NCX-1000, a NO-releasing derivative of ursodeoxycholic acid, selectively delivers NO to the liver and protects against development of portal hypertension

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Portal hypertension resulting from increased intrahepatic resistance is a common complication of chronic liver diseases and a leading cause of death in patients with liver cirrhosis, a scarring process of the liver that includes components of both increased fibrogenesis and wound contraction. A reduced production of nitric oxide (NO) resulting from an impaired enzymatic function of endothelial NO synthase and an increased contraction of hepatic stellate cells (HSCs) have been demonstrated to contribute to high intrahepatic resistance in the cirrhotic liver. 2-(Acetyloxy) benzoic acid 3-(nitrooxymethyl) phenyl ester (NCX-1000) is a chemical entity obtained by adding an NO-releasing moiety to ursodeoxycholic acid (UDCA), a compound that is selectively metabolized by hepatocytes. In this study we have examined the effect of NCX-1000 and UDCA on liver fibrosis and portal hypertension induced by i.p. injection of carbon tetrachloride in rats. Our results demonstrated that although both treatments reduced liver collagen deposition, NCX-1000, but not UDCA, prevented ascite formation and reduced intrahepatic resistance in carbon tetrachloride-treated rats as measured by assessing portal perfusion pressure. In contrast to UDCA, NCX-1000 inhibited HSC contraction and exerted a relaxing effect similar to the NO donor *S*-nitroso-*N*-acetylpenicillamine. HSCs were able to metabolize NCX-1000 and release nitrite/nitrate in cell supernatants. In aggregate these data indicate that NCX-1000, releasing NO into the liver microcirculation, may provide a novel therapy for the treatment of patients with portal hypertension.

Portal hypertension resulting from increased intrahepatic resistance is a common complication of chronic liver diseases and a leading cause of death in patients with liver cirrhosis, a scarring process of the liver that includes components of both increased fibrogenesis and wound contraction (1). A close relationship occurs between the progression of liver fibrosis and development of portal hypertension. In particular, the development of portal-central anastomosis and arterialization/capillarization of liver sinusoids represents the hallmark of the fibrogenic process as well as the key determinant for increased intrahepatic vascular resistance (2). Portal-central anastomoses are embedded in developing scar tissue where a complex interplay between several cell types and soluble mediators occurs. Recent evidence links perisinusoidal hepatic stellate cells (HSCs), also known as Ito cells or lipocytes, which are analogous to tissue pericytes or vascular smooth muscle cells, to a role in portal hypertension (3–5). Indeed, not only are HSCs involved in collagen deposition and development of liver fibrosis, but they also regulate intrahepatic blood flow by sinusoid contraction/constriction (3–5). In the injured liver, HSCs undergo a striking functional transition process termed activation. A critical feature of the activation process is the acquisition of smooth muscle isoforms of actin and myosin and enhanced contractile properties (5). The transition/activation process is associated with an enhanced expression of receptors for endothelins, suggesting that increases in intrahepatic resistance are, at least in part, caused by an enhanced contractile response to these vasoconstrictor agents (6, 7). HSC contraction is counterbalanced by vasorelaxing agents such as nitric oxide (NO) (8). NO is an ubiquitous messenger produced from conversion L-arginine to L-citrulline by either constitutive or inducible NO synthase (NOS) (9, 10). The constitutive NOS isoforms are localized predominantly in neuronal cells and endothelial cells (eNOS) (9, 10). There is evidence that in the injured liver endothelin-1 production is increased (as a result of enhanced production by HSCs) whereas NO release from sinusoidal endothelial cells is reduced, ostensibly owing to impaired function of endothelial cell eNOS (11–13). Because NO relaxes HSCs and antagonizes the effect of endothelin-1, a reduced production of NO will modify the dynamic balance of endothelin-1 and NO, favoring sinusoidal and vascular wall constriction by perisinusoidal stellate cells and hepatic vascular smooth muscle cells (14–16). Given the apparent defect of eNOS function in sinusoidal endothelial cells of the cirrhotic liver, several pharmacological approaches have been attempted to deliver NO directly to the liver and reduce intrahepatic resistance (17–20). Nitrovadilators have been the focus of current therapeutic approaches aimed at intrahepatic vasodilation. Although these agents reduce portal pressure in experimental animals and humans, recent studies indicate that their systemic vasodilatory actions may have detrimental effects on portal hypertension patients, suggesting that developing a compound(s) that selectively releases NO in the liver without affecting systemic arterial pressure could represent a major advance in the treatment of this life-threatening disease (21).

NO-releasing nonsteroidal anti-inflammatory drugs (NO-NSAIDs) are a recently described class of NSAID derivatives

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Abbreviations: CCl_4, carbon tetrachloride; eNOS, endothelial NO synthase; HSC, hepatic stellate cell; iNOS, inducible NO synthase; MCP-1, monocytic chemotactic protein-1; NCX-1000, 2-(acetyloxy) benzoic acid 3-(nitrooxymethyl) phenyl ester; NOS, nitric oxide synthase; NSAID, nonsteroidal anti-inflammatory drug; SNAP, *S*-nitroso-*N*-acetylpenicillamine; TNF-α, tumor necrosis factor α; UDCA, ursodeoxycholic acid.

