

# Nitric Oxide Ameliorates Hydrophobic Bile Acid-induced Apoptosis in Isolated Rat Hepatocytes by Non-mitochondrial Pathways\*

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**Hydrophobic bile acids are toxic to isolated rat hepatocytes by mechanisms involving mitochondrial dysfunction and oxidative stress. In the current study we examined the role of nitric oxide (NO), a potential mediator of apoptosis, during bile acid-induced apoptosis. Freshly isolated rat hepatocytes and hepatic mitochondria generated NO and peroxynitrite (ONOO<sup>-</sup>) in a concentration- and time-dependent manner when exposed to the toxic bile salt glycochenodeoxycholate (GCDC) (25–500 μM), which was prevented by the nitric-oxide synthase (NOS) inhibitors N<sup>G</sup>-monomethyl-N-arginine monoacetate (L-NMMA) and 1400W. Relationships between hepatocyte NO production and apoptosis were examined by comparing the effects of NOS inhibitors with other inhibitors of GCDC-induced apoptosis. Inhibitors of caspases 8 and 9, the mitochondrial permeability transition blocker cyclosporin A, and the antioxidant idebenone reduced NO generation and apoptosis in GCDC-treated hepatocytes. In contrast, NOS inhibitors had no effect on GCDC-induced apoptosis despite marked reduction of NO and ONOO<sup>-</sup>. However, treatment with the NO donors S-nitroso-N-acetylpenicillamine and spermine NONOate [N-(2-aminoethyl)-N-(2-hydroxy-2-nitrohydrazino)-1,2-ethylenediamine] inhibited apoptosis and caspase 3 activity while significantly elevating NO levels above GCDC-stimulated levels. Neither NO donors nor NOS inhibitors affected GCDC-induced mitochondrial permeability transition or cytochrome c release from liver mitochondria or GCDC-induced mitochondrial depolarization from isolated hepatocytes, suggesting that NO inhibits bile acid-induced hepatocyte apoptosis by a non-mitochondrial-dependent pathway. In conclusion, whereas NO produced from GCDC-treated hepatocytes neither mediates nor protects against bile acid-induced apoptosis, higher levels of NO inhibit GCDC-induced hepatocyte apoptosis by caspase-dependent pathways.**

The accumulation and toxicity of hydrophobic bile acids within the liver play an important role in the pathogenesis of

cholestatic liver disorders and congenital defects in bile acid synthesis and transport (1). Although the mechanisms responsible for bile acid-induced hepatotoxicity have not been fully elucidated, exposure of freshly isolated or primary cultured hepatocytes to high concentrations (500 μM–1 mM) of hydrophobic bile acids leads to hepatocyte necrosis (2, 3), whereas exposure to lower concentrations (25–100 μM) induces the morphologic and biochemical features of apoptosis (4, 5). Although the induction and execution of bile acid-induced hepatocyte apoptosis may involve a variety of cell signaling pathways, it is clear that oxidative stress and mitochondrial perturbations are two critical steps in this apoptosis model. Several laboratories demonstrated that hydrophobic bile acids directly stimulate generation of reactive oxygen species (ROS)<sup>1</sup> from hepatocytes (2, 6) and liver mitochondria (2, 7) and that inhibition by antioxidants protects hepatocytes from cell necrosis and apoptosis. Recent attention has also focused on the role of the mitochondrial permeability transition (MPT) during apoptosis. Induction of the MPT, characterized by large amplitude swelling and loss of the electrochemical potential across the inner mitochondrial membrane (8, 9), leads to release of cytochrome c and apoptosis-inducing factor into cytosol, activating downstream caspases and cellular apoptosis (10). The role of the MPT in bile acid-induced hepatocyte necrosis and apoptosis has recently been established (11–15). Evidence from several laboratories indicates that stimulation of the MPT by bile acids is commensurate with increased generation of ROS (7, 11, 14, 15) and that oxidative modification of the MPT pore may mediate its opening (16).

Recently, nitric oxide (NO) has emerged as a regulatory molecule involved in control of a variety of biological processes. Enzymatically produced by constitutive and inducible forms of NO synthases (NOS), NO is found in a variety of cell types (for reviews, see Refs. 17 and 18). In addition to its role as a cell signaling agent, NO functions as an antioxidant by reacting with other free radical species, such as superoxide, in a diffusion-controlled reaction yielding the potent oxidizing and nitrating peroxynitrite (ONOO<sup>-</sup>). Therefore, NO can operationally function as either a prooxidant or antioxidant. Most toxicity studies in rat hepatocytes support a potent protective effect of NO, particularly against cell death by apoptotic pathways (19). Of particular relevance are recent reports demon-

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<sup>1</sup> The abbreviations used are: ROS, reactive oxygen species; MPT, mitochondrial permeability transition; NOS, nitric-oxide synthase; iNOS, inducible NOS; GCDC, glycochenodeoxycholate; BSA, bovine serum albumin; SNAP, S-nitroso-N-acetylpenicillamine; DAF-2/DA, 4,5-diaminofluorescein; NONOate, [N-(2-aminoethyl)-N-(2-hydroxy-2-nitrohydrazino)-1,2-ethylenediamine]; SNN, spermine NONOate; DCDHF-DA, dichlorodihydrofluorescein diacetate; L-NMMA, N<sup>G</sup>-monomethyl-N-arginine monoacetate; CsA, cyclosporin A; KRH, Krebs-Ringers HEPES; MOPS, 4-morpholinepropanesulfonic acid; ASK-1, apoptosis signal-regulating kinase.

strating that NO, administered exogenously or stimulated endogenously via NOS induction, inhibits tumor necrosis factor- $\alpha$  or Fas-dependent hepatocyte apoptosis (19–22). Several mechanisms have been proposed to explain the anti-apoptotic effects of NO including increased cGMP production, nitrosylation of caspases, inhibition of Bid cleavage and translocation to mitochondria, and inhibition of the MPT (for review, see Ref. 19). Finally, it has been reported that NO can either stimulate or inhibit the MPT in liver mitochondria, often depending upon experimental conditions and the source of mitochondria (23, 24).

