An NO derivative of ursodeoxycholic acid protects against Fas-mediated liver injury by inhibiting caspase activity

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Caspases are key mediators in liver inflammation and apoptosis. In the present study we provide evidence that a nitric oxide (NO) derivative of ursodeoxycholic acid (UDCA), NCX-1000 ([2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester]), protects against liver damage in murine models of autoimmune hepatitis induced by i.v. injection of Con A or a Fas agonistic antibody, Jo2. Con A administration causes $CD4^+$ T lymphocytes to accumulate in the liver and up-regulates FasL expression, resulting in FasL-mediated cytotoxicity. Cotreating mice with NCX-1000, but not with UDCA, protected against liver damage induced by Con A and Jo2, inhibited IL-1β, IL-18, and IFN-γ release and caspase 3, 8, and 9 activation. Studies on HepG2 cells demonstrated that NCX-1000, but not UDCA, directly prevented multiple caspase activation induced by Jo2. Incubating HepG2 cells with NCX-1000 resulted in intracellular NO formation and a DTT-reversible inhibition of proapoptotic caspases, suggesting that cysteine S-nitrosylation was the main mechanism responsible for caspase inhibition. Collectively, these data suggest that NCX-1000 protects against T helper 1-mediated liver injury by inhibiting both the proapoptotic and the proinflammatory branches of the caspase superfamily.

Ursodeoxychoic acid (UDCA), a hydrophilic dihydroxylated bile salt, is the only approved treatment for cholestatic and noncholestatic liver diseases (1, 2). However, recently published large multicenter studies demonstrated that long-term treatment with UDCA does not prevent ongoing bile duct destruction nor causes histological, virological, or biochemical improvement in patients with primary biliary cirrhosis or chronic autoimmune or virus C-induced hepatitis (2). Although the mechanism of action is not entirely understood, there is evidence that UDCA exerts its cytoprotective effects by displacing endogenous hydrophobic bile acids from hepatocyte and bile duct epithelial cell membranes (3). Recently, Rodrigues *et al.* (4) provided evidence that UDCA exerts antiapoptotic effects and protects against cell death caused by deoxycholic acid, ethanol, and Fas ligand (FasL) by inhibiting mitochondrial membrane permeability transition (5, 6).

Fas-regulated apoptosis plays a major role in the pathogenesis of immuno-mediated liver diseases including viral and autoimmune hepatitis, alcoholic liver disease, Wilson's disease, acute liver failure, and primary biliary cirrhosis, suggesting that strategies for down-regulating the Fas–FasL system might have therapeutic value in the treatment of these human diseases (7, 8). Fas receptor ligation by FasL or anti-Fas mAb, results in Fas trimerization and IL-1β converting enzyme (ICE)-like cysteine protease activation (9, 10). ICE is a member of the growing family of cysteine endoproteases that share sequence homology with *Ced-3*, a gene essential for apoptosis in nematodes (11). The increasing number of ICE-like proteases have been renamed caspases to indicate cysteine proteases that cleave their substrates after an aspartic acid residue (12). Caspase 1, the original ICE, has the greatest specificity for cleaving pro-IL-1β and pro-IL-18 and together with caspases 4 and 5, is primarily involved in cytokine activation as demonstrated by the fact that ICE knockout mice are unable to release IL-1β, IL-18, and IFN-γ in response to endotoxin but develop normally (13). In contrast, caspases 2, 3, 6, 7, 8, 9, 10, and 13 have been implicated in apoptosis (9, 10, 12). Caspases 2, 8, 9, and 10, which have long prodomains, are thought to be involved in the signaling cascades initiating apoptosis. Caspase 8 is the prominent signaling caspase involved in apoptosis caused by Fas, tumor necrosis factor (TNF) type I, and DR3 receptors (9, 10, 12). Upon ligation, Fas recruits the adaptor molecule FADD/Mort1, and the zymogen form of the apoptosis-initiating protease caspase 8, through homophilic interaction of death effector domains (DD) leading to the assembly of a death-inducing signaling complex at the cytoplasmic DD of Fas. The proximity of caspase 8 zymogens facilitates activation through self-processing, leading to cleavage of downstream effector caspases (caspases 9 and 3) that are largely responsible, directly and indirectly, for dismantling the apoptotic cells from within (9, 10).

Two models of Fas-mediated liver injury recently have been described (14, 15). In these models the Fas receptors at the hepatocyte cell surface are activated, directly or indirectly, by injecting mice with a Fas agonistic mAb, Jo2, or with the plant mitogen Con A. In the later model, assembly of activated $CD4^+$ T cells into the liver results in a time-dependent release of T helper 1-type cytokines, IL-2, IFN-γ, and TNF-α, resulting in liver cell death (15). However, because IFN-γ-, Fas-, and FasL-deficient mice are protected against damage induced by Con A, it appears that an IFN-γ-mediated activation of the Fas/FasL pathway is an absolute requirement for Con A-induced hepatitis (16–18).