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generated by adding an NO-releasing moiety to parental NSAIDs (22–24). NO-NSAIDs not only lack the gastrointestinal damaging effect of classical NSAIDs, but the slow release of NO in a biological microenvironment also confers novel activities not shared by parent molecules (24). We and others have previously demonstrated that, owing to their chemical structure, these NO-NSAIDs release little amounts of NO in the systemic circulation but transport NO to the liver where they are metabolized (24). Based on this background, we have hypothesized that adding a NO-releasing moiety to ursodeoxycholic acid (UDCA), a compound with an almost exclusive liver metabolism, would have conferred to this new chemical entity the property of selectively releasing significant amount of NO directly to the liver and compensated for defective NO production in cirrhosis (25). Indeed, in a previous study we have demonstrated that this compound is metabolized *in vitro* by isolated hepatocytes to release NO and NO-derived compounds and exerts potent anti-apoptotic effects in animal models of liver injury (25).

The present study was designed to investigate whether NO-UDCA, hereon referred to as NCX-1000 [2-(acetyloxy) benzoic acid 3-(nitrooxymethyl) phenyl ester] (25) reduces portal hypertension in an animal model of chronic liver injury and to define the mechanism responsible for the protective effects. Our results demonstrate that NCX-1000 prevents the development of portal hypertension in cirrhotic rats by modulating HSC contractility.

Materials and Methods

Materials. Carbon tetrachloride (CCl_4), UDCA, and urethane were from Sigma, NCX-1000 was from Nicox (Milan, Italy), and phenobarbital was from Bracco (Milan, Italy).

Induction of Cirrhosis. The study was approved by the Animal Study Committee of the University of Perugia. Male Wistar rats (150–180 g) were obtained from Charles River Breeding Laboratories and maintained on standard laboratory rat chow on a 12-h light/dark cycle. They were given phenobarbital sodium (35 mg/dl) with their drinking water for 3 days and then cirrhosis was induced by i.p. injection of CCl_4, 100 μl/100 g body weight in an equal volume of paraffin oil (21).

Study Protocol. Two parallel protocols were carried out. On the first protocol, 54 rats, 12 animals/group unless specified, were randomly allocated to receive one of the following treatments: group 1 had phenobarbital induction and no further treatment; group 2 (16 animals) was treated with CCl_4 twice a week for 8 weeks; group 3 had CCl_4 twice a week plus UDCA (15 mg/kg); and group 4 had CCl_4 twice a week plus NCX-1000 (15 mg/kg). NCX-1000 and UDCA were dissolved in carboxymethyl cellulose and administered daily by gavage. Animal weight was monitored daily through the study period, and the dosage of CCl_4 was adjusted accordingly to the animal weight. At the end of the treatment surviving animals were killed by an overdose of uretane, and blood, ascitic fluid, and livers were collected. A portion of each liver was fixed in 10% formalin for histological evaluation. The remaining tissue was partitioned and immediately stored under frozen liquid nitrogen at −80°C until used. On the second protocol, 74 rats were randomly allocated to receive the same treatments as protocol 1. At the end of the study, surviving animals were tested for portal and arterial pressure measurement (see below).

Liver Histology, Collagen Immunohistochemistry, and Biochemical Assays. At least 2–3 liver samples (10–15 mg/each) from each animal were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. For evaluation of the liver type I collagen content, cryostat sections were prepared from frozen liver, and immunohistochemistry was carried out as described (28) by using a rabbit polyclonal antibody against monkey pIp (procollagen type I N-terminal propeptide), kindly donated by D. Schuppan, University of Erlangen, Erlangen, Germany (16). The immunohistochemical staining for collagen I was analyzed by a computerized video-image analysis system, Leica (Cambridge) Quantiment Q500MC. A total of five sections for each liver tissue sample were observed under a light field microscope equipped with a ×10 lens. The specific signal was acquired by a charge-coupled device videocamera connected to the microscope. The signal was converted to digital and transformed into pixel units. The threshold of specific detection was automatically calibrated on control sections stained with preimmune rabbit immunoglobulins. Results were expressed as percent area occupied by the signal. In each case, the area analyzed extended beyond the central or portal vein into the surrounding lobule. The average of the score taken from 10 random fields was used to generate a single score for each animal's liver. Liver function was assessed by measuring plasma aspartate aminotransferase, bilirubin, albumin, γ-glutamyl transpeptidase, and alkaline fosfatase levels by a 726 Hitachi (Tokyo) automatic analyzer as described (26).

Reverse Transcription–PCR Analysis of Liver eNOS and Aortic Inducible NOS (iNOS). Total RNA was isolated from liver and thoracic aorta by using TRIzol reagent (Life Technologies, Milan, Italy) as described (25). Primers (Sigma) for rat eNOS were: 5′-TTC CGG CTG CCA CCT GAT CCT AA-3′ (sense) and 5′-AAC ATG TGT CCT TGC TCG AGG CA-3′ (antisense) and for iNOS were: AAC AGT GGC AAC ATC AGG TCG G (sense) and CTC CAA TCT CGG TGC CCA TGT A (antisense). The β-actin primers were used as a control (25).