With little evidence assessing the importance of NO as a primary mediator of bile acid-induced hepatocyte apoptosis, the current study was performed to elucidate if NO promoted or protected against this injury. In these studies, freshly isolated rat hepatocytes were used rather than cultured or hepatoma cell lines so that homeostasis of physiologic bile acid uptake (25) and endogenous antioxidant pathways (26) were maintained to better reflect the *in vivo* conditions observed during cholestatic liver diseases and so that physiologic apoptotic pathways were intact. The bile acid chosen for this study was the sodium salt of glycochenodeoxycholate (GCDC), a toxic, hydrophobic bile acid that accumulates in the human liver during cholestasis (27). The objectives of this study were to determine 1) if NO or its reaction product with superoxide, ONOO<sup>-</sup>, was generated from isolated rat hepatocytes or liver mitochondria exposed to concentrations of GCDC previously observed to promote hepatotoxicity and the MPT, 2) if modulation of NO status by employing either NOS inhibitors or NO donors prevented or promoted hepatocyte apoptosis induced by GCDC, and 3) the effect of NOS inhibitors and NO donors on mitochondrial pathways involved in GCDC-induced apoptosis.

#### EXPERIMENTAL PROCEDURES

**Materials**—All chemicals were obtained in reagent grade quality from suppliers. Bovine serum albumin-fraction V (BSA), *S*-nitroso-*N*-acetylpenicillamine (SNAP), spermine NONOate (SNN), and the caspase 8 substrate, Ac-IETD-pNA, were obtained from Calbiochem. DAF-2/DA, 2,7-dichlorodihydrofluorescein diacetate (DCDHF-DA), *N*<sup>G</sup>-monomethyl-*N*-arginine monoacetate (L-NMMA), 1400W, and cyclosporin A (CsA) were purchased from Alexis Biochemicals (San Diego, CA). Z-IETD-FMK (caspase 8 inhibitor) and Z-LEHD-FMK (caspase 9 inhibitor) were purchased from Enzyme Systems Products (Livermore, CA). Glycochenodeoxycholic acid (Na<sup>+</sup> salt), Griess reagent, and 2,7-dichlorofluorescein diacetate were obtained from Sigma and Eastman Kodak Co., respectively. Idebenone was a gift from Takeda Industries (Osaka, Japan), and the fluorescent probe JC-1 was purchased from Molecular Probes (Eugene, OR).

**Isolation of Rat Hepatocytes and Rat Liver Mitochondria**—Hepatocytes were isolated by a recirculating collagenase technique from 175–225-g male Sprague-Dawley rats (Sasco, Inc., Omaha, NE) maintained on a 12-h light-dark cycle and fed standard laboratory rat chow, as previously described (2). Initial hepatocyte viability measured by trypan blue exclusion was always >94%. Fresh hepatocytes were resuspended in a Krebs-Ringers HEPES buffer containing 0.2% BSA (KRH/BSA) to a concentration  $\approx 1 \times 10^6$ /ml. Rat liver mitochondria were isolated by differential centrifugation through a Percoll gradient as described in detail (15).

**Determination of NO and ONOO<sup>-</sup> in Isolated Hepatocytes and Liver Mitochondria**—Hepatocyte generation of NO or ONOO<sup>-</sup> was determined by using specific fluorescent probes prepared as stock solutions in dimethylformamide, as recently described by Kojima *et al.* (28, 29). Cells were preloaded at 37 °C for 30 min with either 10  $\mu$ M DAF-2/DA for NO detection or 8  $\mu$ M DCDHF-DA for detection of ONOO<sup>-</sup>. Upon entering the hepatocyte, intracellular esterases hydrolyze the diacetate moiety, trapping free DAF-2 or DCDHF within the cell (or mitochondria) that are covalently modified by NO or ONOO<sup>-</sup>, respectively. After loading, cells were washed twice and resuspended in KRH/BSA and preincubated for 30 min either with the antioxidant idebenone, the MPT inhibitor CsA, caspase inhibitors, NOS inhibitors, or appropriate solvent vehicle. Neither solvents nor added compounds alone affected any measurements. Cells were then exposed to 0–500  $\mu$ M GCDC for 4 h

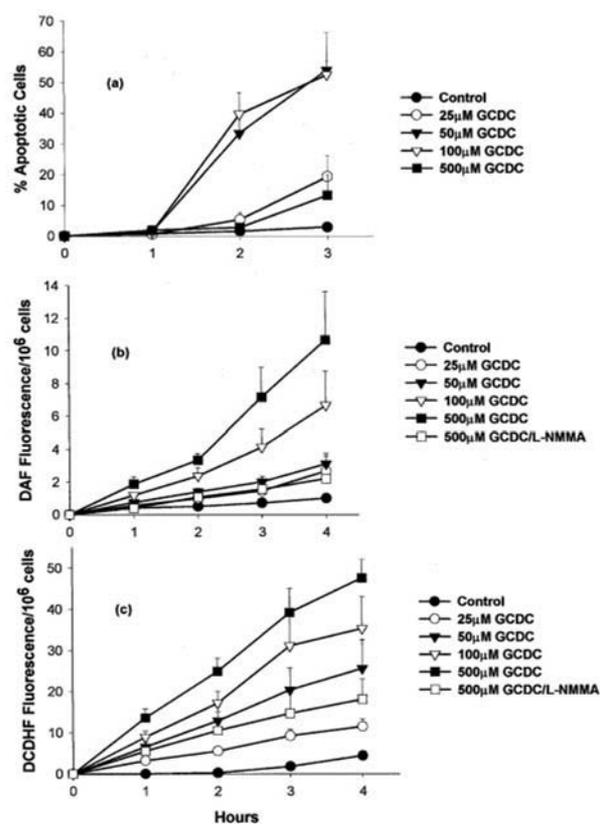


FIG. 1. GCDC stimulates apoptosis (a) and generation of NO (b) and ONOO<sup>-</sup> (c) from isolated rat hepatocytes. Hepatocytes ( $10^6$ /ml) were loaded with either 10  $\mu$ M DAF-2 or 8  $\mu$ M DCDHF (for NO and ONOO<sup>-</sup> determinations, respectively) in KRH/BSA buffer for 30 min at 37 °C before exposure to GCDC (0–500  $\mu$ M). Where indicated, the NOS inhibitor L-NMMA (1 mM) was preincubated for 30 min before the addition of 500  $\mu$ M GCDC. Values are expressed as means  $\pm$  S.E. of at least five experiments.

with hourly aliquots removed for fluorescence determination at 495 nm excitation and 515 nm emission for DAF-2 or 502 nm excitation and 523 nm emission for DCDHF. Generation of NO and ONOO<sup>-</sup> from isolated rat hepatocytes were expressed as relative fluorescence units/ $10^6$  cells. Additionally, in selected experiments hepatocyte NO production was also confirmed by measuring media nitrite levels using the Griess reagent prepared according to the manufacturer's instructions as follows. Hepatocytes ( $1.2 \times 10^6$  cells) were pelleted, and aliquots of supernatant were incubated with Griess reagent for 10 min at room temperature before nitrite quantitation at 540 nm. Results were expressed as  $\mu$ M nitrite based upon a standard curve of sodium nitrite prepared weekly in KRH/BSA.

