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Resistance of rat hepatocytes against bile acid-induced apoptosis in cholestatic liver injury is due to nuclear factor-kappa B activation

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***Background/Aims*: To examine the extent and mechanisms of apoptosis in cholestatic liver injury and to explore the role of the transcription factor nuclear factor-kappa B in protection against bile acid-induced apoptosis.**

***Methods*: Cholestatic liver injury was induced by bile duct ligation in Wistar rats. Furthermore, primary cultures of rat hepatocytes were exposed to glycochenodeoxycholic acid (GCDCA), tauroursodeoxycholic acid (TUDCA), taurochenodeoxycholic acid (TCDCA) and to cytokines. Apoptosis was determined by TUNEL-staining, active caspase-3 staining, activation of caspase-8, -9 and -3.**

***Results*: Limited hepatocyte apoptosis and an increased expression of NF-κB-regulated anti-apoptotic genes A1 and cIAP2 were detected in cholestatic rat livers. Bcl-2 expression was restricted to bile duct epithelium. In contrast to TCDCA and TUDCA, GCDCA induced apoptosis in a Fas-associated protein with death domain (FADD)-independent pathway in hepatocytes. Although bile acids do not activate NF-κB, NF-κB activation by cytokines (induced during cholestasis) protected against GCDCA-induced apoptosis in vitro by upregulating A1 and cIAP2.**

***Conclusions*: GCDCA induces apoptosis in a mitochondria-controlled pathway in which caspase-8 is activated in a FADD-independent manner. However, bile acid-induced apoptosis in cholestasis is limited. This could be explained by cytokine-induced activation of NF-κB-regulated anti-apoptotic genes like A1 and cIAP2.**

***Keywords*: Cholestasis; Bile acids; Hepatocytes; Apoptosis; Caspases; Inflammation; Nuclear factor-kappa B; cIAP2**

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Abbreviations: TNF-α, tumor necrosis factor α; TNFR-1, TNF-α type I receptor; FADD, Fas-associated protein with death domain; GCDCA, glycochenodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; IAP, inhibitor of apoptosis protein; HIAP, human IAP; iNOS, inducible NO synthase; CM, cytokine mixture; IL-1β, interleukin-1β; IFN-γ, interferon-γ; Ad5IκBAA, adenoviral dominant-negative IκB-α; Ad5LacZ, adenoviral β-galactosidase; Ad5dnFADD, adenoviral dominant-negative FADD; AdHIAP1, adenoviral human IAP1; EMSA, electrophoretic mobility shift assay; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; GGT, gamma glutamyltransferase.

1. Introduction

During cholestatic liver injury, the accumulation of bile acids in the liver is thought to play a role in causing hepatocyte damage [1,2]. Toxic hydrophobic bile acids induce hepatocyte swelling and disrupt cell membranes resulting in the release of intracellular constituents [3]. In addition, hydrophobic bile acids may cause hepatocyte injury by non-detergent mechanisms. Exposure of hepatocytes to deoxycholic acid [4] and glycochenodeoxycholic acid [5,6] at concentrations far below their critical micelle concentration, results in apoptotic cell death. However, the extent of hepatocyte apoptosis in cholestatic liver injury remains to be clarified. The present report focuses on apoptotic cell death during cholestasis.

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It has been postulated that toxic bile acids directly activate the Fas death receptor [7,8]. Furthermore, (glycocheno)deoxycholic acid induces a decrease in the mitochondrial membrane potential and cytochrome c release from mitochondria [9,10]. Other studies suggest that oxidative stress is involved in glycochenodeoxycholic acid-induced decrease of the mitochondrial membrane potential in hepatocytes [11].

Besides glycochenodeoxycholic acid, taurine conjugates of chenodeoxycholate and ursodeoxycholate accumulate in rat liver in cholestasis. Taurochenodeoxycholic acid (TCDCA) has been postulated to activate a phosphatidylinositol 3-kinase-mediated survival pathway that involves the induction of nuclear factor-kappa B activation [12]. Ursodeoxycholic acid is used to treat patients with cholestatic liver injury [13], but the protective mechanisms are not fully understood yet [10,14].

An important survival pathway in hepatocytes is the activation of the transcription factor NF-κB [15,16]. This results in the induction of NF-κB-regulated survival genes and inhibition of apoptotic cell death [15]. It is known that endotoxin levels in blood are increased in cholestatic liver diseases. Endotoxin induces cytokine production in Kupffer cells, resulting in the activation of the NF-κB survival pathway in hepatocytes. Therefore, we also investigated the expression of cytokines in cholestatic livers and the role of NF-κB in cholestatic liver injury.

2. Materials and methods

2.1. Animals

Specified pathogen-free male Wistar rats (220–250 g) were purchased from Harlan, Zeist, the Netherlands. They were housed under standard laboratory conditions with free access to standard laboratory chow and water. Each experiment was performed following the guidelines of the local Committee for Care and Use of Laboratory Animals.

2.2. Animal model

Male Wistar rats were anaesthetized with halothane/O_2/N_2O and subjected to bile duct ligation (BDL) [17]. At the indicated times after BDL, the rats (n = 4 per group) were sacrificed, livers were perfused with saline and removed. Control rats ($n = 4$) for each of these time points received a sham operation (SHAM). Specimens of these livers were either snap-frozen in liquid nitrogen for isolation of RNA and protein, or stored in formalin for immunohistochemical studies. For determination of markers of liver damage and cholestasis, heparinized blood samples were obtained by cardiac puncture.