Liver eNOS Activity. Liver eNOS activity was assessed by determining the conversion of ^{3}H-labeled L-arginine to ^{3}H-labeled L-citrulline based on a modified version of the method by Bredt *et al.* (27). Briefly, liver tissue was homogenized in a lysis buffer containing 0.1 mm EGTA, 50 mM Tris·HCl, 0.1 mM EDTA, 5 mM leupeptin, 1 mM PMSF, 1% (vol/vol) Nonidet P-40, 0.1% SDS, and 0.1 mM deoxycholate at pH 7.5. Samples then were incubated with a buffer containing 1 mM reduced nicotinamide adenine dinucleotide phosphate, 3 μM tetrahydropterin, 11 nM calmodulin, 2.5 mM CaCl2, 50 mM valine, 10 μM L-arginine, and 0.2 μCi of L-^{3}H arginine at 37°C. To determine NOS activity, samples were incubated for 20 min and the reaction was stopped by adding 1 ml of cold stop buffer containing 20 mM Hepes, 2 mM EDTA, and 2 mM EGTA at pH 5.5. The mixture then was passed over a Dowex AG 50WX-8X mesh cationic exchanging resin column (Bio-Rad). The column was washed with an additional 1 ml of the same solution, and L-^{3}H citrulline was collected and quantitated by liquid scintillation. Retrieved L-^{3}H citrulline was normalized to liver weight.

Liver Nitrite/Nitrate. Liver samples were homogenated (see above), and nitrite/nitrate concentrations were measured by a fluorimetric detection kit (Cayman Chemicals, Ann Arbor, MI) following the manufacturer's instructions, as described. The lower detection limit was ≈4 pM/well.

Portal Pressure and Isolated Liver Perfusion. *In vivo* portal pressure was measured in urethane anesthetized rats, control animals, and cirrhotic animals, by cannulating the portal vein with a polyethylene catheter (PE-50) and recorded with a strain-gauge transducer connected with PowerLab PC (A.D. Instruments, Milford, MA), according to a previously published method (13, 21). The bile duct was cannulated with polyethylene tubing (PE-10). The hepatic artery was ligated. A ligature was passed around the inferior vena cava above the renal veins, and the vein was injected with 500 units of heparin. The portal vein then was cannulated with a 16-gauge Teflon catheter. The liver was

Table 1. Effects of NO-UDCA and UDCA on body and liver weight, ascites, and liver-related serum parameters after 8 weeks of CCl_4-induced cirrhosis

Treatment (number of animals)	Body weight, g	MAP, mmHg	Albumin, g/dl	γGT, units/liter	AST, units/liter	Ascites, %	Ascitic protein, g/liter
Control (18)	463 ± 18.2	120 ± 4.1	4.4 ± 0.8	38.7 ± 27.7	50.5 ± 19	0	0
CCl_4 (20)	356 ± 7.0	111 ± 5.2	4.5 ± 0.1	39.7 ± 62.4	93 ± 29	75	1.3 ± 0.04
CCl_4 + UDCA (18)	356 ± 3.4	114 ± 2.8	3.9 ± 0.4	27.4 ± 17.9	153 ± 52	62.5	1.2 ± 0.1
CCl_4 + NO-UDCA (18)	386 ± 22.0	116 ± 3.1	4.3 ± 0.2	8.8 ± 6.6*	186 ± 50	28.5*	1.2 ± 0.1

Data are mean ± SE. MAP, mean arterial pressure. AST, aspartate aminotransferase; γGT, γ-glutamyl transpeptidase. *, $P < 0.01$ versus CCL_4 alone.

perfused immediately with Krebs solution containing 2 units/ml of heparin and oxygenated with carbogen gas (95% O_2-5% CO_2) at 37°C. The inferior vena cava then was cut below the ligature, thus allowing the perfusate to escape. Thereafter, the thorax was opened and the supradiaphragmatic portion of the inferior vena cava was cannulated with a 14-gauge Teflon catheter, and the ligature around the vein was tied. The liver was perfused in a nonrecirculating mode with Krebs solution equilibrated with carbogen gas at variable rate (see Fig. 2) by using a peristaltic pump (Gilson). The perfusion pressure was continuously monitored and recorded with the strain-gauge transducer connected to the portal inflow cannula ≈10 cm proximal to the perfusion cannula (13). Four rats for each group were tested for arterial pressure measurement. Briefly, the left common carotid artery was exposed through a cervical incision, and a 2FG polyethylene cannula was introduced into the carotid artery via an arteriotomy. The cannula was connected to strain gauge transducer as described before. After 25-min equilibration arterial and portal pressure was recorded with the same apparatus.