Nitric oxide (NO) exerts immunomodulatory and antiapoptotic activities (19, 20). Previous studies have demonstrated that NO inhibits IL-1β, IL-18, and IFN-γ generation and protects hepatocytes from cytokine-induced apoptosis through a mechanism that involves caspases' S-nitrosylation/inactivation (18, 21, 22). Therefore, delivering appropriate amounts of NO to the liver might be a mechanism to protect against Fas-mediated diseases. NO-releasing nonsteroidal anti-inflammatory drugs (NO-NSAIDs) are a recently described class of NSAID derivatives generated by adding an NO-releasing moiety to parental NSAIDs (23–25). These novel chemical entities not only lack the gastrointestinal damaging effect of classical NSAIDs, but the

Abbreviations: ICE, IL-1β converting enzyme; AMC, 7-amino-4-coumarin; SNAP, *S*-nitroso-*N*-acetyl-D-L-penicillamine; UDCA, ursodeoxycholic acid; NCX-1000, 2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester; FasL, Fas ligand; TNF, tumor necrosis factor; AST, aminotransferase; DAF-DA, difluorescein diacetate.

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slow release of NO in a biological microenvironment confers novel activities not shared by cognate molecules. We recently have demonstrated that an NO-aspirin derivative inhibits T helper 1-type cytokine generation and protects against liver damage induced by Con A (18). Aspirin itself had no effect on Con A-induced hepatitis, suggesting that NO delivery to the liver was responsible for the protection exerted by NO–aspirin. Based on this background, we hypothesized that adding an NO-releasing moiety to UDCA would increase its effectiveness in preventing Fas-mediated liver injury. To test this hypothesis, we synthesized an nitroxy-methylbenzoate derivative of UDCA, hereafter referred to as NCX-1000 ([2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester]) or NO-UDCA (Fig. 1*A*) and tested its effects in animal models of Fas-mediated liver injury.

Materials and Methods

Animals and Treatment Schedules. Pathogen-free, male BALB/c (age 6–8 weeks, 25–30 g) were purchased from Harlan (Milan, Italy) and maintained for at least 1 week at 22°C with 55% relative humidity in a 12-h day/night rhythm with free access to food and water until the day of the experiment. Con A (0.3 mg/mouse) or Jo2 (30 μg/mouse) was injected i.v. NCX-1000 and UDCA were dissolved in ethanol and injected i.p. at doses of 5, 15, or 30 mg/kg simultaneously or 2, 4, and 8 h after Con A administration. At the indicated time points, animals were killed, blood samples, spleens, and the livers were collected, and liver histology, aminotransferase (AST), and plasma cytokine concentrations IL-1β, IL-18, IFN-γ, and TNF-α were assayed by specific ELISA kits (Endogen, Woburn, MA, and MBL International, San Francisco). Standard curves and cytokine concentrations were calculated by linear regression analysis using GRAPHPAD PRISM 3.0 (GraphPad, San Diego) (18).

Reverse Transcriptase–PCR. After the mice were killed, livers were removed and immediately snap-frozen in liquid nitrogen and stored at −80°C until used. Total RNA was isolated by using TRIzol reagent (Life Technologies, Milan, Italy) as described (18). PCR was performed by using specific primers [Sigma, Genosys (The Woodlands, TX), and Stratagene]. For mouse hypoxanthine-guanine phosphoribosyl transferase the sense primer was 5′-GTTGGATACAGGCCAGACTTTGTTG-3′ and antisense was: 5′-GAGGGTAGGCTGGCCTATAGGCT-3′; for mouse IL-1β the sense primer was 5′-GCAACTGTTC-CTGAACTCA-3′ and the antisense was 5′-CTCGGAGCCTG-TAGTGCAG-3′; for mouse IFN-γ the sense primer was 5′-CATTGAAAGCCTAGAAAGTCTG-3′ and the antisense was 5′-CTCATGAATTCCTTTTTCG-3′; for IL-18, the sense primer was 5′-ACTGTACAACCGGAGTAATACGG-3′ and the antisense was 5′-AGTGAACATTACAGATTTATCCC-3′. The cDNAs were amplified with a "hot start" reaction in 20 μl of reaction containing 5 μl of cDNA product, 2 μl of PCR buffer (200 mM Tris·HCl, pH 8.4/500 mM KCl), 200 μM dNTPs, 1 μM primers sense and antisense, 1.5 mM of $MgCl_2$, 1 unit of Platinum *Taq* polimerase (Life Technologies), and water in a Hybaid PCR Sprint thermocycler (Celbio, Milan, Italy). PCR products were then separated on 1.5% agarose gel and stained with 0.5 μg/ml ethidium bromide. The size of PCR products was assessed by comparison with 1 μg 100-bp DNA ladder (Life Technologies). The gel was photographed under UV transillumination and images were digitalized. Each assay was carried out in triplicate.