2.3. Hepatocyte isolation and experimental design

Hepatocytes were isolated and cultured as described previously [15,18]. Twenty hours after isolation, hepatocytes were exposed to 50 μM glycochenodeoxycholic acid (GCDCA, Calbiochem, La Jolla, CA), 50 μM tauroursodeoxycholic acid (TUDCA, Calbiochem) or 50 μM taurochenodeoxycholic acid (TCDCA, Calbiochem) and/or 50 μM of the caspase-8 inhibitor Ac-IETD-CHO (BIOMOL, Plymouth Meeting, USA). In some experiments, hepatocytes were pre-incubated with a cytokine mixture (CM) composed of 20 ng/ml recombinant tumor necrosis factor-α (TNF-α, R&D Systems, Abingdon, UK), 10 ng/ml recombinant interleukin-1β (IL-1β, R&D Systems), 100 U/ml recombinant interferon-γ (IFN-γ, Life Technologies Ltd.) and 10 μg/ml LPS (*Escherichia coli*, serotype 0127:B8, Sigma, St. Louis, MO) as described before [15]. Hepatocytes received adenovirus (MOI of 10) 15 h prior to exposure of bile acids or cytokine mixture. Each experimental condition was performed in triplicate wells. Each experiment was performed three times, using hepatocytes from different isolations.

Cells were harvested at the indicated time-points and rinsed three times with ice-cold phosphate buffered saline (PBS) prior to the addition of Trizol reagent (RNA isolation) (Life Technologies Ltd.) or hypotonic cell lysis buffer (protein analysis, caspase-3 assay) as described previously [15]. For measurement of caspase-8 activity, cells were harvested in cell lysis buffer using a caspase-8 fluorometric Protease Assay Kit (BioVision, Mountain View, USA).

2.4. Adenoviral constructs

Adenoviral constructs have been described previously [15,19,20,21].

2.5. Electrophoretic mobility shift assay (EMSA)

To demonstrate the presence of NF-κB in the nucleus upon activation, an EMSA was performed. Nuclear extracts and EMSA for NF-κB were prepared using a final concentration of 0.25% Nonidet P-40 as described previously [22]. The probe containing the NF-κB consensus sequence is 5′-AGCTGCGGGGATTTTCCCTG-3′.

2.6. Caspase-3 and -8 enzyme activity assay

Caspase-3 enzyme activity was assayed as described before [15]. Caspase-8 activity was measured using a Caspase-8 Fluorometric Protease Assay Kit (BioVision) according to the manufacturer's instructions. Assays were performed with 20 μg of protein.

2.7. Immunohistochemical evaluation

TUNEL staining was performed according to manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Livers of D-Gal/LPS treated mice were used as positive control [23]. Staining of Bcl-2 and active caspase-3 was performed on formalin fixed, paraffin-embedded portions of the liver as described previously [24] and according to the manufacturer's instructions. Bcl-2 antibody (DAKO, Glostrup, Denmark) and active caspase-3 antibody (New England Biolabs, Beverly, USA) were used for 1 h at 1:50 and 1:100, respectively. Goat-anti-rabbit horseradish peroxidase and rabbit-anti-goat horseradish peroxidase were used to detect active caspase-3 antibody.

2.8. RNA isolation and reverse-transcriptase polymerase chain reaction (RT-PCR)

RNA isolation, reverse transcription and PCR were performed as described previously [15]. For every PCR, expression of 18S was used as internal control. Primers are listed in Table 1.

2.9. Western blot analysis

Western blot analysis of cell lysates was performed using antibodies against Bcl-2 (Dako) and cleaved caspase-9 (Cell Signaling Technology, Beverly, MA) both at a dilution of 1:1000. Equal loading was confirmed by Ponceau S staining.

2.10. Immunocytochemistry

Analysis of active caspase-9 was performed on hepatocytes cultured on coverslips and exposed to 50 μM GCDCA for 4 h. Coverslips were washed in PBS, fixed in 4% paraformaldehyde for 10 min followed by incubation in 1% Triton-X100 for 5 min. Before adding primary antibodies, cells were washed twice with PBS. Antibody against active caspase-9 was used at a dilution of 1:50 for 30–60 min. Goat-anti-Rabbit antibody coupled to FITC (GAR-FITC, Molecular Probes, Eugene, OR, USA) was added at a dilution of 1:600 for 45 min. Slides were evaluated on a Leica confocal laser scanning microscope.

Table 1
Oligonucleotide primers used for RT-PCR analysis

Primers (rat)	Sense and antisense
18S	5′-GTATTGCGCCGCTAGAGGTG-3′
	5′-CTGAACGCCACTTGTCCCTC-3′
TNF-α	5′-CACCATGAGCACGGAAAGCA-3′
	5′-GCAATGACTCCAAAGTAGACC-3′
IL-1β	5′-CAGGAAGGCAGTGTCACTCA-3′
	5′-GGGATTTTGTCGTTGCTTGT-3′
IFN-γ	5′-GCCCTCTCTGGCTGTTTACTG-3′
	5′-CTTTTCCGCTTCCTTAGGCT-3′
Collagen type I	5′-GCCCTGCTGGTCCCAAAGGTTC-3′
	5′-CATCTTTGCCAGCGGGACCAAC-3′

2.11. Statistical analysis

Results are presented as the mean of at least three independent experiments ± standard deviation. A Mann–Whitney test was used to determine the significance of differences between two experimental groups. A P value of less than 0.05 ($P < 0.05$) was considered to be statistically significant.