HSC Contraction. HSCs were isolated from rat liver by the pronase-collagenase method (28, 29). HSCs then were cultured on uncoated plastic dishes where they spontaneously acquired an activated phenotype, characterized by the expression of α-smooth muscle actin and loss of vitamin A droplets. After reaching confluency (about 14 days after plating), activated HSCs were detached by incubation with trypsin (0.025% trypsin/0.5 mM EDTA), split in a 1:5 ratio, and subcultured as described. Experiments were performed on cells between the first and third serial passages. Contraction of HSCs on collagen lattices was performed in 24-well flat-bottom tissue culture plates or 25-mm plastic culture dishes according to the method described by Rockey *et al.* (29). Briefly, culture plates (Costar) were preincubated with PBS containing 1% BSA (500 μl per well) for at least 1 h at 37°C, then washed twice with PBS and air-dried. A mixture of eight parts Vitrogen 100 (Nutacon, Amsterdam, The Netherlands), 1 part 10 × MEM, and 1 part 0.2 M Hepes (resulting in a final collagen concentration of 2.4 mg/ml) was made at 4°C, added to the culture plates, and incubated for 1 h at 37°C to allow gelation. HSCs were suspended in a standard medium containing 20% FCS and layered on top of formed lattice. Cell contraction then was induced by adding FCS to the HSC monolayers (29). To assess whether NCX-1000 inhibited FCS-induced contraction, 100 μM NCX-1000, UDCA, or *S*-nitroso-*N*-acetylpenicillamine (SNAP) were added to the HSC monolayers, lactice then was detached by gentle circumferential dislodgment with a 200-μl micropipet tip, and contraction was measured by monitoring the change in lattice area over 24 h. To assess whether NCX-1000 releases NO, HSCs were incubated with UDCA, NCX-1000 or SNAP at 100 μM. At the indicated time point (see Fig. 4) samples of supernatants were collected and nitrite/nitrate was measured by a fluorimetric detection kit as described above. Intracellular NO formation in NCX-1000-treated HSCs was carried out as described (25). Briefly, HSCs cells (1×10^5/ml) were loaded by suspending them in PBS in the presence of 10 μM 4,5-diaminofluorescein diacetate (Calbiochem). Cells were washed twice in iced buffer solution, and samples were added to a quartz cuvette while stirring continuously, and the temperature was thermostatically maintained at 37°C by using a Hitachi 2000 fluorescence spectrophotometer. Samples were preincubated with 1 mM L-N6-(1-iminoethyl) lysine for 30 min to suppress endogenous NO generation and then incubated with 100 μM SNAP, NCX-1000, or UDCA, and excited at 395 nm, and the intensity of fluorescence was emitted at 515 nm recorded. NO generation was expressed in arbitrary units of absorbance.

Monocyte Chemotactic Protein-1 (MCP-1). HSC activation also was assessed by measuring MCP-1 concentrations (R & D Systems) in supernatants of HSCs (1×10^5/ml) incubated with 100 ng/ml tumor necrosis factor α (TNF-α) and IFN-γ for 24 h (30).

Statistical Analysis. ANOVA or Student's *t* test were used for statistical comparisons. For calculation of mean values and statistical variation, *n* indicates the number of separate experiments each with an individual cell preparation. Data are means ± SEM.

Results

NCX-1000 Prevents Ascite Formation in CCl_4-Treated Rats. Long-term CCl_4 administration resulted in extensive liver damage and nodular transformation. At laparotomy all CCl_4-treated rats showed macroscopically macro/micronodular cirrhosis of the liver. The CCl_4-treated animals had signs of portal hypertension and splenomegaly increased diameter of the portal vein and its intestinal afferents and ascites (Table 1). The weight of cirrhotic rats was found to be significantly lower than that of sex- and age-matched control animals (356.0 ± 27.0 g vs. 463.0 ± 18.0 g, respectively; $P < 0.0001$) (Table 1). As shown in Table 1, treating animals with UDCA or NCX-1000 had no effect on serum albumin or bilirubin, although NCX-1000 caused a statistically significant reduction of γ-glutamyl transpeptidase. In contrast to UDCA, NCX-1000 significantly reduced the percentage of animals with ascites from 75% to 28.5% ($P < 0.001$) (Table 1). The analysis of protein content in the ascitic fluid confirmed the noninflammatory origin of the transudates. Moreover, in contrast to UDCA, NCX-1000 significantly decreased the amount of nitrite/nitrate content in the ascitic fluid ($P < 0.01$). Thus NCX-1000, but not UDCA, reduces ascite formation in a rat model of chronic liver injury.

NCX-1000 and UDCA Reduce Collagen Deposition. At immunohistochemical analysis and semiquantitative scoring evaluation, liver specimens obtained from rats treated with CCl_4 showed extensive central-portal, central-central, and portal-portal bridging fibrosis (Fig. 1). In the CCl_4-treated rats the total amount of type I collagen was ≈22.5% of total area ($P < 0.01$ versus controls). UDCA and NCX-1000 decreased total liver collagen contents of ≈50%, compared with the livers of animals treated with CCl_4

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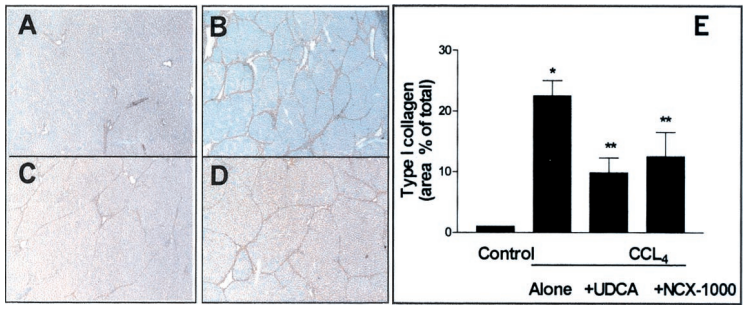

Fig. 1. Liver collagen content in histological sections of rat liver stained with anticollagen 1 antibody. Representative photomicrographs of liver obtained from control (*A*) or cirrhotic rats. Rats were treated for 8 weeks with CCl_4 alone (*B*), CCl_4 plus 15 mg/kg per day UDCA (*C*), or 15 mg/kg per day NCX-1000 (*D*). In UDCA- and NCX-1000-treated animals, lobular collagen is reduced. Collagen fibrils were counted in ×10 high-power fields per section. (*E*) Quantification of liver collagen content. Values represent the mean ± SEM of at least eight rats. *, $P < 0.01$ versus control; **, $P < 0.01$ versus CCl_4 alone.

alone ($P < 0.01$); however, there were no rats with a histologically normal liver (Fig. 1).

