

BASIC STUDIES

Effect of bile duct ligation on bile acid composition in mouse serum and liver

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Keywords

bile acid toxicity – bile duct ligation – inflammation – obstructive cholestasis

Abbreviations

BA, bile acid; Bcrp, breast cancer resistance protein; Bsep, bile salt export pump; CA, cholic acid; CA7S, cholic acid 7-sulphate; CDCA, chenodeoxycholic acid; CDCA7S, chenodeoxycholic acid 7-sulphate; Conj BA, conjugated bile acid; Cyp, cytochrome P450; DCA, deoxycholic acid; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; GCA, glycocholic acid; CA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; HDCA, hyodeoxycholic acid; HO-1, heme oxygenase 1; Icam-1, intercellular adhesion molecule 1; IL, interleukin; LCA, lithocholic acid; α MCA, α -muricholic acid; β MCA, β -muricholic acid; ω MCA, ω -muricholic acid; MDCA, murideoxycholic acid; Mip-2, macrophage-inflammatory protein-2; Mrp, multidrug resistance-associated protein; Ntcp, Na⁺-taurocholate cotransporting polypeptide; Oatp, organic anion transporting polypeptide; Ost, organic solute transporter; Rpl13a, ribosomal protein L13a; Serpine1, serpin peptidase inhibitor clade Ememmer 1; Spp1, secreted phosphoprotein 1; TCA, taurocholic acid; TCDCa, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; THDCA, taurohyodeoxycholic acid; TLCA, tauroolithocholic acid; T α MCA, tauro- α -muricholic acid; T β MCA, tauro- β -muricholic acid; T ω MCA, tauro- ω -muricholic acid; TMDCA, tauromurideoxycholic acid; TNF α , tumour necrosis factor alpha; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid; Unconj BA, unconjugated bile acid; Vcam-1, vascular cell adhesion molecule 1.

Abstract

Background: Cholestatic liver diseases can be caused by genetic defects, drug toxicities, hepatobiliary malignancies or obstruction of the biliary tract. Cholestasis leads to accumulation of bile acids (BAs) in hepatocytes. Direct toxicity of BAs is currently the most accepted hypothesis for cholestatic liver injury. However, information on which bile acids are actually accumulating during cholestasis is limited. **Aim:** To assess the BA composition in liver and serum after bile duct ligation (BDL) in male C57Bl/6 mice between 6 h and 14 days and evaluate toxicity of the most abundant BAs. **Results:** Bile acid concentrations increased in liver (27-fold) and serum (1400-fold) within 6 h after surgery and remained elevated up to 14 days. BAs in livers of BDL mice became more hydrophilic than sham controls, mainly because of increased 6 β -hydroxylation and taurine conjugation. Among the eight unconjugated and 16 conjugated BAs identified in serum and liver, only taurocholic acid (TCA), β -muricholic acid (β MCA) and T β MCA were substantially elevated representing >95% of these BAs over the entire time course. Although glycochenodeoxycholic acid and other conjugated BAs increased in BDL animals, the changes were several orders of magnitude lower compared with TCA, β MCA and T β MCA. A mixture of these BAs did not cause apoptosis or necrosis, but induced inflammatory gene expression in cultured murine hepatocytes. **Conclusion:** The concentrations of cytotoxic BAs are insufficient to cause hepatocellular injury. In contrast, TCA, β MCA and T β MCA are able to induce pro-inflammatory mediators in hepatocytes. Thus, BAs act as inflammagens and not as cytotoxic mediators after BDL in mice.