3. Results

3.1. Apoptosis is very limited in cholestatic liver injury

To investigate the extent of hepatocyte apoptosis in cholestatic liver injury, rats were sacrificed 4 days and 1, 2,

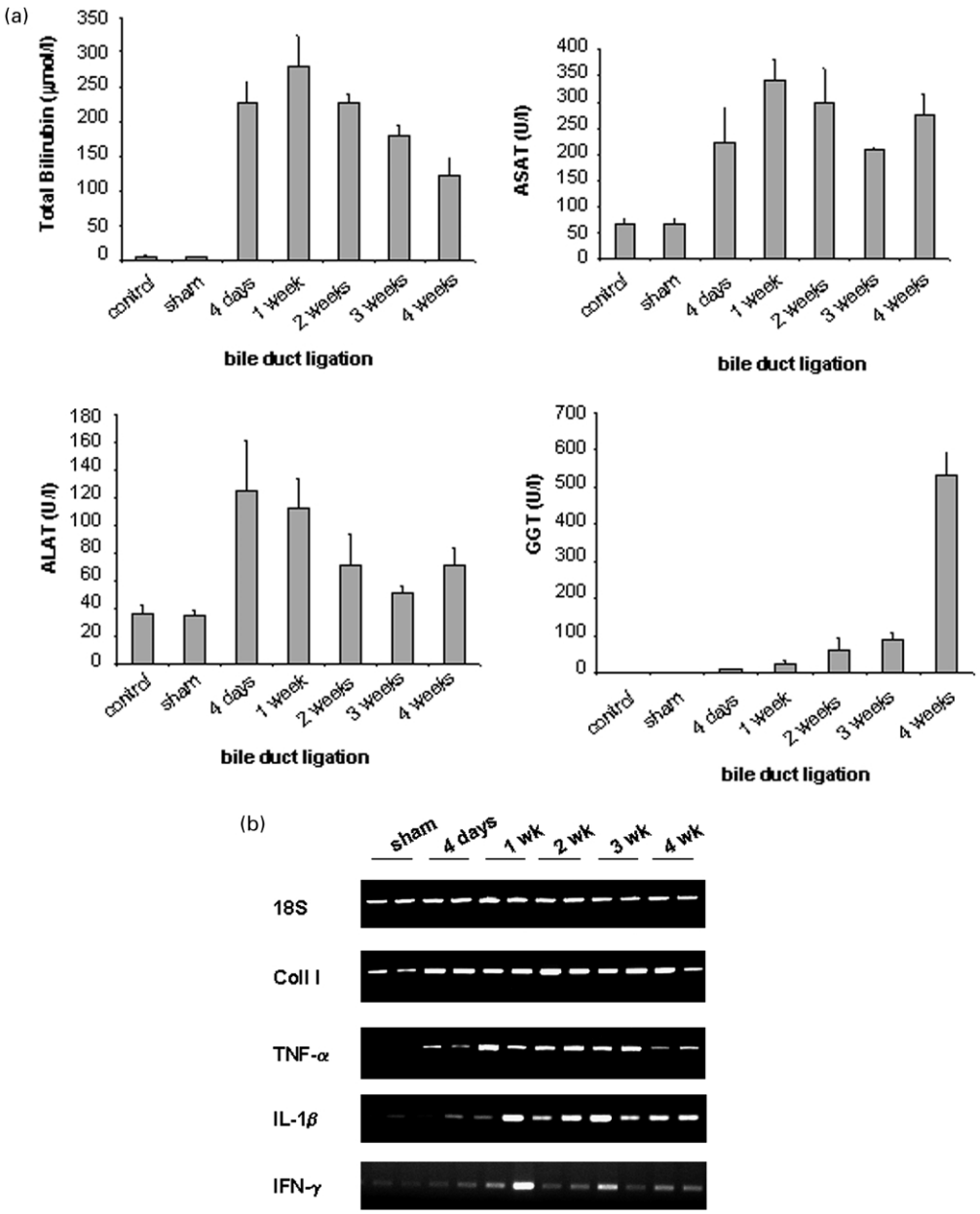

Fig. 1. Liver injury and inflammation after bile duct ligation in rats. (A) Serum levels of total bilirubin, ASAT, GGT and ALAT. (B) mRNA levels of Collagen type I (Coll I), TNF-α, IL-1β and IFN-γ. 18S mRNA served as internal control. Data represent mean of four animals a time point ± SD.