NCX-1000 Reduces Portal Pressure in Cirrhotic Rats. Because these data demonstrated that NCX-1000 and UDCA reduced liver fibrosis, but UDCA had no effect on the percentage of animals that developed ascites, we have reasoned that the NO-releasing derivative should had protected against ascite development by reducing portal pressure in cirrhotic rats. As shown in Fig. 2, cirrhotic rats exhibited greater increases in perfusion pressure than control rats in response to incremental increases in flow as evidenced by a significantly greater perfusion pressure at each flow rate ($P < 0.01$ in comparison with control rats; control, $n = 10$; cirrhosis, $n = 7$) (Fig. 2). NCX-1000 administration almost completely reverted portal hypertension induced by CCl_4 as demonstrated by the fact that incremental increases in flow failed to increase portal perfusion pressure ($P < 0.001$ in comparison with CCl_4 alone; NCX-1000, $n = 8$). In contrast, incremental increases in flow perfusion of portal vein of UDCA-treated rats increased portal perfusion pressure to the same level documented in rats treated with CCl_4 alone ($P > 0.05$ in comparison with CCl_4 alone; UDCA, $n = 7$). Thus, NCX-1000 significantly reduces intrahepatic resistance in cirrhotic rats as measured by assessing portal perfusion pressure.

To gain insight on the mechanism through which NCX-1000 reduces portal pressure in cirrhotic rats, we measured eNOS activity. Indeed, as shown in Fig. 3 *A–C*, although eNOS mRNA expression was unchanged, eNOS activity was significantly reduced in cirrhotic rats in comparison with control rats. Treating rats with NCX-1000 or UDCA had no effect on eNOS protein expression or activity (Fig. 3*C*). However, liver nitrite/nitrate concentrations were significantly higher in rats treated with NCX-1000 in comparison with animals treated with CCl_4 alone or CCl_4 plus UDCA (Fig. 3*C*). In contrast NCX-1000 administration markedly decreased nitrite/nitrate contents in the ascitic fluid (Fig. 3*C*). Because ascitic nitrite/nitrate concentrations reflect peripheral NO production, we then assessed eNOS and iNOS expression in the aorta. In contrast to the liver, aortic iNOS was significantly induced by CCl_4 administration, an effect that was completely reversed by cotreating the animals with NCX-1000, but not with UDCA (Fig. 3*D*). No change were measured on eNOS expression (data not shown).

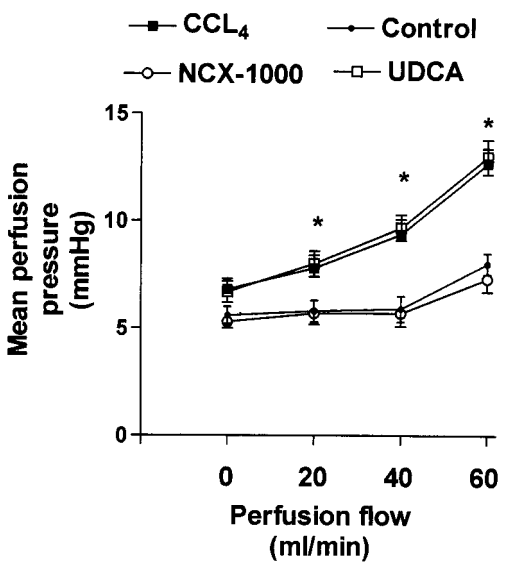

Fig. 2. Flow-induced increases in perfusion pressure in control and cirrhotic rats. The increase in perfusion pressure was greater in cirrhotic animals as demonstrated by a significantly greater perfusion pressure at each flow rate. *, $P < 0.01$ versus control and NCX-1000-treated rats.

NCX-1000 Modulates HSC Contraction. Having found that NCX-1000 had no effect on eNOS activity but reduced portal hypertension and increased liver NO content, we then assessed whether NCX-1000 directly modulated HSC contraction. As shown in Fig. 4*A*, NCX-1000 reverted HSC contraction induced by FCS, indicating that HSCs are able to directly metabolize NCX-1000. Indeed, as shown in Fig. 4 *B* and *C*, incubating HSC with NCX-1000, 0.1 mM, resulted in time-dependent increase in NO-related intracellular fluorescence in HSCs loaded with 4,5-diaminofluorescein diacetate, an NO-reactive fluorochrome, as well as release of nitrite/nitrate in cells supernatants. Similarly to NCX-1000, the NO donor SNAP inhibited FCS-induced contraction of HSCs and caused a time-dependent formation of NO (Fig. 4 *A–C*). NCX-1000 also inhibited MCP-1 release from HSCs stimulated with TNF-α and IFN-γ (Fig. 3*D*). In contrast to NCX-1000, UDCA had no effect on HSCs contraction, NO generation, and cytokine-induced MCP-1 release (Fig. 4).