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Cholestatic liver diseases can be caused by genetic defects, drug toxicities, hepatobiliary malignancies or bile duct obstruction by gallstones (1). Acute and chronic cholestasis causes hepatocellular injury, bile duct proliferation, fibrosis, cirrhosis and eventually liver failure (2). The reduction or complete blockage of bile flow during cholestasis results in the hepatic retention of products normally excreted into bile, such as cholesterol and bile acids (BAs). Some of the BAs, especially hydrophobic BAs, can cause cell injury in cultured hepatocytes (3). Therefore, the predominant hypothesis of the mechanism of hepatocellular injury during cholestasis assumes that BAs accumulating in hepatocytes are the main cause of cell death (3). Although BAs such as tauroolithocholic acid (TLCA), deoxycholic acid (DCA), glycodeoxycholic acid (GDCA) and glycochenodeoxycholic acid (GCDCA) can cause necrosis through promotion of mitochondrial oxidant stress (4), the more recent focus was on apoptotic cell death by these BAs (3, 5). Consistent with a role of apoptotic cell death, exposure of a hepatocyte cell line to GCDCA stimulates apoptosis that is prevented by the pan-caspase inhibitor, z-VAD-FMK (6). Pretreatment of mice with z-VAD-FMK, however, does not prevent hepatocellular injury after bile duct ligation (BDL), an animal model of cholestasis, indicating that apoptosis is not important for liver injury *in vivo* during obstructive cholestasis (7). In addition, neither in rat nor mouse models of BDL was there morphological evidence of apoptosis or activation of caspases (7–10). The reason for the discrepancy between *in vitro* and *in vivo* studies is not fully understood, however, *in vitro* studies generally involve high concentrations of a specific BA whereas hepatocytes *in vivo* are exposed to a mixture of pro- and anti-apoptotic BAs (9). Most importantly, because of the limited knowledge what specific BAs accumulate in hepatocytes or serum during cholestasis, the selection of BAs for *in vitro* studies is more based on achieving a toxic effect rather than on which BAs hepatocytes are exposed to *in vivo*.

In contrast to direct cytotoxicity by BAs, it was hypothesized that hepatocellular injury during cholestasis results from an inflammatory response initiated by pro-inflammatory mediators released from hepatocytes exposed to pathological concentrations of BAs (11). Consistent with this hypothesis, cholestatic liver injury is substantially reduced in bile duct-ligated mice that are deficient in CD18 or intercellular adhesion molecule-1 (ICAM-1) (12, 13). These findings suggest that most of the injury is actually caused by neutrophils and not directly by BAs. Furthermore, exposure of primary mouse hepatocytes to taurocholic acid (TCA) does not cause cell death, but upregulates ICAM-1 and stimulates release of pro-inflammatory chemokines, both of which are important for neutrophil-dependent injury during cholestasis (11).

In both hypotheses, it is proposed that BAs are the key mediators initiating apoptosis or instigating production of inflammatory mediators. Whether concen-

trations of BAs that stimulate these processes are increased *in vivo* during cholestasis, however, is not known. Accordingly, the purpose of the present study was to quantify concentrations of individual BAs in serum and livers of mice subjected to bile duct ligation, and to assess if the BAs with the highest concentrations affect viability of hepatocytes. This information could provide important reference data for the interpretation of past *in vitro* studies and for future *in vivo* and cell culture experiments aimed at studying hepatocellular injury during cholestasis.

Materials and methods

Chemicals and reagents

Bile acid standards were either purchased from Steraloids, Inc. (Newport, RI, USA), Sigma-Aldrich (St Louis, MO, USA), or synthesized in our lab (14). All other chemicals, unless indicated, were purchased from Sigma-Aldrich.

Animal experiments

Adult male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All mice were fed a Teklad Rodent Diet #8604 (Harlan Laboratories, Madison, WI, USA) *ad libitum*, and housed according to the American Animal Association Laboratory Animal Care guidance. Studies were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee. Under Nembutal anaesthesia (10% Nembutal in saline), the abdominal cavity was opened and the common bile duct was ligated twice with 5-0 surgical silk, and the bile duct was cut between the ligatures. The gall bladder was left intact. The abdominal muscle was sutured with Ethicon 5-0 dissolvable suture material and the wound was closed with surgical staples. Sham surgeries were performed similarly without BDL. Serum and livers (gallbladder removed) were collected 6 h, 12 h, 1, 2, 3, 5, 7 and 14 days after BDL. Livers were snap frozen in liquid nitrogen and stored at -80°C until analysis.