Caspase Activity. Liver lysates were prepared by homogenization in hypotonic buffer (25 mM Hepes, pH 7.5/5 mM $MgCl_2$/1 mM EGTA/1 mM PMSF/1 mg/ml leupeptin and aprotinin). Homogenates were centrifuged at 15,000 rpm for 15 min, and extracted proteins (50 μg) were tested in duplicate experiments by measuring the proteolytic cleavage of specific fluorogenic substrates: Ac-YVAD-7-amino-4-coumarin (AMC), Ac-DEVD-AMC, Ac-IEDT-AMC, and Ac-LEHD-AMC (Alexis, San Diego), respectively, for caspases 1, 3, 8, and 9 (18, 25). AMC released by enzyme reaction was measured spectrofluorometrically with an excitation wavelength of 360 nm and an emission wavelength of 460 nm (18, 25).

Cell Culture and Treatments. The human hepatoblastoma cell line, HepG2, was obtained from the American Type Cell Collection. Cells were maintained in 25-cm^2 polystyrene culture flasks with 5 ml grown at 37°C in 5% CO_2 in DMEM containing high glucose levels (GIBCO/BRL) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine and antibiotics, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 37°C humidified incubator under an atmosphere of 5% CO_2 in air.

Assessment of Apoptosis. Cells were treated with Jo2 alone or in combination with 1–100 μM UDCA or NCX-1000 for various time intervals. The percentage of apoptotic cells was determined by quantitation of DNA fragmentation, through analysis of propidium iodide-stained nuclei by flow cytometry as described by Nicoletti *et al.* (26). Apoptosis also was assessed by examining the morphology of 4′,6-diamidino-2-phenylindole-stained nuclei by fluorescence microscopy (25).

Detection of NO Formation. Intracellular NO formation in NCX-1000-treated HepG2 was carried out according to the method of Kojima *et al.* (27) using difluorescein diacetate (DAF-DA). Briefly, HepG2 cells (1×10^6/ml) were loaded by suspending them in PBS in the presence of 10 μM DAF-DA. Cells were washed twice in iced buffer solution, samples were added to a quartz cuvette while stirring continuously, and the temperature was thermostatically maintained at 37°C by using a Hitachi 2000 (Hitachi) fluorescence spectrophotometer. Samples were pre-incubated with 1 mM L-N^6-(1-iminoethyl)-lysine for 30 min to suppress endogenous NO generation and then incubated with 100 μM *S*-nitroso-*N*-acetyl-D-L-penicillamine (SNAP), NCX-1000, or UDCA, excited at 395 nm, and the intensity of fluorescence emitted at 515 nm was recorded. NO generation was expressed in arbitrary units of absorbance. NO generation also was assessed by measuring nitrite/nitrate concentrations in cell supernatants (Cayman Chemicals, Ann Arbor, MI).

Assessment of Caspase S-Nitrosation. Lysates obtained from HepG2 cells incubated with Jo2 and/or 10 μM NCX-1000 and UDCA were exposed to DTT (20 mM) for 1 h on ice. The DTT and excess NO were removed by passing the sample through a Sephadex G-25 column pre-equilibrated with the lysis buffer, and caspase activity was measured (25). Formation of nitrosylated thiol was investigated by exposing cell lysates to 5 mM $HgCl_2$ for 10 min on ice and NO released was measured by using DAF-DA as described (18, 21, 28).

Data Analysis. All values in the figures and text are expressed as mean ± SE of n observations. Data sets were compared with a ANOVA and Student's t test for unpaired data when appropriate (29).

Results

NCX-1000 Protects Against Acute Liver Injury Induced by Con A. Injecting mice with 30 mg/kg of Con A caused a time-dependent increase in AST plasma levels. Consistent with previous reports, elevated plasma levels of AST were observed 4 h after Con A administration although the highest values were reached at 8–24 h (Fig. 1*B*). Cotreating animals with NCX-1000 resulted in a dose- and time-dependent protection against damage induced by Con A. At the dose of 15 mg/kg, NCX-1000 completely protected against damage induced by Con A (Fig. 1*C*). In contrast