Discussion

Clinical trials have demonstrated that the combination of β-blockers and nitro-vasodilators is effective in reducing mortality caused by gastrointestinal bleeding in patients with chronic liver diseases. The rationale behind the use of these two drugs is that by acting at separate points in the chain of pathophysiologic events that lead to portal hypertension their combination increases the portal pressure-lowering effect of either drug used separately (17). A practical limitation of this approach, however, derives from the fact that conventional NO donors, nitroglycerin and isosorbide-5-mononitrate, release NO in the blood stream, causing systemic hypotension and progression of the vasodilatory syndrome, aggravating renal dysfunction and sodium retention in cirrhotic patients with ascites (19, 20).

The increased intrahepatic vascular resistance in cirrhotic patients is due not only to fixed anatomical changes inherent to cirrhosis but also to active contraction of vascular smooth muscle cells and, possibly, activated HSCs (3–6). This active contraction is caused mainly by an impaired production of NO that acts in

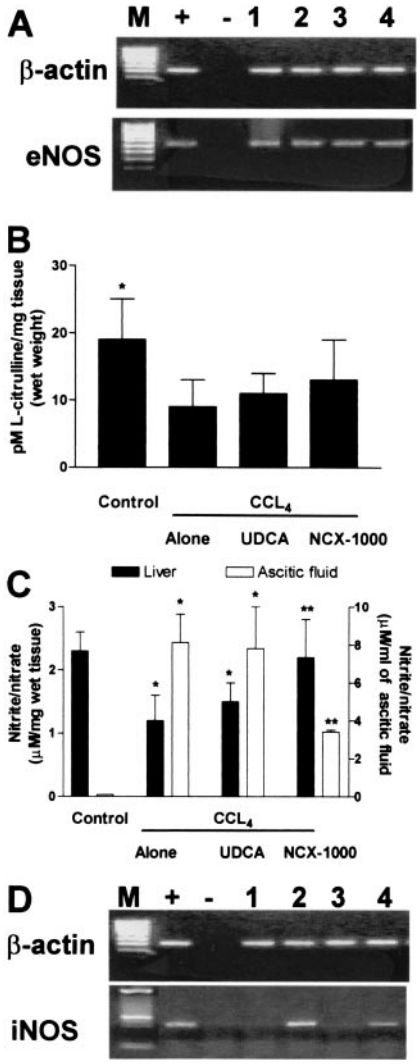

Fig. 3. (*A*) Reverse transcription–PCR analysis of liver eNOS expression. M, molecular markers; +, positive control; −, negative control; lane 1, control liver; lane 2, CCL4-treated rats; lane 3, UDCA-treated rats, and lane 4, NCX-1000-treated rats. (*B*) eNOS activity (see *Materials and Methods*). Data are mean ± SEM of at least four determinations. *, $P < 0.01$ versus all other groups. (*C*) Nitrite/nitrate contents in liver extract and ascitic fluid. Data are mean ± SEM of 4–8 determinations. *, $P < 0.01$ versus all other groups. (*D*) Reverse transcription–PCR analysis of iNOS expression in homogenates obtained from thoracic aortae. The lane markers are the same as in *A*.

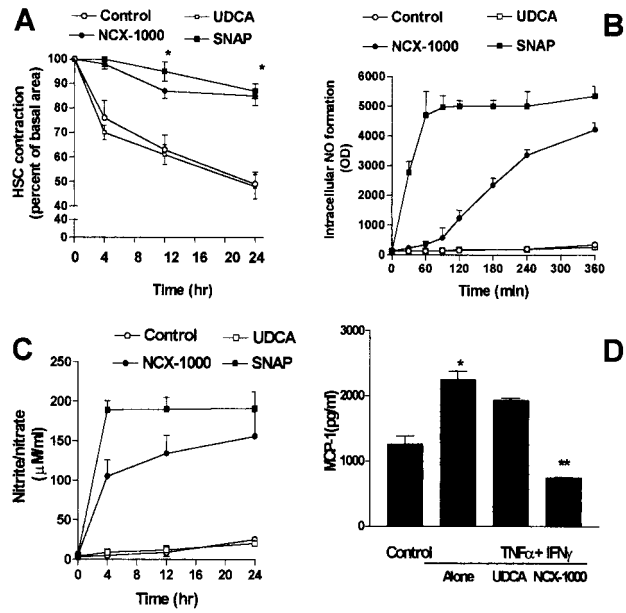

Fig. 4. NCX-1000 inhibits HSC contraction. (*A*) HSC contraction induced by FCS is inhibited by NCX-1000 and SNAP. Cells were incubated with 100 μM of NCX-1000, UDCA, or SNAP, and contraction was induced by FCS (see *Materials and Methods*). $P < 0.01$ versus control (FCS-treated cells) or UDCA-treated cells. (*B* and *C*) Incubating HSCs with NCX-1000 and SNAP but not UDCA (100 μM) increases nitrite/nitrate in cell supernatants (*B*) and intracellular NO-related fluorescence (*C*). (*D*) NCX-1000, but not UDCA (100 μM), inhibits MCP-1 release from activated HSCs. *, $P < 0.01$ versus control; **, $P < 0.01$ versus cell incubated with TNF-α plus IFN-γ.

