, a condition in which the CO effect was lower with respect to control rats, even after SNP pretreatment. These results, taken together, not only support the hypothesis that the HO/CO system plays a role in the vasodilatation of mesenteric arteries in ascitic cirrhosis but also suggest that in ascitic cirrhotic rats, the effect of CO is mainly through BKCa. They also emphasize that, in cirrhotic rats, there are other endothelial factors, apart from NO, permitting the vasodilating effect of CO on mesenteric arteries. It is not clear what these factors are. It is possible that a role is played by the endothelial-derived hyperpolarizing factor and/or by the myoendothelial gap junctions. Further studies are needed to clarify this issue.

Western blot data confirmed and supported the results of the

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**Fig. 8.** Western blot analysis of the α- and of β1-subunits of BKCa in the small resistance mesenteric vessels of controls rats and of cirrhotic rats, with and without ascites. The reported blots are representative of three to five experiments. Lanes 1 and 2, cirrhotic rats with ascites; lanes 3 and 4, cirrhotic rats without ascites; lanes 5 and 6, control rats. Lower, densitometric analysis of α-subunit (graph A) and of β1-subunit (graph B). *, \( p < 0.05 \) with respect to control rats. In cirrhotic rats with and without ascites, the expression of BKCa α-subunit was increased, whereas the expression of BKCa β1-subunit was decreased.
hemodynamic experiments. There was an increase in protein expression of HO-1 and of BKCa, α-subunit in mesenteric arteries of cirrhotic rats, particularly in those with ascites, associated with a decrease in the expression of β-subunit. This finding supports the hypothesis that CO could play a key role in the regulation of mesenteric vasorelaxation in cirrhosis because the α-subunit is the target of CO on BKCa. CO action on BKCa has been identified in the capacity of enhancing the coupling of Ca2+ sparks to BKCa. (Jaggar et al., 2002). More recently, Jaggar et al. (2005) have demonstrated that CO activates BKCa by binding to heme and modifying its interaction with an α-subunit heme-binding domain. The effect of CO on BKCa is independent of the regulatory β-subunit (Wu et al., 2002; Jaggar et al., 2005). Indeed, the presence of BKCa, β-subunit is not necessary for the effect of CO, whereas on the contrary, it is essential for the stimulating effect of NO (Wu et al., 2002). The decreased expression of β-subunit may be due to activation of the Renin-Angiotensin-Daldoisterone system present in ascitic cirrhosis (Wilkinson and Williams, 1980; Schrier et al., 1988) because β-subunit synthesis is inhibited by high levels of angiotensin II (Amberg et al., 2003b). However, the decreased presence of β-subunit seems not sufficient to inhibit the action of BKCa because of the increased expression of the α-subunits and of the increased expression of HO, which through CO stimulates BKCa independently from β-subunit. Why the expression of α-subunit is increased in cirrhotic rats remains to be clarified. Dubuis et al. (2002, 2005) and Wu and Wang (2005) have hypothesized that the expression of BKCa, channels may be induced by high CO levels, a condition that might be present in the mesenteric circulation of cirrhotic rats. In conclusion, an overexpression of BKCa, α-subunits, together with HO-1 up-regulation and increased CO production, participates in the endothelium-dependent alterations and mesenteric arterial vasodilatation of ascitic cirrhotic rats.

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References
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