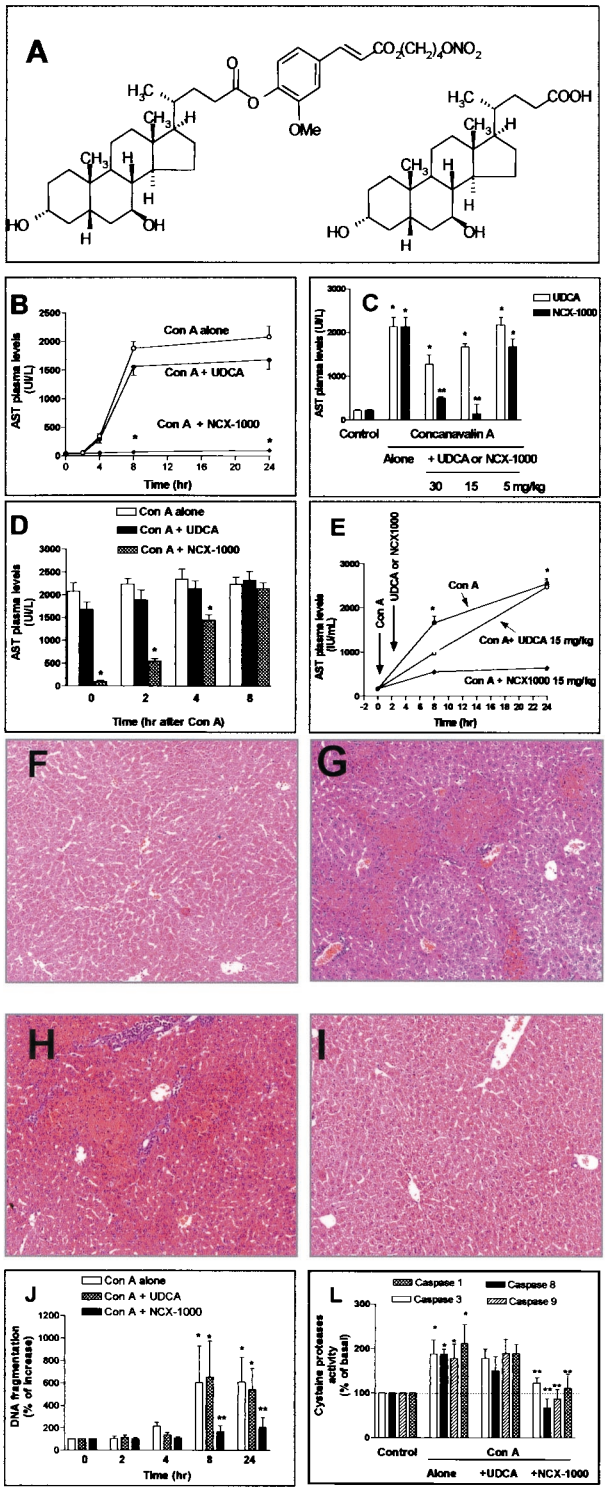

Fig. 1. (*A*) Structure of NCX-1000 (*Left*) and UDCA (*Right*). (*B*) Time course of AST plasma levels in mice treated with Con A and NCX-1000 or UDCA. *, $P <$ 0.01 versus Con A alone. (*C*) NCX-1000 causes a dose-dependent reduction of AST release in Con A-induced hepatitis. Data are mean ± SE of five mice for each data point. *, $P < 0.001$ versus control. **, $P < 0.01$ versus Con A alone. (*D*) Effect of treating mice with 15 mg/kg NCX-1000 or UDCA at various time intervals after Con A. *, $P < 0.01$ versus Con A alone. (*E*) Time course of AST plasma levels in mice injected with NCX-1000 or UDCA 2 h after Con A. *, $P <$ 0.01 versus con A alone. (*F–I*) Histological examination of liver sections from control and Con A-treated mice. Paraffin sections were stained with hematoxylin/eosin. (Original magnifications: ×200.) (*F*) Control mouse. (*G*) Liver appearance 24 h after Con A administration. (*H*) Liver section from a mouse treated with 15 mg/kg UDCA, 2 h after Con A. (*I*) Liver section from a mouse treated with 15 mg/kg NCX-1000, 2 h after Con A. (*J*) Time course of liver DNA fragmentation. *, $P < 0.01$ versus basal values. **, $P < 0.01$ versus Con A alone. (*L*) Liver caspase activity 24 h after Con A injection. *, $P < 0.01$ versus control; **, $P <$0.01 versus Con A alone.

to NCX-1000, treating animals with UDCA failed to protect against liver injury (Fig. 1 *B* and *C*). Furthermore, NCX-1000, at the dose of 15 mg/kg, was effective in preventing liver damage even when injected 2 h after Con A ($P < 0.01$ versus Con A alone) and caused an approximately 40% reduction of AST levels when injected 4 h after the plant mitogen (Fig. 1 *D* and *E*). Histopathologic analysis demonstrated extensive lesions, characterized by massive hepatocyte necrosis with cytoplasmic swelling of most surviving hepatocytes and frequent nuclear chromatin condensation, indicative of apoptosis in mice treated with Con A (Fig. 1*G*). A moderate infiltration of mononuclear and polymorphonuclear cells in the portal area and around the central vein was also observed. Injecting mice with 15 mg/kg NCX-1000 2 h after Con A completely prevented these changes (Fig. 1 *H* and *I*).