Mitochondrial generation of NO and ONOO<sup>-</sup> was also determined using DAF-2/DA and DCDHF-DA as described above with the following modifications. Washed and Percoll gradient-purified mitochondria were resuspended in a buffer containing 5 mM HEPES, 50 mM KCl, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 125 mM sucrose, pH 7.4, treated with 1% Chelex 100, and loaded with either 10  $\mu$ M DAF-2/DA or 3  $\mu$ M DCDHF-DA at 28 °C for 30 min. After 2 washes in 5 mM MOPS, 100 mM KCl, 1 mM EGTA, pH 7.4, treated with 1% Chelex 100, mitochondria was centrifuged at  $10,000 \times g$  for 10 min. The final mitochondrial pellet was resuspended in the same buffer and preincubated with or without NOS inhibitors for 10 min before the addition of GCDC. After the addition of GCDC, aliquots of mitochondrial suspensions were removed for fluorescence determination of NO or ONOO<sup>-</sup> as described above.

To confirm the specificity of DAF-2 and DCDHF toward NO and ONOO<sup>-</sup>, respectively, studies were carried out with mitochondria loaded with dichlorofluorescein diacetate, a probe utilized to detect intracellular hydroperoxide generation (30). Previous studies from our laboratory showed that mitochondria exposed to GCDC generate hydroperoxides (31). If DAF-2 and DCDHF specifically bound NO/ONOO<sup>-</sup> and not hydroperoxides, we would expect no effect of NOS inhibitors upon hydroperoxide (dichlorofluorescein fluorescence) generation. For these studies, dichlorofluorescein fluorescence was determined at

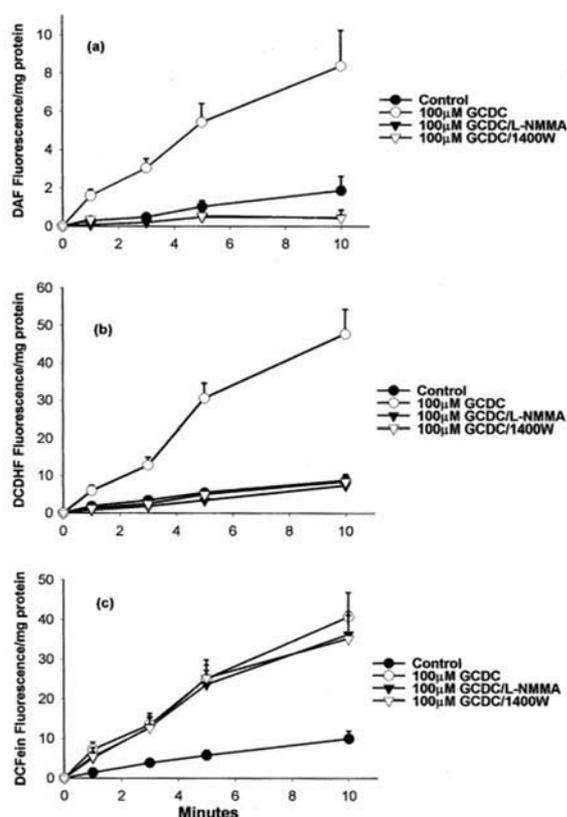


FIG. 2. NOS inhibitors prevent the GCDC-induced generation of NO (a), ONOO<sup>-</sup> (b), but not hydroperoxides (c) from rat liver mitochondria exposed to GCDC. Liver mitochondria ( $\approx 1.0$  mg/ml) were loaded with either 10  $\mu$ M DAF-2, 3  $\mu$ M DCDHF, or 8  $\mu$ M dichlorofluorescein diacetate (for NO, ONOO<sup>-</sup>, and hydroperoxide determinations, respectively) for 30 min at 28 °C and exposed to GCDC (100  $\mu$ M) for 10 min. NOS inhibitors were preincubated for 5 min before the addition of GCDC. As shown in the figure, NOS inhibitors reduced NO and ONOO<sup>-</sup> levels from GCDC-treated mitochondria without affecting hydroperoxide generation, supporting the specificity of DAF and DCDHF toward NO and ONOO<sup>-</sup>, respectively. Values are expressed as means  $\pm$  S.E. of at least five experiments.

490-nm excitation and 520-nm emission and under identical conditions as DAF-2 and DCDHF, as previously described in detail (2). Results were compared with a standard curve using 2',7'-dichlorofluorescein as the standard and were expressed as pmol of dichlorofluorescein/mg of mitochondrial protein.

**Determination of Hepatocytes Apoptosis**—Hepatocyte apoptosis was quantitated by determining the percentage of hepatocytes with nuclear morphologic changes of apoptosis detected by fluorescence microscopy of 4,6-diamidino-2-phenylindole-stained fixed hepatocytes, as previously described (5).

**Enzymatic Activity of Caspase 3**—Caspase 3 activity in hepatocytes was analyzed as follows. Hepatocytes ( $4\text{--}5 \times 10^6$  cells) were pelleted, washed with cold KRH buffer, and resuspended in 1.2 ml of 100 mM HEPES, pH 7.4, containing a protease inhibitory mixture (P-8340 from Sigma). Cells were lysed by three freeze-thaw cycles, and a post-mitochondrial supernatant was obtained by centrifugation at  $12,000 \times g$  for 30 min at 4 °C. Caspase activity was measured by incubating supernatants ( $\approx 300\text{--}600$   $\mu$ g of protein) with the chromogenic substrate Ac-DEVD-pNA (150  $\mu$ M) at 37 °C for 1 h, and the cleavage product was determined at 405 nm. Results were expressed as absorbance/mg protein/h.