Bile acid extraction and quantification

Serum BA extraction and quantification were described previously (15). Liver BA concentrations were quantified by a recent method using ultra performance liquid chromatography – tandem mass spectrometry (UPLC/MS/MS) (14). BA stock solutions were diluted with 50% methanol and spiked with internal standards ($^2\text{H}_4$ -GCDCA and $^2\text{H}_4$ -CDCA) to construct standard curves between 5 and 20 000 ng/ml. All standard curves were constructed using a $1/\text{concentration}^2$ weighted quadratic regression, and the correlation coefficient (r^2) for all BAs was above 0.99. The limit of detection (signal/noise ratio = 3) for the various BAs was in the range of

5–10 ng/ml, which equals 0.01–0.02 nmol/ml. For a preliminary analysis of hydroxylated BA, we obtained taurine-conjugated and unconjugated 3 α , 6 α , 7 α , 12 α -tetrahydroxyl BA from Dr Mary Vore (University of Kentucky, Lexington, KY, USA). We optimized and obtained the multiple reaction monitoring (MRM) conditions for these two standards. Then we used the same MRM conditions to obtain chromatographs of tetrahydroxy BAs in BDL samples.

Total RNA isolation

Total RNA was isolated using RNA-Bee reagent (Tel-Test Inc., Friendswood, TX, USA) according to the manufacturer's protocol. Total RNA concentrations were quantified spectrophotometrically at 260 nm. One microgram per microlitre solutions were prepared by dilution in diethyl pyrocarbonate-treated deionized water. Integrity of RNA samples was determined by formaldehyde-agarose gel electrophoresis with visualization by ethidium bromide fluorescence under ultraviolet light.

Multiplex suspension array

Liver mRNA was quantified by multiplex suspension array (Panomics-Affymetrix, Inc., Fremont, CA, USA). Individual gene accession numbers can be accessed at www.panomics.com (sets #21021 and #21151). Samples were analysed using a Bio-Plex 200 System Array reader with Luminex 100 xMAP, and the data were acquired using Bio-Plex Data Manager version 5.0 (Bio-Rad, Hercules, CA, USA). Assays were performed according to each manufacturer's protocol. During the preliminary experiment, the mRNA of three housekeeping genes, namely ribosomal protein 13a (Rpl13a), glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and beta-actin (β -actin) were quantified in livers of BDL mice. BDL did not alter Rpl13a or Gapdh, but increased β -actin in mouse livers. Rpl13a in BDL mice appeared to be more consistent than Gapdh. Therefore, RNA data in the present study are presented as relative light units (RLU) normalized to Rpl13a mRNA.

Bile acid toxicity in isolated hepatocytes

Hepatocytes were isolated from C57BL/6 mice and treated with a mixture of bile acids for 6 h as described (11). The bile acids used in the mixture were 341 μ M β MCA, 1 mM T β MCA and 1 mM TCA. This approximates the serum concentrations measured at 6 h after BDL. Only bile acids with concentrations >50 μ M were considered and included in the mixture. As positive control for caspase-dependent apoptosis, cells were treated with 5 mM galactosamine and 100 ng/ml recombinant murine TNF- α (Genzyme, Cambridge, MA, USA) for 6 h (16). Caspase-3 activity based on Z-VAD-fmk inhibitable Ac-DEVD-AMC fluorescence was measured as described (17) and alanine aminotransferase (ALT)

activity was determined in the cells and in the culture supernatant using a commercial kit (18). The mRNA levels of proinflammatory genes in cultured hepatocytes were determined as described in detail (11).

Statistical analysis

Data are represented as mean \pm SEM ($n = 5$ –6). Differences between mean values were tested for statistical significance ($P < 0.05$) by the two tailed Student's *t*-test.

Results

Serum BA concentrations

Total BA concentrations in mouse serum were quantified at 0 h, 6 h, 12 h, 1, 2, 3, 5, 7 and 14 days after sham and BDL surgeries (Fig. 1). Sham-operated mice maintained their serum BA concentrations in a range of 0.5–2.3 nmol/ml during the study. After BDL, unconjugated and conjugated BAs in mouse serum increased 300- and 4000-fold, respectively, within 6 h. Thereafter, unconjugated BAs in serum of BDL mice decreased about 99% between 6 h and 14 days, but were still about three-fold higher than sham-operated mice at 14 days. In contrast, conjugated BAs in serum of BDL mice decreased about 60% between 6 h and 1 day followed by a tripling between 1 and 5 days, and were maintained at concentrations of \sim 3000 nmol/ml thereafter. Two weeks after surgery, more than 99% of BAs in serum of BDL mice were conjugated, whereas only 60% of BAs in serum of sham-operated mice were conjugated. Thus, changes in total BA concentration in serum of BDL mice followed a similar kinetics as conjugated BAs (Fig. 1).