Using an ELISA that specifically identifies histone-associated DNA fragments, an increased rate of DNA fragmentation was detected as early as 4 h after Con A administration. The increase after 4 h was greater than 50% of background level. After the time course of AST plasma levels, maximal DNA fragmentation occurred at 8 h, exceeding the pretreatment levels by more than 6-fold and remained significantly higher than basal values until 24 h ($P < 0.001$ vs. basal). NCX-1000, but not UDCA, at the dose of 15 mg/kg completely protected against DNA fragmentation induced by Con A (Fig. 1*J*). As shown in Fig. 1*L*, Con A administration significantly increased the liver activity of caspase 1, 3, 8, and 9. Cotreating mice with NCX-1000 prevented these changes.

NCX-1000 Down-Regulates Systemic and Liver Cytokine Response. Confirming previous observations, injecting mice with Con A caused a time-dependent increase in IL-1β, IL-18, IFN-γ, and TNF-α plasma concentrations (Fig. 2 *A–D*). Although treating mice with 15 mg/kg UDCA 2 h after Con A had no effect on this cytokine pattern, NCX-1000 caused a ≈40–70% reduction of IL-1β, IL-18, and IFN-γ plasma levels without affecting liver (Fig. 2) and spleen (data not shown) cytokine mRNA expression.

Although Fas is constitutively expressed in the liver, and its expression was unmodified by Con A, the plant lectin strongly up-regulated FasL mRNA expression. Injecting mice with 15 mg/kg NCX-1000 or UDCA had no effect on liver expression of Fas/FasL mRNA (Fig. 2*E*).

NCX-1000 Inhibits Liver Caspases in Jo2-Treated Mice. Liver Fas crosslinking in Jo2-injected mice, 50 μg/mouse, resulted in an extensive liver damage, with a marked increase in AST plasma levels as early as 4 h after the mAb injection and a severe hepatitis resulting in ≈50% mortality within the first 8 h. In this experimental setting, pretreating mice with 15 mg/kg NCX-1000, 30 min before Jo2, resulted in ≈50–60% reduction in AST plasma levels and caused a 50% reduction in Jo2-induced lethality. In contrast, UDCA administration did not protect against liver injury induced by Jo2 nor did it prevent lethality. Jo2 injection caused a ≈6- to 8-fold increase in caspase 8-like activities (Fig. 3). NCX-1000 (15 mg/kg), but not UDCA, prevented caspase activation. Thus, NCX-1000 exerts its protective effect acting downstream to Fas/FasL crosslinking and inhibits caspase activation in Jo2-treated mice.

NCX-1000 Directly Inhibits Caspase Activity in HepG2 Cells. Incubating HepG2 cells with Jo2 resulted in a time-dependent increase in

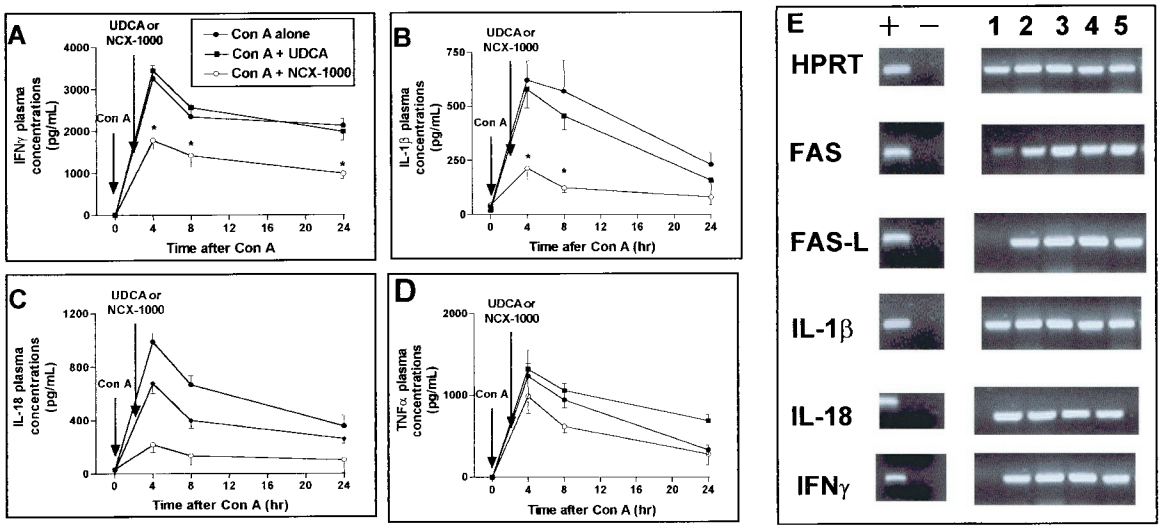

Fig. 2. (*A–D*) NCX-1000 (15 mg/kg) reduces IL-1β, IL-18, and IFN-γ release in Con A-treated mice. Data points represent the mean ± SE for five mice/group. *, $P < 0.01$ versus Con A alone. (*E*) Reverse transcription–PCR analysis of hypoxanthine-guanine phosphoribosyl transferase (HPRT), Fas, FasL, IL-1β, IL-18, and IFN-γ mRNA transcripts on liver lysates. Lane 1, control; lane 2, 8 h after Con A injection; lane 3, 24 h after Con A injection; lane 4: Con A plus UDCA, 15 mg/kg; lane 5, Con A plus NCX-1000, 15 mg/kg.