**Measurement of the Mitochondrial Permeability Transition**—The MPT was measured spectrophotometrically as described in detail (15). Briefly, hepatic mitochondria (1.5–3.0 ml) were incubated at 25 °C for 5 min alone or in the presence of a NOS inhibitor or SNAP. After the preincubation period, 100  $\mu$ M CaCl<sub>2</sub>, 5 mM sodium succinate, and 5  $\mu$ M rotenone (in dimethylformamide) were added to mitochondria, and the absorbance at 540 nm was monitored for 5 min. The MPT was then induced by the addition of 100–200  $\mu$ M GCDC, and absorbance was monitored for an additional 5 min. After the MPT experiment, some mitochondrial samples were centrifuged immediately at  $10,000 \times g$  for 30 min at 4 °C to isolate the mitochondrial pellet for immunoblot

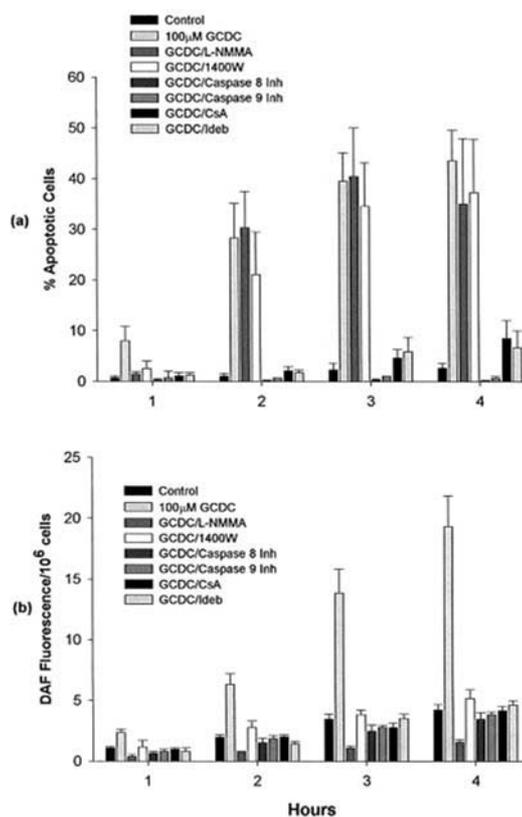
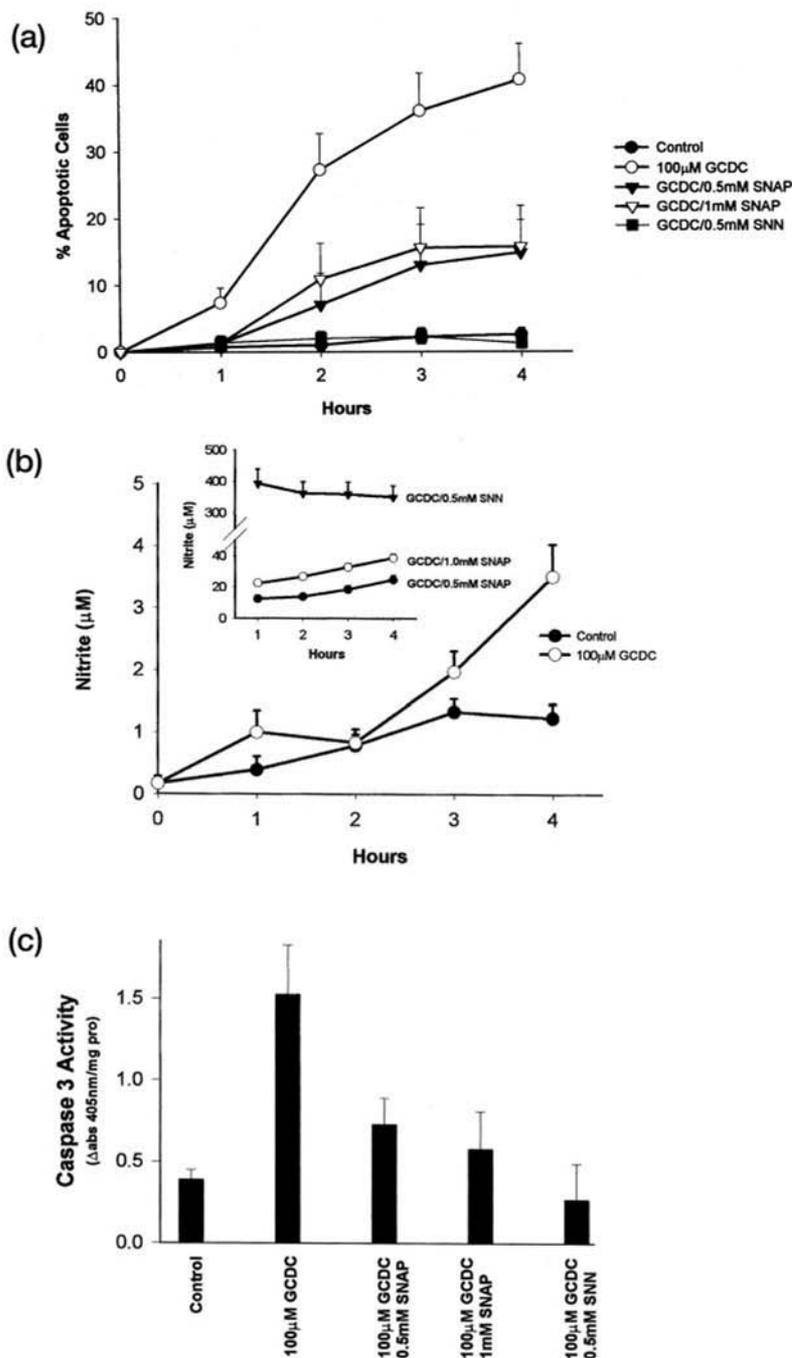


FIG. 3. Effect of NOS inhibitors on GCDC-induced apoptosis (a) and NO generation (b) from isolated rat hepatocytes. Rat hepatocytes were prepared as described in Fig. 1 and pretreated for 30 min with NOS inhibitors (1 mM L-NMMA or 100  $\mu$ M 1400W) before induction of apoptosis with GCDC (100  $\mu$ M). For comparative purposes, additional inhibitors of GCDC-induced apoptosis were also employed. GCDC-induced apoptosis and NO generation were all significantly reduced by the antioxidant idebenone (*Ideb*; 100  $\mu$ M), the MPT blocker CsA (5  $\mu$ M), and inhibitors of caspase 8 (25  $\mu$ M Z-IETD-FMK) and caspase 9 (20  $\mu$ M Z-LEHD-FMK). However, NOS inhibitors had no effect upon GCDC-induced apoptosis, despite significantly reducing NO generation.

analysis of cytochrome *c* as described below.

**Flow Cytometry**—Flow cytometric analysis was performed to determine the effect of NO donors on GCDC-induced MPT in freshly isolated hepatocytes as previously described in detail (14). Briefly, hepatocytes were treated with 0 or 100  $\mu$ M GCDC in the absence or presence of 0.5 mM SNAP or SNN for 3 h. Hourly aliquots of cells were removed, loaded with 7.6  $\mu$ M JC-1 or 3  $\mu$ M propidium iodide for 15 min at 22 °C in the dark, and washed with KRH buffer at 4 °C before flow cytometry on a BD PharMingen FACSCalibur using CELLQuest software. In actively respiring mitochondria, JC-1 aggregates form, and the intensity of their fluorescence at 590 nm is proportional to the mitochondria  $\Delta\psi$  and indicative of a closed MPT pore. Approximately 10,000 cells were analyzed for each time point and treatment. The content of JC-1 aggregates was determined only in live cells, as indicated by their lack of uptake of propidium iodide.