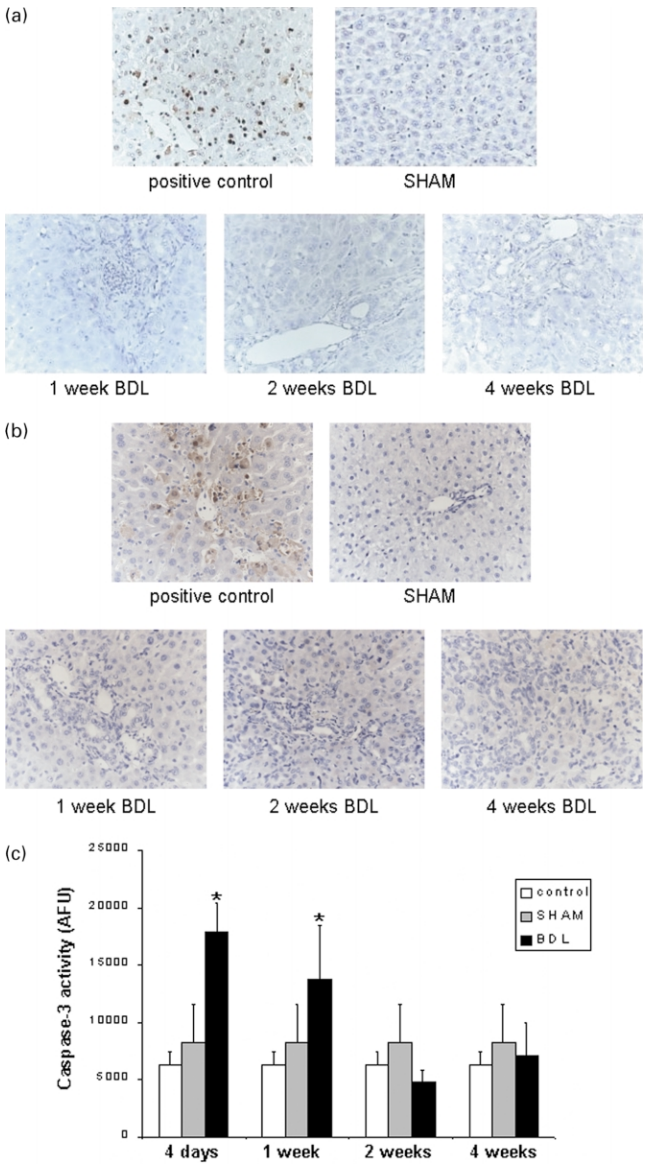

Fig. 2. Limited apoptosis in rat livers beyond 1 week of bile duct ligation (BDL). (A) TUNEL staining. (B) Active caspase-3 staining. SHAM animals served as control. D-Gal/LPS treated livers served as positive control. (C) Caspase-3 activity in liver homogenates. Data represent mean of four animals a time point ± SD. *$P < 0.05$ for BDL 4 days and 1 week versus control and SHAM animals.

3 and 4 weeks after bile duct ligation. Sham operated animals of all time points were averaged and served as control. Total bilirubin, alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and gamma glutamyltransferase (GGT) levels were elevated within the first week after BDL and still persisted 4 weeks after BDL (Fig. 1A). Furthermore, mRNA levels of collagen type I, TNF-α, IL-1β and IFN-γ were clearly increased in cholestatic livers (Fig. 1B).

TUNEL staining was performed to detect apoptotic nuclei. In cholestatic livers, TUNEL staining demonstrated hardly any apoptosis while apoptotic hepatocytes were

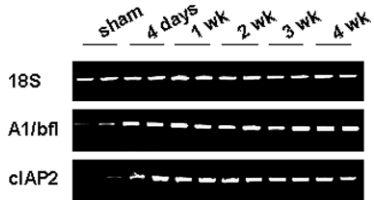

Fig. 3. Induced mRNA expression of NF-κB-regulated genes A1/Bfl-1 and cIAP2 in rat livers after bile duct ligation (BDL). 18S mRNA was used as internal control. SHAM animals of 1 week served as control. Two of four representative animals are shown.

clearly present in D-Gal/LPS-treated livers (Fig. 2A). To confirm these data, a more specific staining was performed demonstrating active caspase-3. Again, only positive control livers were stained (Fig. 2B). Finally, we determined caspase-3 activity in liver homogenates. As shown in Fig. 2C, there is a 3-fold increase of caspase-3 activity within the first week after bile duct ligation, which declines after 1 week and remains at control level up to 4 weeks after BDL. All together, these data demonstrate that apoptotic cell death in cholestatic liver injury is limited and suggests adaptation against the apoptotic actions of bile acids.

In previous work, we demonstrated that cIAP2 (inhibitor of apoptosis protein 2) and A1 are important NF-κB-regulated anti-apoptotic genes in hepatocytes [15]. Since TNF-α, IL-1β and IFN-γ mRNA levels are increased in cholestatic livers (Fig. 1B), we investigated whether cIAP2 and A1 are induced during cholestatic liver injury. The mRNA expression of both anti-apoptotic cIAP2 and A1 genes was strongly increased (Fig. 3). Bcl-2, an anti-apoptotic protein was clearly induced in cholestatic livers (Fig. 4A), but immunohistochemical evaluation demonstrated that expression of this protein was restricted to bile duct epithelium (Fig. 4B).

3.2. GCDCA, but not TUDCA, induces apoptosis in a FADD-independent manner

A time course study on isolated rat hepatocytes exposed to the toxic bile acid GCDCA confirmed that apoptosis was induced 1 h after exposure, peaking at 4 h and returning to control levels within 24 h (Fig. 5). This confirmed previous reports [6]. TUDCA and TCDCA did not induce apoptosis.

In hepatocytes, a 3-fold increase of caspase-8 activity was demonstrated 2 h after GCDCA exposure which was still elevated after 4 h (Fig. 6A). TUDCA did not induce caspase-8 activity (data not shown). Interestingly, GCDCA-induced activation of caspase-8 (Fig. 6A) and caspase-3 (Fig. 6B) was shown to be FADD-independent. Functionality of Ad5dnFADD was demonstrated by its complete inhibition of cytokine-induced caspase-3 activation in hepatocytes in which NF-κB activation is inhibited. Caspase-8 inhibition by Ac-IETD-CHO (Fig. 6A) had no effect on GCDCA-induced activation of caspase-3 (Fig. 6B).