the number of apoptotic cells, as assessed by measuring propidium iodide incorporation, DNA fragmentation, nuclei morphology by 4′,6-diamidino-2-phenylindole staining and caspase 8, 9, and 3 activity (Fig. 4 *A–D*). Cotreating the cells with NCX-1000 concentration dependently prevented these changes. Although UDCA was also effective in reducing apoptosis in this experimental setting, it was ≈100-fold less potent that NCX-1000 (Fig. 4). Incubating HepG2 cells lysates with 20 mM DTT prevented caspase inhibition caused by NCX-1000, resulting in ≈70% recovery of protease activity (Fig. 4*C*). Moreover, exposure of lysates obtained from cells treated with NCX-1000 to $HgCl_2$ resulted in a detectable release of NO as measured by assessing DAF fluorescence in the medium (Fig. 4*E*). Because experiments with DTT indicate that exposure to NCX-1000 causes caspase S-nitrosation/inactivation we assessed whether incubating HepG2 cells with NCX-1000 results in intracellular NO formation. Indeed, incubating untreated HepG2 cells with 100 μM NCX-1000 or SNAP resulted in a time-dependent increase in intracellular NO-related fluorescence in DAF-DA-loaded hepatocytes as well as nitrite/nitrate concentrations in cell supernatants (Fig. 4 *F* and *G*). Thus, NCX-1000 penetrates HepG2 cell membranes and is metabolized to release intracellular NO.

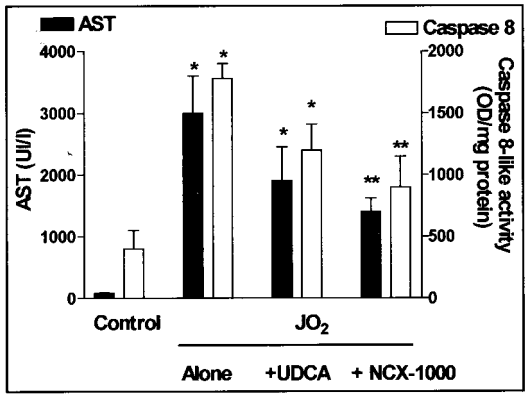

Fig. 3. NCX-1000 protects against liver damage induced by Fas agonistic mAb, Jo2. NCX-1000 reduces AST plasma levels and caspase 8-like activity in mice treated with Jo2. Data are mean ± SE of five experiments. *, $P < 0.01$ versus control. **, $P < 0.01$ versus Jo2 alone.

Discussion

Con A-induced hepatitis is a murine model of Fas-dependent autoimmune disease in which acute liver injury develops in response to massive liver infiltration by $CD4^+$ T cells (15). In the present study we showed that at the dose of 15 mg/kg, NCX-1000 completely protected against necro-apoptotic damage induced by the plant lectin by inhibiting liver margination of $CD4^+$ T lymphocytes as well as IL-1β, IL-18, and IFN-γ release. Previous studies have demonstrated that hepatitis induced by Con A requires the cooperation of numerous effector mechanisms including IFN-γ, the Fas/FasL and perforin/granzyme system, IL-4, and/or TNF-α. However, because IFN-γ-deficient mice are protected against liver damage induced by Con A, and IFN-γ drives hepatocytes to apoptosis, enhances cytotoxic $CD8^+$ T lymphocyte differentiation, up-regulates FasL expression, and mediates CD4/CD8-dependent fulminant hepatitis in transgenic mice expressing the hepatitis B surface antigen, this cytokine bodes well as a key mediator in this model of liver injury (15, 17, 18, 30–32). The fact that NCX-1000 inhibits IFN-γ release may, therefore, contribute to the protective effect exerted by this compound in animals treated with Con A. The present results suggest that NCX-1000 inhibits IFN-γ release indirectly by down-regulating IL-18 generation. IFN-γ release from T lymphocytes and NK cells in response to mitogenic and antigenic stimulation is regulated by the availability of two IFN-γ-releasing cytokines: IL-12 and IL-18 (formerly indicated as IGIF, IFN-γ inducing factor) (33–36). Although *in vitro* studies have documented that IL-12 and IL-18 act sinergistically to release IFN-γ, we previously have demonstrated that IL-18 immunoneutralization prevents IFN-γ release and protects against Con A-induced liver injury (18). Because NCX-1000 completely abolished IL-18 release *in vivo* and *in vitro* (S.F. and A.M., unpublished observation) it seems likely that IGIF inhibition was responsible for IFN-γ suppression. Although NCX-1000 inhibits IL-18 release, it has no effect on IL-18 mRNA expression, suggesting a site of action downstream to IL-18 mRNA synthesis. IL-18 is synthesized as a precursor molecule (pro-IL-18) that lacks a signal