the liver as an endogenous vasodilator (2). In experimental models of liver cirrhosis, as well as in cirrhotic patients, simultaneously with the excessive synthesis of NO in the systemic vasculature there is an impaired enzymatic function of liver eNOS despite the fixed expression of eNOS mRNA and protein (11, 13). In endothelial cells eNOS catalytic activity is modulated by dynamic regulatory processes including posttranslational protein modifications, interactions with regulatory proteins, and availability of essential cofactors (13). It is well established that the binding of eNOS with the ubiquitous calcium-regulatory protein calmodulin promotes NO production, whereas caveolin-1, the coat protein of nonclathrin-coated transport vesicles and putative signaling molecule, decreases the catalytic activity of the enzyme (31). Liver expression of calveolin-1 is increased in cirrhotic rats, suggesting that an abnormal protein–protein interaction is responsible for the defective eNOS function (13). Moreover, because the cirrhotic liver is unable to respond to incremental shear stress with an increased generation of NO, a defect of mechanotransduction pathways responsible for flow-mediated activation of eNOS also has been postulated (12, 13, 21). Whatever mechanism is responsible for presumed defect in posttranslational handling of eNOS, transfecting cirrhotic rats with a recombinant adenovirus carrying the neuronal NOS gene, which undergoes lesser posttranslational modifications than eNOS, leads to liver neuronal NOS overexpression and intrahepatic resistance reduction (21).

NCX-1000 is a NO-releasing derivative of UDCA generated by adding a nitroxybutyl moiety to UDCA (25). We have previously demonstrated that this compound, but not UDCA, protects against liver damage induced by Con A in mice by directly modulating liver resident immune system (25). Indeed in comparison with currently available NO donors, NCX-1000 has several potential advantages: (*i*) it is stable and releases NO very slowly in the blood stream, a property that should minimize its effect on arterial pressure, a major drawback of currently available nitrates; and (*ii*) it is almost exclusively metabolized in the liver, a property that should allow delivery of NO directly to hepatic cells.

In the present study we have provided evidence that NCX-1000 protects against development of portal hypertension in an animal model of liver cirrhosis by reducing intrahepatic resistance. Several of our results indeed support the concept that NCX-1000 modulates both anatomical and dynamic component of portal hypertension. First, UDCA and NCX-1000 exert similar antifibrotic effects, but only NCX-1000 protects against portal hypertension and ascite development induced by CCl_4, thereby demonstrating that NCX-1000 reverses the dynamic component of portal hypertension, i.e., HSC contraction. Second, similarly to UDCA, NCX-1000 has no effect on liver eNOS expression and activity (Fig. 2), demonstrating that its effect is unrelated to change in endogenous NO production. Third,

NCX-1000 administration significantly increases liver content of nitrite/nitrate, demonstrating that liver cells are able to use NCX-1000 as a source of NO despite a defect in posttranslational handling of eNOS. Fourth, as a further index of its ability to protect against development of portal hypertension, NCX-1000 reduces the nitrite/nitrate content in the ascitic fluid and prevents iNOS expression induction in the thoracic aorta, suggesting that similar to other compounds generated with the same technology (i.e., NO-NSAIDs), the NO-UDCA derivative modulates iNOS expression (32). Fifth, NCX-1000 has no effect on mean arterial pressure, both in control and cirrhotic rats, indicating that it releases a low amount of NO in the blood stream (33). Sixth, NCX-1000 directly modulates HSC functions as demonstrated by our *in vitro* experiments. Indeed NCX-1000 reduces HSC contraction as well as MCP-1 release induced by TNF-α and IFN-γ (30). The finding that SNAP also inhibits HSC contraction (31) and that both compounds increase nitrite/nitrate release in cell supernatants as well as intracellular fluorescence in cells loaded with the NO-reactive fluorochrome 4,5-diaminofluorescein diacetate, whereas UDCA itself has no effect, strongly supports the view that NCX-1000 inhibits HSC functions through an NO-dependent mechanism (25).

The results of the present study are important for another reason, which is the demonstration that HSCs are able to directly metabolize NCX-1000 to generate NO. This result is relevant because hematogenous compounds must traverse the sinusoidal endothelium as well as the sinusoidal space of Disse (wherein reside stellate cells) before coming into contact with hepatocytes, making it possible for NCX-1000 to directly reach HSCs from the perisinusoidal space (i.e., independently of hepatocyte handling). If this is the case it cannot be excluded that hepatic sinusoidal cells can use NCX-1000 as a source of NO.

In summary, the results presented in this study are consistent with previous data indicating that nitrogenous compounds are capable of affecting portal vascular resistance. Moreover, it establishes that whatever mechanism is responsible for presumed eNOS dysfunction it is possible to restore normal intrahepatic resistance by a compound that selectively releases NO in the liver. Finally, our data demonstrated that UDCA can be used as a carrier to transport other biologically active molecules to the liver with minimal systemic effects.