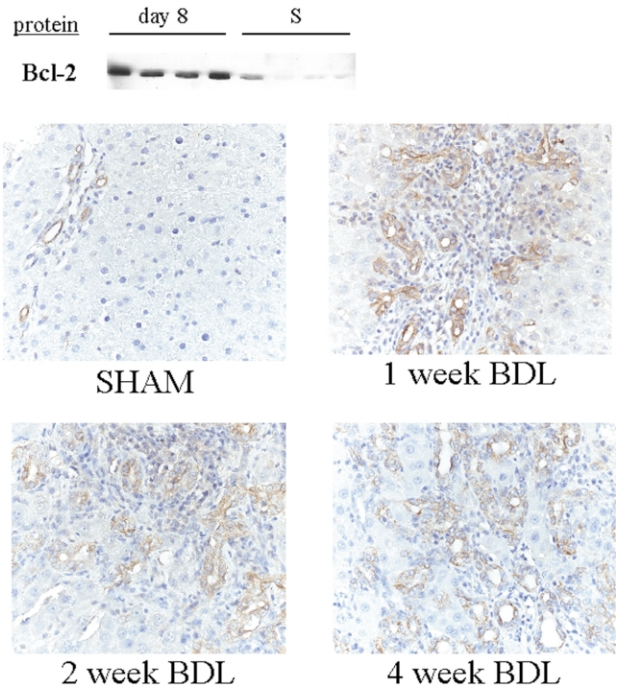

Fig. 4. Bcl-2 expression in cholestatic rat livers is restricted to bile duct epithelium. SHAM animals of 1 week served as control. (A) protein expression after 8 days of bile duct ligation (BDL). (B) Immunohistochemistry on formalin fixed, paraffin-embedded liver sections after 1–4 weeks of BDL.

Immunocytochemistry on GCDCA-exposed hepatocytes demonstrated a clear caspase-9 staining after 4 h which was not observed in control hepatocytes or TUDCA-exposed hepatocytes (Fig. 7A). Western blot confirmed these results, demonstrating active caspase-9 only in GCDCA-exposed hepatocytes, but not in control or TUDCA-exposed hepatocytes (Fig. 7B).

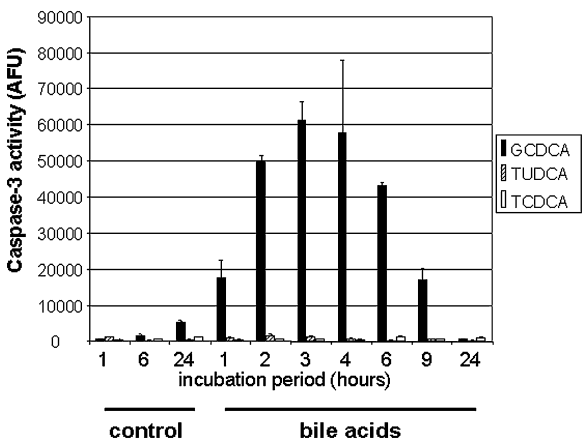

Fig. 5. GCDCA induces caspase-3 activity. Time course study of caspase-3 activity in primary hepatocytes exposed to 50 μM of GCDCA, TUDCA or TCDCA. Representative data of three independent experiments are shown and presented as mean of $n = 3$ per condition.

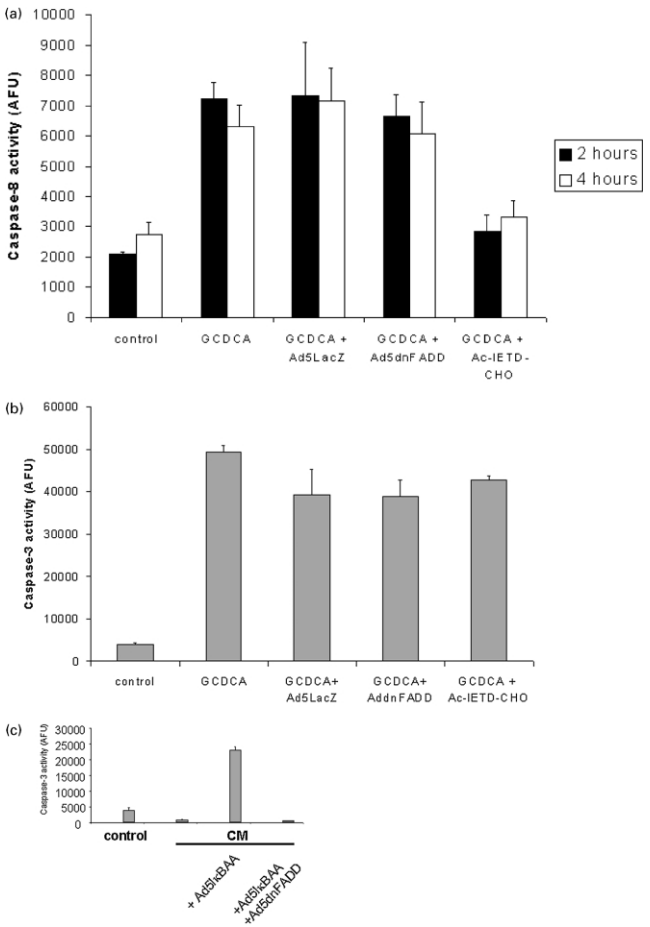

Fig. 6. GCDCA induces FADD-independent caspase-3 and -8 activation in primary hepatocytes. (A) Caspase-8 activity after 2, and 4 h of exposure to GCDCA with or without a caspase-8 inhibitor (Ac-IETD-CHO) or adenoviral expression of dominant negative FADD (AD5dnFADD). LacZ virus (Ad5LacZ) served as control. (B) Caspase-3 activity after 4 h of exposure to GCDCA with or without Ad5LacZ, Ad5dnFADD or Ac-IETD-CHO. (C) Caspase-3 activity after 6 h of exposure to cytokines (CM) and specific inhibition of NF-κB (Ad5IkBAA) plus or minus Ad5dnFADD. One representative of three independent experiments is shown. Mean of $n = 3$ per condition is presented.