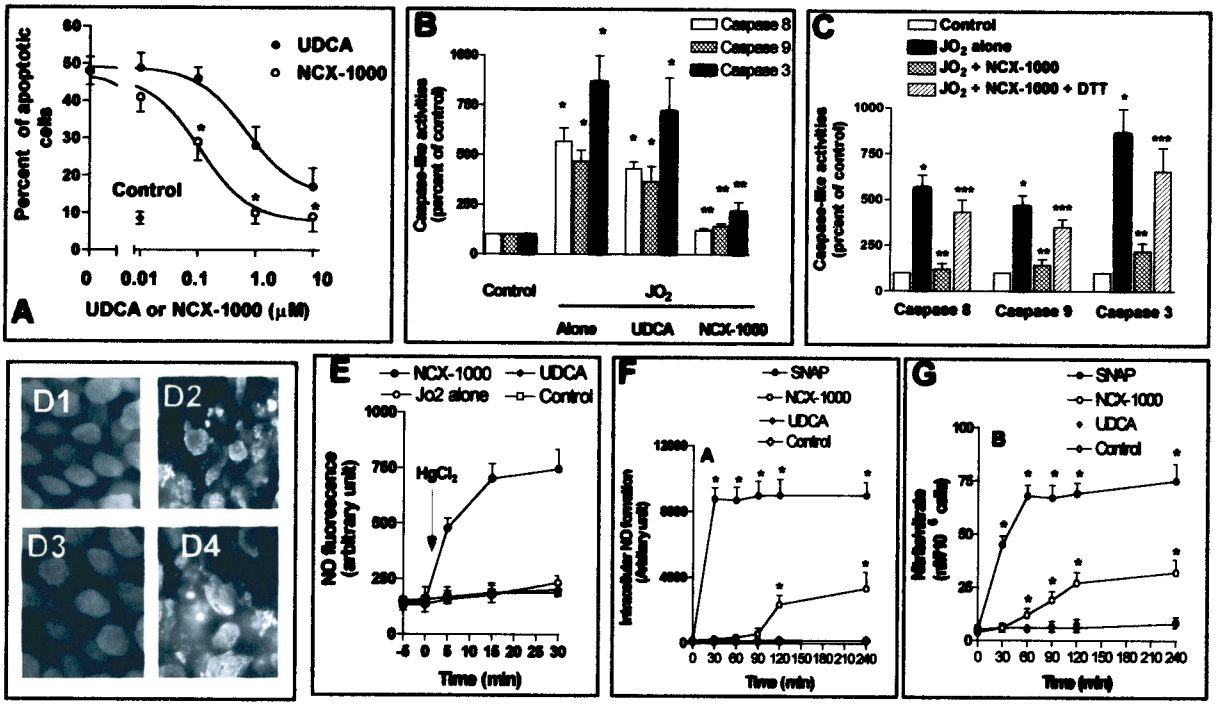

Fig. 4. NCX-1000 protects HepG2 cells from Jo2-induced apoptosis. (*A*) UDCA and NCX-1000 causes a concentration-dependent inhibition of Jo2-induced apoptosis. Data are mean ± SE of six experiments carried out in duplicate. *, $P < 0.01$ versus Jo2 alone. (*B*) NCX-1000 inhibits caspase 3, 8, and 9 activity in HepG2 cells treated with Jo2. Data are mean ± SE of six experiments carried out in duplicate. *, $P < 0.01$ versus control; **, $P < 0.01$ versus Jo2 alone. (*C*) Reversibility of caspase inhibition by DTT. Data are mean ± SE of six experiments carried out in duplicate. *, $P < 0.01$ versus control; **, $P < 0.01$ versus Jo2 alone; ***, $P < 0.01$ versus Jo2 plus NCX-1000. (*D*) 4′,6-Diamidino-2-phenylindole staining of HepG2 nuclei. D1, control cells; D2, cells treated with Jo2. Note extensive nuclear fragmentation. D3, cells treated with Jo2 + 10 μM NCX-1000; D4, cells treated with Jo2 + 10 μM UDCA. (*E*) Release of NO from cell lysates incubated with NCX-1000. Data are mean ± SE of six experiments carried out in duplicate. (*F* and *G*) Time course of intracellular NO formation and nitrite/nitrate release from HepG2 cells incubated in the absence of added agent (control) or 100 μM of SNAP, UDCA, or NCX-1000. Data are mean ± SE of six different experiments. *, $P < 0.01$ versus baseline.

peptide and requires ICE/caspase 1-like proteases for cleavage into a mature peptide (34). We and others have previously demonstrated that exogenous and endogenous NO inhibits ICE-dependent processing of pro-IL-18 (18, 37). In contrast to UDCA, NCX-1000 reduces liver ICE activity. This finding strongly supports the concept that NO released by NCX-1000 causes inhibition of cysteine proteases involved in pro-IL-18 processing. These results are in line with previous studies demonstrating that NO exerts immunomodulatory functions, leading to a specific impairment of T helper 1-like cytokine (IL-2, TNF-α, and IFN-γ) production (19).