**Immunoblot Analysis of Cytochrome *c* Release from Liver Mitochondria**—Immunoblot analysis of cytochrome *c* was performed by separating mitochondrial proteins on a 12.5% SDS-PAGE gel before transferring proteins onto an Immobilon-P membrane (Fisher) in a buffer consisting of 25 mM Tris-HCl, 192 mM glycine, 10% methanol (32). After transfer, the blots were incubated with 1% nonfat dry milk (Bio-Rad) followed by incubation with 1  $\mu$ g/ml mouse anti-cytochrome *c* monoclonal antibody (BD PharMingen). After 3 washes with phosphate-buffered saline containing 0.1% Tween 20, blots were incubated with 1:1000 dilution of horseradish peroxidase polyclonal anti-mouse Ig (BD PharMingen). After three washes with phosphate-buffered saline with Tween, cytochrome *c* reactivity was developed colorimetrically using the Opti-4CN substrate kit according to the manufacturer's instruction, scanned, and quantitated by densitometry using UN-SCAN IT gel software (Silk Scientific Inc., UT). Cytochrome *c* content was expressed as relative densitometry units.



**FIG. 4. Effect of NO donors on GCDC-induced apoptosis (a), media nitrite levels (b), and caspase 3 activity (c).** Hepatocytes were preincubated for 30 min with either SNAP (0.5–1.0 mM) or SNN (0.5 mM) before induction of apoptosis with GCDC (100  $\mu$ M). Cells were removed hourly for quantitation of apoptosis (4,6-diamidino-2-phenylindole staining) and nitrite levels and after 3 h for measurement of caspase 3 activity. *a*, SNAP and SNN significantly reduced GCDC-induced apoptosis by ~64 and 100%, respectively, by 4 h. *b*, SNAP and SNN increased nitrite levels from hepatocytes by 20–30-fold for SNAP and ~400-fold for SNN, by 4 h when compared with untreated hepatocytes. GCDC (100  $\mu$ M) also significantly increased nitrite levels by 4 h compared with untreated cells. *c*, GCDC (100  $\mu$ M) stimulated caspase 3 activity after 3 h incubation, which was prevented by pretreatment with either SNAP or SNN. *abs*, absorbance.

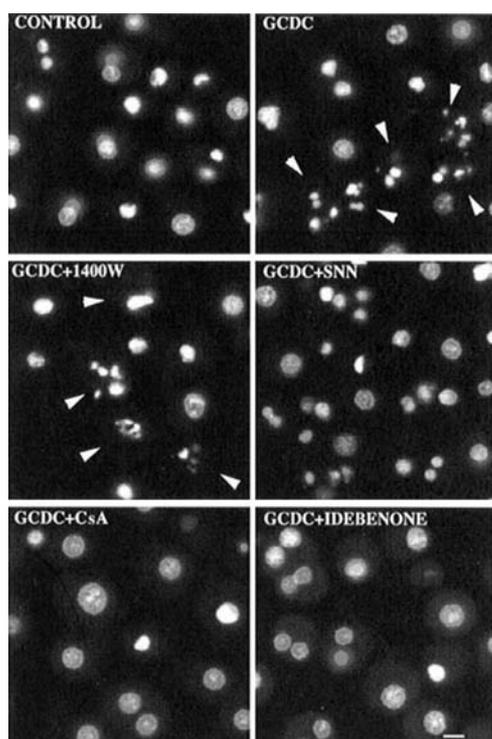
**Statistical Analysis**—Mean and S.E. were calculated for each time point. Comparisons among groups were performed by analysis of variance and the Scheffe test or *t* test where appropriate. A *p* value of < 0.05 was considered statistically significant.

## RESULTS

**GCDC Stimulates NO and ONOO<sup>-</sup> Generation from Isolated Rat Hepatocytes and Liver Mitochondria**—GCDC caused significant apoptosis in hepatocytes after 2 h of exposure to 50 and 100  $\mu$ M GCDC (Fig. 1*a*), the latter concentration being chosen for subsequent experiments. To determine whether GCDC stimulated NO or ONOO<sup>-</sup> generation in rat hepatocytes or liver mitochondria, the fluorescent probes DAF-2/DA and DC-DHF-DA were utilized for detection of NO and ONOO<sup>-</sup>, respectively (28, 29). Rat hepatocytes incubated in the absence of GCDC generated very low levels of NO and ONOO<sup>-</sup> (Fig. 1, *b–c*). Exposure to GCDC (25–500  $\mu$ M) resulted in concentration-

and time-dependent increases of cellular NO and ONOO<sup>-</sup> production beginning at 1 h of incubation, indicating that GCDC stimulation of NO/ONOO<sup>-</sup> generation preceded induction of apoptosis. Evidence that the observed increases of DAF-2 and DC-DHF fluorescence represented hepatocyte NO/ONOO<sup>-</sup> generation was provided by the significant reduction of NO and ONOO<sup>-</sup> (90% and >75%, respectively) in the presence of the NOS inhibitor L-NMMA (1 mM) (Fig. 1) and 1400W (Fig. 2), a more selective inhibitor of inducible NOS (33).

GCDC stimulated NO/ONOO<sup>-</sup> production from liver mitochondria within 1 min, increasing NO/ONOO<sup>-</sup> levels by 4–6-fold by the end of the 10-min incubation (Fig. 2, *a–b*). Because NOS activity is also detectable in mitochondria (34), we examined the effect of L-NMMA and 1400W on mitochondria exposed to GCDC. As seen from Fig. 2, *a–c*, inhibition of NOS reduced NO and ONOO<sup>-</sup> production in mitochondria. Because GCDC