3.3. Bile acids do not activate the transcription factor NF-κB in primary hepatocytes

We demonstrated that NF-κB-regulated genes are induced in cholestatic livers (Fig. 3). Therefore, we investigated whether NF-κB is involved in the protection against bile acid-induced apoptosis. EMSA performed on nuclear extracts of primary hepatocytes demonstrated that GCDCA, TCDCA and TUDCA did not activate NF-κB, whereas cytokines clearly activated this transcription factor (Fig. 8A). mRNA expression of NF-κB-regulated anti-apoptotic A1/Bfl-1 and cIAP2 is not induced by GCDCA, TCDCA and TUDCA as shown in Fig. 8B. NF-κB regulated inducible Nitric Oxide synthase (iNOS) was included as positive control. Finally, inhibition of NF-κB by recombinant adenovirus expressing dominant

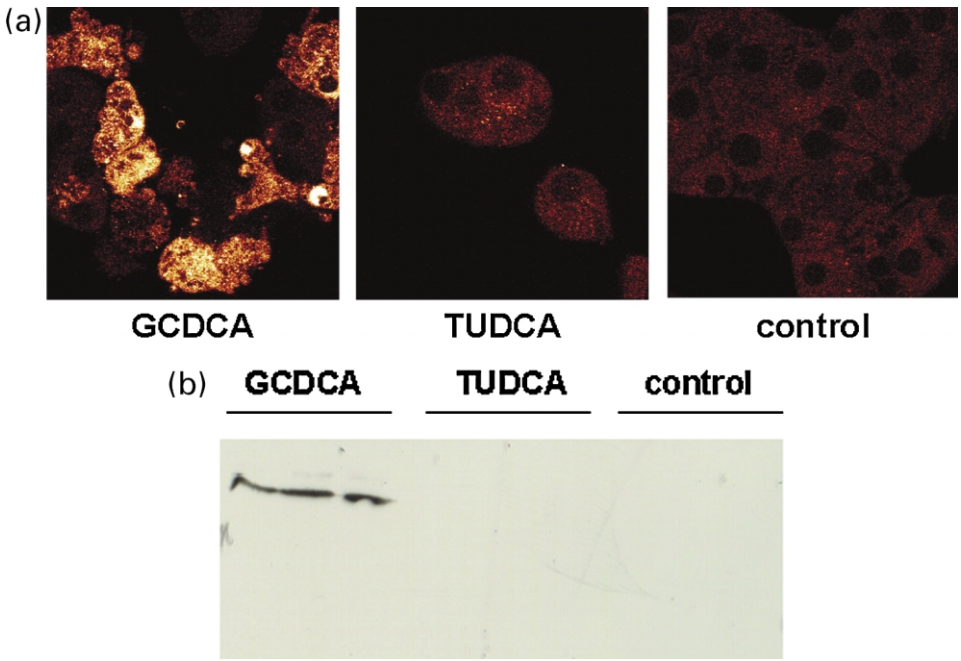

Fig. 7. Caspase-9 is activated in GCDCA-induced primary hepatocytes. (A) Immunocytochemistry; and (B) Western blot on primary hepatocytes after 4 h of exposure to GCDCA and TUDCA. One representative of three independent experiments is presented.

negative IκB did not increase GCDCA-induced caspase-3 activity and did not influence the effects of TUDCA and TCDCA on caspase-3 activity (Fig. 8C).

3.4. Cytokines inhibit glychochenodeoxycholic acid-induced apoptosis of hepatocytes

The protective role of cytokine-induced NF-κB activation against bile acid-induced apoptosis was investigated. Since in chronic cholestatic livers TNF-α, IL-1β, and IFN-γ are elevated (shown in Fig. 1C), we used these cytokines in vitro together with LPS. Primary hepatocytes were pre-incubated for different time intervals with cytokines prior to the addition of GCDCA. Pre-stimulation with cytokines for 3 h significantly inhibited GCDCA-induced apoptosis (Fig. 9A). As shown in Fig. 9B, adenoviral overexpression of the human homologue of rat cIAP2 in primary hepatocytes completely inhibited GCDCA-induced caspase-3 activation. These results indicate that cytokine-induced activation of NF-κB inhibits GCDCA-induced apoptosis in primary hepatocytes and that this protection is at least partly due to cIAP2.

4. Discussion

Bile acids are in part responsible for liver injury during cholestasis and apoptosis has been postulated as an important mechanism for this liver injury [25]. However, in the present study using three different methods to detect apoptosis, we could not demonstrate apoptotic hepatocytes in cholestatic livers. In previous studies [25], TUNEL staining was used to demonstrate apoptosis. Since the TUNEL assay frequently yields false-positive results [26], we have also used a more apoptosis-selective staining directed against active caspase-3 and an assay to measure caspase-3 activity. In contrast to previous studies investigating apoptosis only within 1 week after BDL [27,28], we investigated apoptotic cell death up to 4 weeks after BDL. We demonstrate that caspase-3 activity is only elevated within 1 week after BDL confirming previous results. However, after 1 week, caspase-3 activity rapidly declines to control levels. Taken these data together, we conclude that hepatocytes are well protected against apoptosis during cholestasis. Increased levels of ASAT and ALAT (Fig. 1) after bile duct ligation must be due to necrotic cell damage.

During cholestasis, the NF-κB pathway is activated [27] and we demonstrated in this report that the NF-κB-dependent anti-apoptotic genes A1 and cIAP2 [15] are highly induced in BDL livers. However, we found that none of the bile acids tested activates NF-κB in hepatocytes and that inhibition of NF-κB activation did not result in increased bile acid-induced apoptosis. This contrasts to reports describing NF-κB activation by TCDCA [12]. However, these latter results were obtained in hepatoma cells and therefore, conclusions from these studies cannot be extrapolated to primary hepatocytes.