Hepatocyte cell death induced by Con A is caused by necrosis and apoptosis. Earlier studies demonstrated that Con A injection leads to rapid up-regulation of liver FasL expression, resulting in Fas activation at the hepatocyte cell surface (15–18, 30–32). However, because reverse transcription–PCR analysis demonstrated that NCX-1000 had no effect on liver Fas/FasL expression, it seems likely that NCX-1000 protects against liver cell apoptosis by inhibiting intracellular steps downstream to Fas activation. Several lines of evidence suggest that NCX-1000 acts as a cysteine protease inhibitor: (*i*) Con A administration caused a time-dependent activation of ICE-like endoproteases, an effect that was completely prevented by cotreating mice with NCX-1000; (*ii*) NCX-1000 reduced acute liver damage and inhibited caspase 8, 9, and 3 activity in Jo2-treated mice, i.e., in a model characterized by direct Fas activation; and (*iii*) NCX-1000 inhibited caspase activation in HepG2 cells treated with Jo2.

Inhibition of caspase activity caused by NCX-1000 was likely because of the release of NO from the nitro moiety of the compound as demonstrated by the fact that UDCA itself had no effect on cytokine generation and caspase activation in mice treated with Con A or Jo2. Indeed, the results of our *in vitro* experiments suggest that cysteine S-nitrosation was the main mechanism involved in caspase inhibition (18, 21, 22, 37–39). Supporting this view we demonstrated that: (*i*) similar to the "conventional" NO-donor SNAP, NCX-1000 penetrates HepG2 cell membranes to release free NO and/or NO-derived compounds as assessed by measuring intracellular NO formation in cells loaded with the NO-specific fluorochrome, DAF-DA, and nitrite/nitrate concentrations in cell supernatants (18, 27). The kinetics of NO generation from NCX-1000, however, is different from that of SNAP, because the NO moiety dissociates slowly from UDCA, resulting in a lower, but sustained, release of NO. (*ii*) Inhibition of caspase 3, 8, and 9 activities caused by NCX-1000 was prevented by incubating HepG2 lysates with DTT, an agent that effectively removes the thiol-bound NO groups from proteins (18, 21, 22, 37–39). (*iii*) Exposure to $HgCl_2$, an agent that displaces NO from cysteine residues, resulted in NO release from lysates obtained from HepG2 cells incubated with NCX-1000 but not with UDCA (18, 21, 22, 37–39). (*iv*) Inhibition of caspase activity caused by $HgCl_2$ was prevented by DTT, indicating that NO removed by $HgCl_2$ was bound to a cysteine group (21). Caspases are a plausible target for S-nitrosylation because these endoproteases possess highly conserved and functionally essential cysteine residues within their catalytic site. Previous studies by Dimmeler *et al.* (22) carried out with purified caspase 1 and 3 subunits have demonstrated that the p20 subunit of caspase 1 and the p17 subunit of caspase 3 are selective targets for NO compounds, and that S-nitrosylation of these two subunits is associated with profound inhibition of enzyme ac-

tivity. Generally, it is recognized that the S-nitrosylation is extensively involved in caspase regulation (40). A recent report from Mannick *et al.* (41) indicates that in resting human cell lines, caspase 3 zymogens are S-nitrosylated and denitrosylated on Fas/FasL crosslinking, indicating that caspase activation requires both denitrosylation and zymogen cleavage.

Although our data indicate S-nitrosation is the main mechanism responsible for caspase inhibition, it cannot be excluded that NCX-1000 interferes with the Fas/FADD/caspase 8 pathway by down-regulating caspase 8 recruitment into the death-inducing signaling complex. Supporting this view we demonstrated that NCX-1000 was more effective in reducing liver damage induced by Con A than by Jo2. Because in the former model NCX-1000 also reduced circulating levels of IFN-γ, where IFN-γ increases Fas expression at the hepatocyte cell surface, it is likely that down-regulation of IFN-γ production interferes with death-inducing signaling complex assembly. The finding that exposure to DTT allowed only 70% recovery of caspase 8 activity supports this concept.

In summary, the present data indicate that the addition of an NO-releasing moiety to UDCA confers effective immunoregulatory and antiapoptotic activities to this compound, and that these effects are caused mainly by the S-nitrosation/inhibition of both the proapoptotic and proinflammatory branches of the caspase superfamily. Given the limited efficacy of currently used drugs in treating immunomediated liver injury, assessment of the effects of NCX-1000 in a clinical setting appears to be warranted.

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