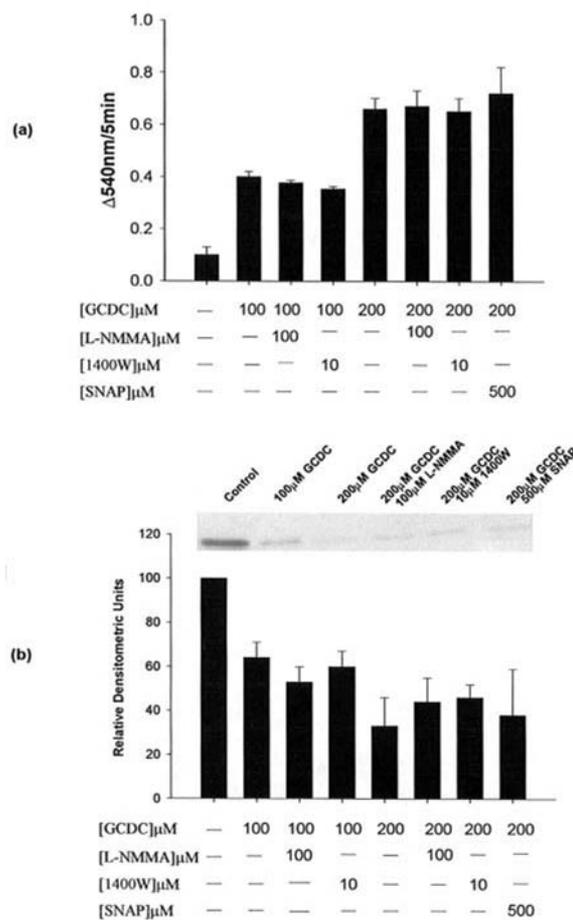


**FIG. 5. Nuclear morphology of 4,6-diamidino-2-phenylindole-stained rat hepatocytes exposed to 100  $\mu\text{M}$  GCDC (or vehicle) for 3 h and pretreated with either an NO donor SNN (0.5 mM) or NOS inhibitor 1400W (100  $\mu\text{M}$ ).** Also shown are hepatocytes pretreated with CsA (5  $\mu\text{M}$ ) or idebenone (100  $\mu\text{M}$ ). The arrowheads identify fragmented nuclei of apoptotic cells. The figure indicates that GCDC is a potent inducer of hepatocyte apoptosis, which is prevented by elevated NO, CsA, and idebenone but was not affected by NOS inhibition. The bar in the bottom right panel is 10  $\mu\text{m}$ .

also stimulated hydroperoxide generation from liver mitochondria (15, 31), we determined whether NOS inhibitors affected hydroperoxide generation from mitochondria exposed to GCDC. GCDC caused significant hydroperoxide formation in mitochondria, which was resistant to NOS inhibitors (Fig. 2c). These findings support prior observations (28, 29) that DAF-2 and DCDHF react poorly with reactive oxygen species other than NO and ONOO<sup>-</sup> and can be utilized as effective probes to detect hepatocyte generation of NO and ONOO<sup>-</sup>. Because of the close correlation between NO and ONOO<sup>-</sup> generation and their suppression by NOS inhibitors, in subsequent experiments only NO levels (DAF-2 fluorescence) were determined.

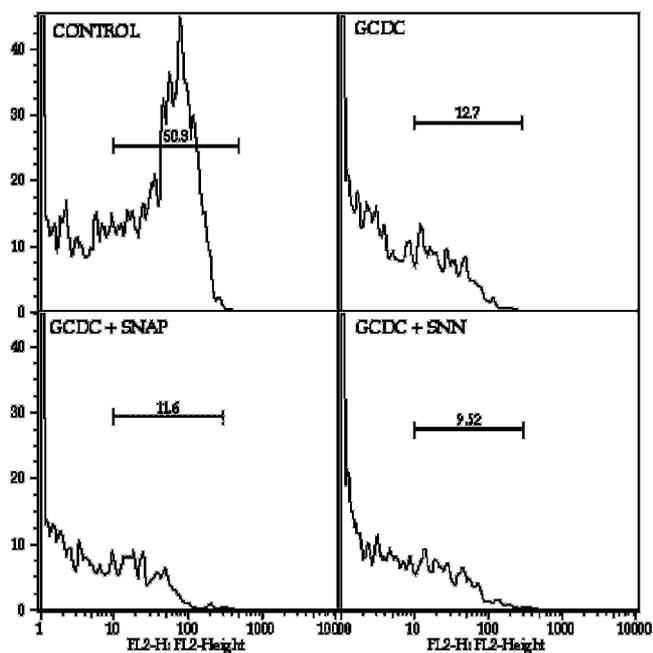
**Effect of NOS Inhibition on Apoptosis**—The increased generation of NO in rat hepatocytes and liver mitochondria exposed to GCDC raised the possibility that NO or its metabolites could function as an important mediator or cell signal responsible for the cellular changes observed during GCDC-induced hepatocyte injury (5, 14, 15). Therefore, we examined whether modulation of NO status prevented or potentiated GCDC-induced apoptosis compared with other inhibitors of hepatocyte apoptosis. GCDC (100  $\mu\text{M}$ ) caused substantial hepatocyte apoptosis by 4 h ( $40.8 \pm 5.4\%$ ), which was reduced by >75% by inhibitors of either caspase 8 (Z-IETD-FMK) or 9 (Z-LEHD-FMK), CsA, or idebenone (Fig. 3a). These inhibitors of apoptosis also completely suppressed GCDC-induced generation of NO (Fig. 3b). In contrast, GCDC-induced apoptosis was unaffected by pretreatment with NOS inhibitors despite complete inhibition of cellular NO generation (Fig. 3a). These findings indicate that endogenously produced NO in GCDC-treated rat hepatocytes does not mediate nor stimulate apoptosis.

**Effect of NO Donors on Apoptosis and Mitochondrial Pertur-**



**FIG. 6. NO modulation fails to prevent the GCDC-induced MPT (a) or release of cytochrome c (b) from rat liver mitochondria.** Modulation of mitochondrial NO by NOS inhibitors (100  $\mu\text{M}$  L-NMMA or 10  $\mu\text{M}$  1400W) or the NO donor SNAP (500  $\mu\text{M}$ ) was performed by preincubating mitochondria for 10 min before induction of the MPT by GCDC (100–200  $\mu\text{M}$ ). GCDC caused rapid, high amplitude swelling of mitochondria, which was quantitated by the change in absorbance at 540 nm over a 5-min incubation. After the 5-min exposure to GCDC, mitochondria were isolated by centrifugation, analyzed for mitochondrial cytochrome c content by immunoblotting, and expressed as relative densitometric units compared with untreated (control) mitochondria. GCDC promoted mitochondrial cytochrome c loss compared with untreated mitochondria, which was unaffected by modulators of NO. Values are means  $\pm$  S.E. of three experiments. The inset shows a typical immunoblot of mitochondrial cytochrome c remaining after the specific treatments.