The major primary BAs in serum of sham-operated mice were CA, TCA, α/ω MCA, β MCA and TMCA, which all ranged in concentration between 0.2 and 1.4 nmol/ml (Fig. 2A). After BDL, most primary BAs, except GCDCA, underwent a marked increase at 6 h followed by a rapid decrease from 6 h to 1 day. After that, serum CA and CDCA in BDL mice decreased to the sham level, whereas α/ω MCA, β MCA and GCA were maintained at slightly higher concentration than sham controls. In contrast, TCA, TCDCA and TMCA increased markedly from 1 to 5 days. As a result, after 1 day of BDL, TCA and TMCA are the predominant conjugated BAs identified in mouse serum.

Figure 2B shows the time course of secondary BAs in serum of mice after surgery. The predominant secondary BAs in serum of sham-operated mice were TDCA (0.02–0.05 nmol/ml) and UDCA (0.09–0.13 nmol/ml). BDL had little effect on DCA, TDCA, LCA and UDCA levels, although it increased TDCA and TLCA mainly at 6 h after surgery. Interestingly, BDL caused a marked increase in TUDCA concentrations at 6 h; this was followed by a decrease of TUDCA levels from 12 h to 2 days and another increase thereafter (Fig. 2B). Therefore, after BDL, TUDCA became the predominant sec-

ondary BA in mouse serum with levels between 10- and 100-fold higher than all other secondary BAs. Whereas the early spike of secondary BAs at 6 h may be because of absorption of residual BAs from the intestine and reduced clearance by the liver, the later increase in UDCA may originate from the liver. Consistent with previous data (14), glucuronidation and sulphation are only minor metabolic pathways for BAs in livers of mice also after BDL (data not shown).

Liver BA concentrations

Sham-operated mice maintained their liver BAs in a range of 55–254 nmol/g liver weight throughout the study, with about 80% of the BAs conjugated (Fig. 3). At 6 h after BDL, unconjugated and conjugated BAs increased about 26- to 27-fold in the liver. Thereafter, unconjugated BAs gradually decreased between 12 h and 14 days after BDL with levels at

14 days being 90% lower than peak levels. In contrast, the hepatic content of conjugated BAs remained close to peak levels throughout the entire time course. As a result, the percentage of conjugated BAs in livers of BDL mice increased from 80% at 6 h to 99% at 14 days. Mainly because of the increase in conjugated BAs, the total BA concentration in livers of mice increased about 27-fold at 6 h after BDL, and remained at 70–80% of peak levels during the course of the study (Fig. 3).

The major primary BAs in livers of sham-operated mice were TCA and T β MCA (Fig. 4A). After 6 h of BDL, major increases in BA levels were observed for CA (nine-fold), GCA (14-fold) and TCA (17-fold). CA and GCA decreased to sham levels within 2 days after BDL, whereas TCA underwent a decrease from 6 h to 1 day, but was maintained at higher concentrations than sham controls thereafter. BDL had little effect on CDCA and GCDCA, whereas it gradually increased TCDCA concentrations about four-fold within 2 weeks. After BDL, the concentration of α MCA in the liver underwent a three-fold increase at 6 h, followed by a decrease to sham concentrations at 12 h, and became lower than sham controls after 7 days. In contrast, T α MCA in livers of mice increased about 12-fold at 6 h after BDL, and remained at higher levels than sham controls thereafter. After BDL, β MCA increased about 35-fold at 6 h, and thereafter decreased gradually to similar concentrations as in livers of sham-operated mice after 7 days. In contrast, T β MCA in livers of BDL mice underwent a 150-fold increase at 6 h, followed by a 45% decrease from 6 h to 1 day, and increased gradually about 1.5-fold from 1 to 14 days. TCA7S and TCDCA7S were the two predominant BA sulphates in livers of sham-operated mice. After BDL, both TCA7S and TCDCA7S increased about 17-fold during the first 3 days, but thereafter decreased rapidly to similar concentrations as in the livers of sham-operated mice. Therefore, sulphation is not a prominent pathway of BA metabolism in livers of BDL mice. Although nine primary BAs were identified in livers of BDL mice, TCA, β MCA and T β MCA together are the predominant primary BAs identified throughout the study (Fig. 4A).