Activation of the NF-κB pathway in cholestasis can be explained by the elevated expression of cytokines as part of the inflammatory response. We observed increased expression of TNF-α, IL-1β and IFN-γ mRNA in BDL livers. Furthermore, in TNF-receptor type I knock-out mice, the activation of NF-κB is not inhibited after BDL [27], again indicating that besides TNF-α, the onset of this

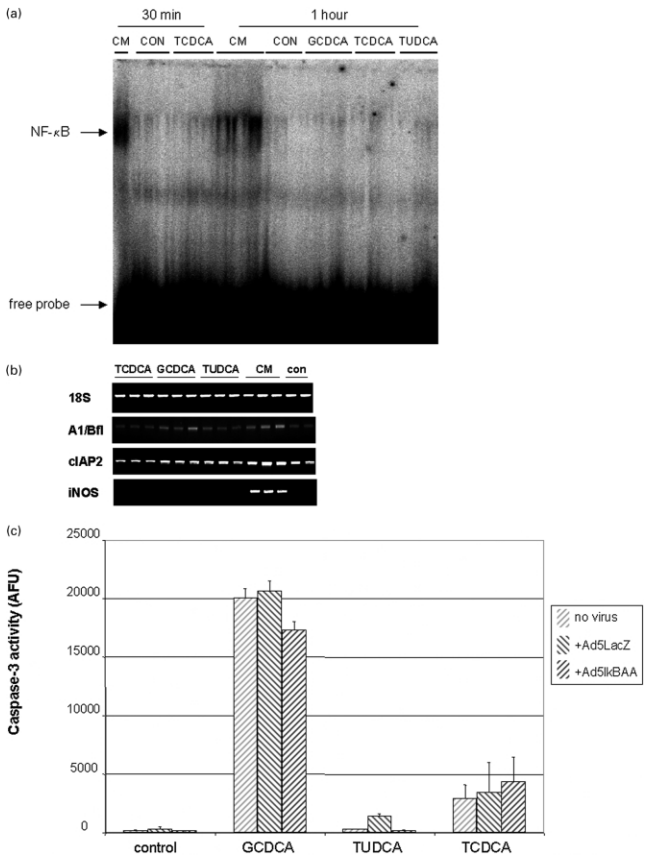

Fig. 8. Bile acids do not activate NF-κB in primary hepatocytes. See Section 2 for details. One representative (mean of $n = 3$ per condition) of three independent experiments is shown. (A) EMSA on nuclear extracts of hepatocytes treated for 30 min to 1 h with 50 μM GCDCA, TUDCA, TCDCA, or cytokines (CM). (B) mRNA expression of NF-κB-regulated genes (A1/Bfl and cIAP2) 10 h after incubation with bile acids or cytokines as indicated in the figure. 18S and iNOS mRNA served as internal control and positive control, respectively. (C) Caspase-3 activity, 4 h after bile acid incubation. Adenoviral expression of dominant negative IκB-α (Ad5IκBAA) was used to inhibit NF-κB. LacZ virus (Ad5LacZ) served as control.

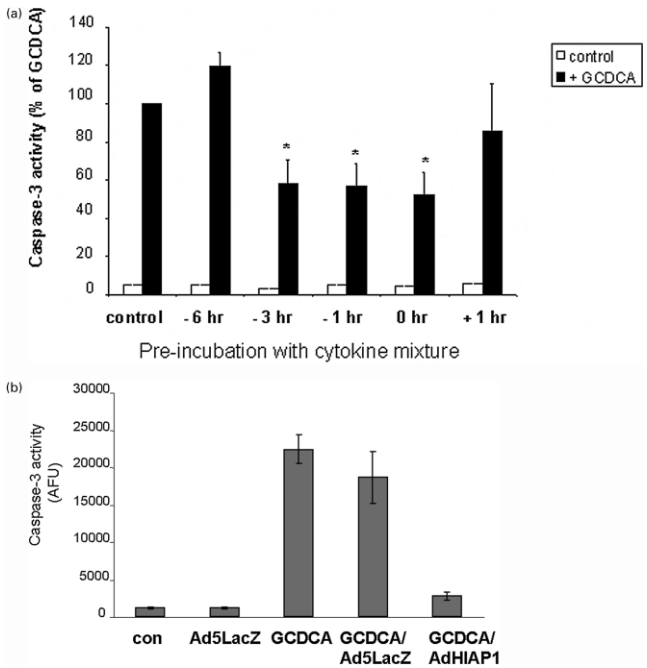

Fig. 9. Cytokines inhibit GCDCA-induced apoptosis. (A) Primary hepatocytes were pre-incubated for different time intervals (hours (h)) with cytokines (TNF-α, IL-1β, IFN-γ and LPS), before adding glycochenodeoxycholic acid (GCDCA). See Materials for details. Caspase-3 activity is presented as percentage of GCDCA alone. Data represent mean of three independent experiments ± SD. *$P < 0.05$ for −3, −1 and 0 h versus GCDCA alone. (B) Caspase-3 activity in primary hepatocytes exposed to GCDCA with or without adenoviral overexpression of the human homologue cIAP2 (AdHIAP1). LacZ virus served as control (Ad5LacZ). Representative data of three independent experiments are shown and presented as mean of $n = 3$ per condition.

survival pathway during cholestasis can be explained by IL-1β and IFN-γ [29,30]. A protective effect of NF-κB activation in cholestasis is supported by our observations that in vitro overexpression of cIAP2 inhibited GCDCA-induced apoptosis. Furthermore, cytokine-induced activation of NF-κB in hepatocytes prior to GCDCA exposure significantly reduced GCDCA-induced apoptosis. Therefore, we postulate that cytokine induced NF-κB-regulated A1/Bfl-1 and cIAP2 contribute to the protection of hepatocytes against bile acid-induced apoptosis during cholestasis.