**bations**—Although inhibition of cellular NO generation by NOS inhibitors failed to alter the course of GCDC-induced apoptosis, we performed additional studies to determine whether further elevating NO levels by exogenous administration of NO donors (SNAP and SNN) influenced this process, as demonstrated in other models of cellular toxicity (20, 22). Indeed, preincubation with NO donors significantly reduced GCDC-induced apoptosis by ~65% for SNAP (0.5–1.0 mM) and 100% in the case of 0.5 mM SNN (Figs. 4a and 5). To compare NO production with hepatocyte apoptosis, we measured extracellular nitrite production, a primary metabolite of NO (Fig. 4b). Compared with untreated cells, GCDC stimulated a 3-fold increase in nitrite levels by 4 h of incubation, approximately the increase in DAF-2 fluorescence observed between untreated and 100  $\mu\text{M}$  GCDC-treated hepatocytes (Fig. 1a). Moreover, cells pretreated with NO donors had substantially elevated nitrite levels, 20–30-fold by SNAP (0.5–1.0 mM) and 400-fold by SNN (0.5 mM) (Fig. 4b, inset). These data suggesting that high levels of NO were necessary for inhibition of apoptosis. One postulated mecha-



**FIG. 7. The Effect of NO on GCDC-induced collapse of mitochondrial  $\Delta\psi$  in freshly isolated hepatocytes.** Isolated rat hepatocytes were exposed to 100  $\mu\text{M}$  GCDC in the absence or presence of 0.5 mM SNAP or SNN. Hourly aliquots were removed, loaded with 7.6  $\mu\text{M}$  JC-1 for 15 min at 22  $^{\circ}\text{C}$ , and washed with KRH buffer before fluorescence determination at 590 nm. JC-1 aggregates, which represent an intact mitochondrial  $\Delta\psi$ , were significantly reduced by 2 h of treatment with GCDC compared with untreated cells. Preincubation of NO donors with hepatocytes failed to prevent this bile acid-induced mitochondrial depolarization from isolated rat hepatocytes. NO donors alone failed to affect JC-1 aggregate production when compared with control cells (not shown). The numbers above the bars indicate the % of the population of live cells, which contain the amount of fluorescence under the bar.

nism responsible for NO-dependent inhibition of apoptosis is the nitrosylation and inactivation of caspases (19, 20). Therefore, we determined caspase 3 activity in cells exposed to GCDC with and without NO donors. Caspase 3 is a downstream, effector caspase involved in the execution of apoptotic cell death (35). Increased hepatocyte caspase 3 activity measured after 3 h of incubation with 100  $\mu\text{M}$  GCDC was inhibited by SNAP (Fig. 4c).

Finally, we determined if NO modulation had any direct effect on mitochondrial pathways involved in bile acid-induced apoptosis. We specifically examined the effect of NOS inhibitors and SNAP on the MPT and cytochrome *c* release in liver mitochondrial suspensions exposed to GCDC and on GCDC-induced depolarization of isolated rat hepatocytes by flow cytometry. Both inhibition of NO production (by L-NMMA and 1400W) and enhancement of mitochondrial NO levels by (SNAP) had no effect on the induction of MPT or cytochrome *c* release by GCDC (100–200  $\mu\text{M}$ ) in isolated mitochondria (Fig. 6). Similarly, flow cytometry demonstrated that fluorescence of JC-1 aggregates, indicative of an intact mitochondrial  $\Delta\psi$  and a closed MPT pore, decreased significantly by 2 h in hepatocytes treated with GCDC (100  $\mu\text{M}$ ) compared with untreated cells and that mitochondrial depolarization occurred early in the induction of bile acid-induced hepatocyte apoptosis (Fig. 7). However, preincubation of cells with either 0.5 mM SNAP or SNN failed to prevent this bile acid-induced mitochondrial depolarization. Thus, the inhibition of apoptosis by NO was associated with reduced caspase 3 activity but without any direct effect on mitochondrial pathways involved in GCDC-induced apoptosis.

## DISCUSSION

In the present study, we have demonstrated that neither NO nor ONOO<sup>-</sup> play a role in mediating hepatocellular apoptosis induced by hydrophobic bile acids and that elevated NO levels may have an anti-apoptotic effect that does not involve mitochondrial pathways of apoptosis. By using fluorescent probes specific for NO and ONOO<sup>-</sup>, we have provided evidence that GCDC stimulates NO/ONOO<sup>-</sup> production from both freshly isolated rat hepatocytes and liver mitochondria by a NOS-dependent pathway. These probes offer many benefits for assessing NO/ONOO<sup>-</sup> status including the ability to detect low intracellular concentrations (which may allow detection of NO from constitutively produced NO) and their lack of reactivity toward other reactive species such as superoxide and hydroperoxides (28, 29, 36). Corroboration of the lack of reactivity of these probes toward hydroperoxides was demonstrated in our experimental model.

These results are the first to demonstrate stimulation of NO and ONOO<sup>-</sup> generation in both isolated rat hepatocytes and liver mitochondria by a hydrophobic bile acid under conditions that may exist in the cholestatic liver. Similar results were recently reported in endothelial vascular cells exposed to bile acids, in which the hydrophobicity of the bile acids correlated with their capacities to increase NO production (37). In addition to NO production, ONOO<sup>-</sup> generation in rat hepatocytes and liver mitochondria further demonstrates the GCDC stimulation of superoxide. We have previously shown that ROS are generated by the respiratory chain of mitochondria exposed to bile acids (38) and have proposed that impaired electron transport (39) was responsible for mitochondrial superoxide generation. The mechanisms responsible for the increased generation of NO from rat hepatocytes or liver mitochondria exposed to GCDC are unclear. Under normal physiological conditions, basal low levels of NO within the liver are controlled by endothelial NOS, which in turn is primarily regulated by intracellular calcium flux and calmodulin binding (40). However, upon stimulation by cytokines or microbial products such as lipopolysaccharides, induction of a distinct NOS, inducible NOS (iNOS), results in rapid synthesis of NO. Regulation of this NOS isoform occurs at both transcriptional and post-translational steps by a variety of signal-transducing agents including phosphatidylinositol 3-kinase (reviewed in Ref. 41). Inasmuch as phosphatidylinositol 3-kinase activation has been shown to prevent hepatocyte apoptosis induced by hydrophobic bile acids (but not GCDC) (42, 43), it is unclear whether the NO stimulated by GCDC is the result of phosphatidylinositol 3-kinase regulation of either iNOS or “constitutive” NOS isoforms. Further characterization of iNOS-dependent NO production from rat hepatocytes was recently reported by Fariss and co-workers (44, 45). Their studies revealed time-dependent increases of nitrite formation and expression of iNOS and NOS enzymatic activity after 3 h of incubation. Because we were unable to demonstrate increased iNOS expression by GCDC (data not shown), it is unlikely that iNOS induction was responsible for the observed GCDC-stimulated NO that appeared within 1 h of incubation.