The predominant secondary BAs in livers of sham-operated mice were T ω MCA and TDCA (Fig. 4B). BDL had little effect on DCA concentration in liver, but significantly decreased TDCA, LCA and TLCA after 1 day. Within 6 h after BDL, UDCA, HDCA and MDCA increased about five- to eight-fold, whereas TUDCA, THDCA and TMDCA increased about 27- to 66-fold in livers of mice. Among these secondary BAs, UDCA, HDCA, THDCA, MDCA and TMDCA decreased to similar concentrations as sham controls within 1 day and became lower than sham controls after 5 days. In contrast, TUDCA underwent a 75% decrease between 6 and 12 h, but remained at higher levels than sham controls thereafter. After BDL, ω MCA increased about 18-

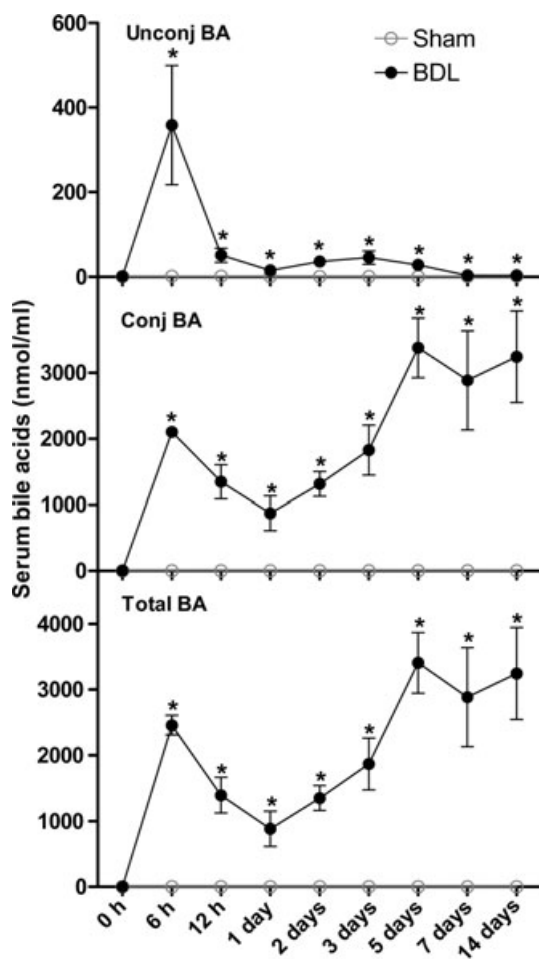


Fig. 1. Unconjugated -, conjugated - and total bile acid (BA) concentrations in serum of sham-operated controls and bile duct-ligated (BDL) mice. All BA data are expressed as mean \pm SE of six mice in each group. * $P < 0.05$ [sham-operated controls (open circles) vs. BDL mice (closed circles)].