We also observed a clear induction of the anti-apoptotic Bcl-2 in total liver homogenates. In contrast to studies describing Bcl-2 expression in hepatocytes [31], we found that this expression was restricted to bile duct epithelium and was not detected in hepatocytes in BDL livers. Our results are in accordance with studies reporting absence of Bcl-2 expression in hepatocytes in various liver diseases [32,33]. Therefore, we conclude that Bcl-2 is not involved in the protection of hepatocytes against bile acid-induced apoptosis.

The present report demonstrated that only GCDCA induces apoptosis in primary hepatocytes, whereas the taurine conjugates of CDCA and UDCA do not. An intestesting observation is the FADD-independent activation of caspase-8 and caspase-3 in GCDCA-exposed primary hepatocytes. Although dominant-negative FADD did completely abolish cytokine-induced apoptosis, we did not detect inhibition of GCDCA-induced apoptosis. This is in contrast to results obtained in hepatocytes exposed to DCA. Blocking of FADD did inhibit apoptosis in these cells [8]. However, in this study, apoptosis is induced by another unconjugated bile acid in the presence of a MAPK inhibitor. Not all bile acids induce apoptosis in hepatocytes: the glycine-conjugated bile acid GCDCA induces apoptosis, whereas the taurine-conjugated bile acids TUDCA and TCDCA do not. Therefore, it is likely that DCA behaves differently compared to glycine-conjugated CDCA. Contrary to our data using primary cultures of rat hepatocytes, others observed FADD-dependent apoptosis in rat

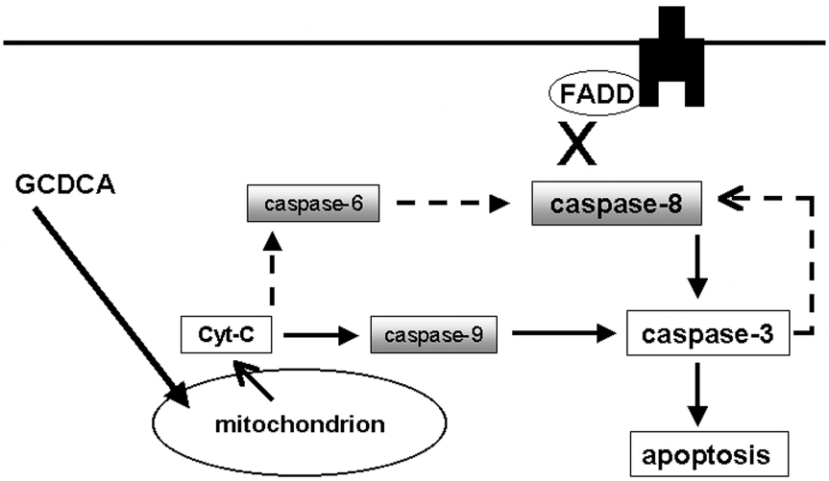

Fig. 10. Mechanisms of GCDCA-induced apoptosis in primary rat hepatocytes. GCDCA induces apoptosis in a mitochondria-controlled pathway in which caspase-8 is activated in a FADD-independent manner. See Section 4 details. Dotted arrows are postulated events in primary hepatocytes.

hepatoma cells [7]. However, it is known that the regulation of cell survival and apoptosis in continuously proliferating hepatoma cells is different from non-proliferating primary non-transformed hepatocytes. In cancer cell lines like Jurkat and Burkitt's lymphoma, caspase-8 is activated in a FADD-independent manner [35,36]. Besides FADD, another adaptor protein could be involved in caspase-8 activation, e.g. FLASH, a protein that interacts with a dead-effector domain of caspase-8. Whether this protein is also present in hepatocytes has to be clarified [36]. A role for Fas in GCDCA-induced apoptosis has been proposed using hepatocytes from Fas-deficient mice [7]. These and other authors also demonstrated GCDCA-induced Fas trafficking and aggregation in rat hepatoma cells [7,34]. Our data demonstrating FADD-independent apoptosis of GCDCA appear to rule out an important role of Fas in GCDCA-induced apoptosis in primary cultures of rat hepatocytes. At present, apart from differences in species and cell type, we have no explanation for these discrepant results. Another explanation for FADD-independent activation of caspase-8 after exposure to GCDCA is postmitochondrial processing of caspase-8. Indeed, one of the key findings of our study is that caspase-8 inhibition had no effect on GCDCA-induced caspase-3 activation. Hepatocytes are type II cells in which formation of the death inducing signaling complex is strongly reduced [37,38]. In type II cells, apoptosis is dependent on the release of pro-apoptotic factors from mitochondria, which activate caspase-9 and subsequent caspase-3 activation. Recently, it has been demonstrated that caspase-6 is the direct activator of caspase-8 in the cytochrome c-induced apoptosis pathway [39]. We demonstrated GCDCA-induced caspase-9 activation, indicating the release of pro-apoptotic factors from mitochondria. Therefore, postmitochondrial activation of caspase-8 is very plausible and in accordance with existing literature [39,40,41]. This hypothesis is supported by two facts: mitochondria play a key role in bile acid-induced apoptosis [42] and GCDCA-induced apoptosis is inhibited by blockers of the mitochondrial permeability transition [11]. In summary, we postulate that in primary hepatocytes, GCDCA induces apoptosis in a mitochondria-controlled pathway in which caspase-8 is activated in a FADD-independent manner (Fig. 10).

Overall, we conclude that hepatocytes are protected against bile acid-induced apoptosis during cholestatic liver injury due to induction of cytokine-induced, but not bile acid-induced, NF-κB-dependent anti-apoptotic genes like A1 and cIAP2. These findings are relevant for the treatment of patients with inflammation inhibitors resulting in a-selective inhibition of NF-κB.

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