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1. Graham, D. Y. & Smith, J. L. (1981) *Gastroenterology* **80,** 800–809.
2. Shah, V., Garcia-Cardena, G., Sessa, W. C. & Groszmann, R. J. (1998) *Hepatology* **27,** 279–288.
3. Friedman, S. L., Roll, F. J., Boyles, J. & Bissell, D. M. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 8681–8685.
4. Friedman, S. L. (1993) *N. Engl. J. Med.* **328,** 1828–1835.
5. Rockey, D. C., Housset, C. N. & Friedman, S. L. (1993) *J. Clin. Invest.* **9,** 1795–1804.
6. Zhang, J. X., Pegoli, W. J. & Clemens, M. G. (1994) *Am. J. Physiol.* **266,** G624–G632.
7. Pinzani, M., Failli, P., Ruocco, C., Casini, A., Milani, S., Baldi, E., Giotti, A. & Gentilini, P. (1992) *J. Clin. Invest.* **90,** 642–646.
8. Rockey, D. C. & Chung, J. J. (1995) *J. Clin. Invest.* **95,** 1199–1206.
9. Bredt, D. S. (1999) *Free Radical Res.* **31,** 577–596.
10. Ignarro, L. J. (1990) *Annu. Rev. Pharmacol. Toxicol.* **30,** 535–560.
11. Rockey, D. C. & Chung, J. J. (1998) *Gastroenterology* **114,** 344–351.
12. Gupta, T. K., Toruner, M., Chung, M. K. & Groszmann, R. J. (1998) *Hepatology* **28,** 926–931.
13. Shah, V., Toruner, M., Haddad, F., Cadelina, G., Papapetropoulos, A., Choo, K., Sessa, W. C. & Grozman, R. J. (1999) *Gastroenterology* **117,** 1222–1228.
14. Pinzani, M., Milani, S., De Franco, R., Grappone, C., Caligiuri, A., Gentilini, A., Tosti-Guerra, C., Maggi, M., Failli, P., Ruocco, C. & Gentilini P. (1996) *Gastroenterology* **110,** 534–548.
15. Housset, C., Rockey, D. C. & Bissell, D. M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 9266–9270.
16. Rockey, D. C. & Weisiger, R. (1996) *Hepatology* **24,** 233–240.
17. Lebrec, D. (1998) *J. Hepatol* **28,** 896–907.
18. Jones, A. L. & Hayes, P. C. (1994) *Am. J. Gastroenterol.* **89,** 7–14.
19. Garcia-Pagan, J. C., Feu, F., Bosh, J. & Rodes, J. (1991) *Ann. Intern. Med.* **114,** 869–873.
20. Garcia-Pagan, J. C., Feu, F., Navasa, M., Bru, C., Ruiz de Arbol, L., Bosch, J. & Rodes, J. (1990) *J. Hepatol.* **11,** 189–195.
21. Yu, Q., Shao, R., Qian, H. S., George, S. E. & Rockey, D. C. (2000) *J. Clin. Invest.* **105,** 741–748.
22. Wallace, J. L., Reuter, B., Cicala, C., McKnight, W., Grisham, M. & Cirino, G. (1994) *Eur. J. Pharmacol.* **257,** 249–255.
23. Elliott, S. N., McKnight, W., Cirino, G. & Wallace, J. L. (1995) *Gastroenterology* **109,** 524–530.
24. Fiorucci, S., Santucci, L., Antonelli, E., Distrutti, E., Del Sero, G., Morelli, O., Romani, L., Federici,B., Del Soldato, P. & Morelli, A. (2000) *Gastroenterology* **118,** 404–421.
25. Fiorucci, S., Mencarelli, A., Palazzetti, B., Morelli, A. & Ignarro, L. J. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 2652–2657 (First Published February 13, 2001; 10.1073/pnas.041603898)
26. Santucci, L., Fiorucci, S., Chiorean, M., Brunori, P. M., Di Matteo, F. M., Sidoni, A., Migliorati, G. & Morelli, A. (1996) *Gastroenterology* **111,** 736–744.
27. Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R. & Snyder, S. H. (1991) *Nature (London)* **351,** 714–718.
28. Casini, A., Pinzani, M., Milani, S., Grappone, C., Galli, G., Jezequel, A. M., Schuppan, D., Rotella, C. M. & Surrenti, C. (1993) *Gastroenterology* **105,** 245–253.
29. Rockey, D. C., Housset, C. N. & Friedman, S. L. (1993) *J. Clin. Invest.* **92,** 1795–1890.
30. Marra, F., Valente, A. J., Pinzani, M. & Abboud, H. E. (1993) *J. Clin. Invest.* **92,** 1674–1680.
31. Failli, P., De Franco, R. M., Caligiuri, A., Gentilini, A., Romanelli, R. G., Marra. F., Batignani, G., Guerra, C. T., Laffi, G., Gentilini, P. & Pinzani, M. (2000) *Gastroenterology* **119,** 479–492.
32. Cirino, G., Wheeler-Jones, C. P., Wallace, J. L., Del Soldato, P. & Baydoun, A. R. (1996) *Br. J. Pharmacol.* **117,** 1421–1426.
33. Wallace, J. L., McKnight, W., Del Soldato, P., Baydoun, A. R. & Cirino, G. (1995) *J. Clin. Inv*. **96,** 2711–2718.