Production of NO in both isolated rat hepatocytes and liver mitochondria exposed to GCDC suggested a possible important regulatory role of NO during bile acid-induced hepatotoxicity. Although others have shown that bile acids may promote hepatocyte apoptosis through pathways involving activation of caspase 8 (46), protein kinase c (47), and Fas aggregation (43), our laboratory has reported ROS generation and MPT as key steps in bile acid-induced apoptosis. As a cell-signaling agent and biologically relevant free radical, NO has been found to either promote or prevent apoptosis. For example, stimulation

of NO generation by induction of iNOS may promote apoptosis in macrophages and hepatocytes (48, 49) and cause release of cytochrome *c* from mitochondria (50, 51). Despite conflicting data, however, the preponderance of evidence strongly supports the notion that NO, supplied exogenously or stimulated via iNOS, acts as an anti-apoptotic or cell survival factor, particularly during tumor necrosis factor- $\alpha$  or Fas ligand-dependent hepatocyte apoptosis (19–22). Proposed mechanisms for the anti-apoptotic effects of NO include 1) increased cGMP production, 2) induction of cytoprotective genes such as heat shock proteins, and 3) inhibition of caspase activities (for review, see Ref. 19). Of particular relevance to the current study, Billiar and co-workers (19, 20) provided evidence that NO can inhibit caspases through direct nitrosylation of key sulfhydryl moieties found in all caspases, thus controlling activation of these proteases by redox modification. In the current study, by utilizing two distinct NOS inhibitors, L-NMMA, a nonspecific inhibitor, and 1400W, a specific inhibitor of iNOS activity (52), we observed that suppression of GCDC-stimulated endogenous NO production (as measured by both intracellular DAF fluorescence and extracellular nitrite accumulation), failed to alter induction of hepatocyte apoptosis. One likely explanation for the lack of effect of NOS inhibitors is that the amount of NO produced was insufficient to modulate pathways critical to the execution of apoptosis. However, in concordance with hepatocyte apoptosis induced by tumor necrosis factor- $\alpha$  or Fas ligand (19–22), substantially elevating NO concentrations with two NO donors afforded significant protection against GCDC-induced apoptosis by a mechanism likely involving inhibition of caspase 3. Furthermore, these data show that SNN, a rapidly yielding NO donor, produced greater quantities of NO and inhibition of apoptosis and caspase 3 activity compared with SNAP, a slower, less “efficient” NO donor (53), consistent with a relationship between NO concentration and protection against apoptosis. An additional mechanism for the protection of NO against bile acid-induced apoptosis could be by preventing mitochondrial perturbations. In this regard, NO has been shown to both induce (50, 54) and inhibit MPT and/or cytochrome *c* release (22, 24) in a variety of experimental conditions and cell types. Results from the present study clearly demonstrate that modulating NO levels with either NOS inhibitors or NO donors had no effect on two key mitochondrial factors involved in GCDC-induced apoptosis (14), the MPT and cytochrome *c* release. Thus the protective effects of NO in our model do not appear to involve mitochondria directly, but rather, interactions with essential cytosolic caspases.

In our experiments, NO generation was also completely suppressed by a variety of inhibitors of hepatocyte apoptosis that do not directly affect NOS activity. The choice of these inhibitors allowed examination of the potential mechanisms and locations of NO production relative to mechanisms of bile acid-induced hepatocyte apoptosis. For instance, inhibition of NO and apoptosis by CsA raises the intriguing possibility that the NO generated from GCDC-treated hepatocytes is contingent upon induction of the MPT. Interestingly, one report describes mitochondrial NOS-dependent stimulation of cytochrome *c* release, which was not mediated by the MPT but rather through formation of ONOO<sup>-</sup> (50). Clearly, the prevention of NO generation by multiple inhibitors of GCDC-induced apoptosis at both upstream (caspase 8 inhibition) and downstream (caspase 9 inhibition) locations suggests that NO generation occurs as a consequence of cellular apoptotic events in this model.

Another potential site of regulation of hepatocyte apoptosis by NO are protein kinases and mitogen-activated protein kinases. Hydrophobic bile acids have been shown to promote hepatocyte apoptosis by ligand-independent activation of Fas

and TRAIL death receptor cascades (55, 56) and subsequent activation of a variety of signal transduction pathways including caspases (46) and protein kinases (47). Among those members of the protein kinase family involved in cell apoptosis, mitogen-activated protein kinases have been reported to protect hepatocytes against bile acid-induced apoptosis (57, 58), potentially explaining the therapeutic effects of the hydrophilic bile acid, tauroursodeoxycholic acid, in cholestatic liver diseases (59). Qiao *et al.* (58) recently showed in primary hepatocytes that deoxycholic acid caused ligand-independent activation of epidermal growth factor receptor, which led to mitogen-activated protein kinase activation via phosphatidylinositol 3-kinase. Activation of this pathway afforded cytoprotection through enhanced expression c-FLIP isoforms that inhibit procaspase 8 cleavage. Conversely, inasmuch as ROS generation has been shown to activate c-Jun NH<sub>2</sub>-terminal kinase and other protein kinases (60), it is proposed that bile acid-stimulated ROS generation may be an upstream event in hepatocytes exposed to hydrophobic bile acids that may trigger activation of protein kinases involved in promoting cellular apoptosis. Finally, a recently characterized member of the mitogen-activated protein kinase family, apoptosis signal-regulating kinase (ASK-1) can be activated by cytotoxic stimuli including Fas, tumor necrosis factor- $\alpha$ , or ROS and is essential for the induction of apoptosis in a number of differentiated cell lines (61, 62). Overexpression of ASK-1 leads to activation of JNK and other mitogen-activated protein kinases causing induction of apoptosis in certain cell types (63), although there is no evidence for the role of ASK-1 activation during hepatocyte apoptosis. Because NO has been shown to regulate protein kinase expression (64), determining the role of NO interactions with these various protein kinase families in bile acid-induced apoptosis will require further investigation.

In summary, our findings support a potential protective role of high levels of NO during bile acid-induced hepatocyte apoptosis. Although GCDC did stimulate NO generation in hepatocytes and mitochondria, the low levels of NO did not play a role in mediating or protecting against hepatocyte apoptosis. However, exogenous addition of NO supplied by NO donors or up-regulation of cellular iNOS by chemical or gene transfer techniques or by stimulation of NF $\kappa$ B might provide an effective cell survival strategy for reducing bile acid-induced cellular injury. Other factors that regulate NO synthesis and NOS expression during bile acid toxicity and cholestasis require further investigation. If accumulation of hydrophobic bile acids is indeed a primary factor in the pathogenesis of cholestatic liver diseases, then results from this study may provide a potential therapeutic strategy in addition to reducing oxidant stress and inhibiting the MPT (14, 15) for preventing or reducing the hepatocellular damage observed clinically in cholestatic liver injury.

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