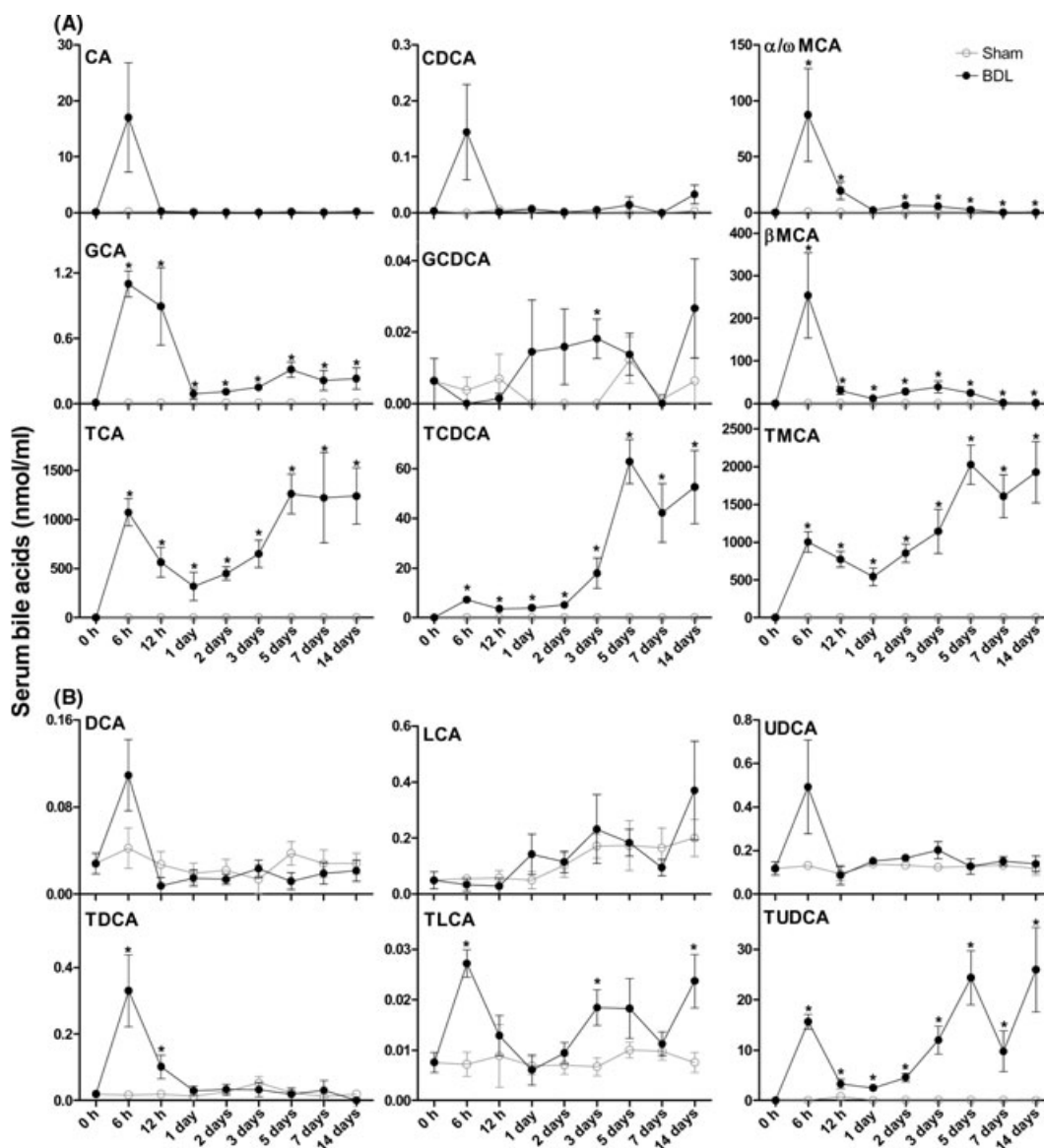


Fig. 2. Individual primary bile acids (BAs) (A) and secondary BAs (B) in serum of sham-operated controls and bile duct-ligated (BDL) mice. All BA data are expressed as mean \pm SE of six mice in each group. * $P < 0.05$ [sham-operated controls (open circles) vs. BDL mice (closed circles)].

fold at 6 h, and decreased rapidly to become lower than sham controls after 5 days. In contrast, T ω MCA in livers of BDL mice increased about 46-fold at 6 h, decreased thereafter, but remained about three-fold higher than sham controls at the end of 2 weeks. Therefore, the predominant secondary BAs in livers of BDL mice were TUDCA, ω MCA and in particular T ω MCA (Fig. 4B).

BA transporters and synthetic enzymes

The impact of BDL on mRNA expression of BA-uptake and – efflux transporters and enzymes involved in BA

synthesis were determined (Fig. S1–S3). In general, the BA-uptake transporters Ntcp, Oatp1a1, Oatp1a4 and Oatp1b2 in mouse liver showed a minor increase at 6 h after BDL, but then were consistently down-regulated (except Oatp1a4) during chronic BDL (Fig. S1). In contrast, BDL had little effect on the canalicular BA-efflux transporters, such as Bsep, Mrp2 and Bcrp (Fig. S2). However, BDL had limited effects on Ost α (data not shown) or Mrp3, whereas it significantly increased Ost β and Mrp4 (Fig. S2). Cyp7a1 is the rate-limiting enzyme of BA synthesis. Cyp7a1 appeared to vary markedly in livers of sham-operated mice and no consistent difference to BDL livers was observed (Fig. S3). In contrast,

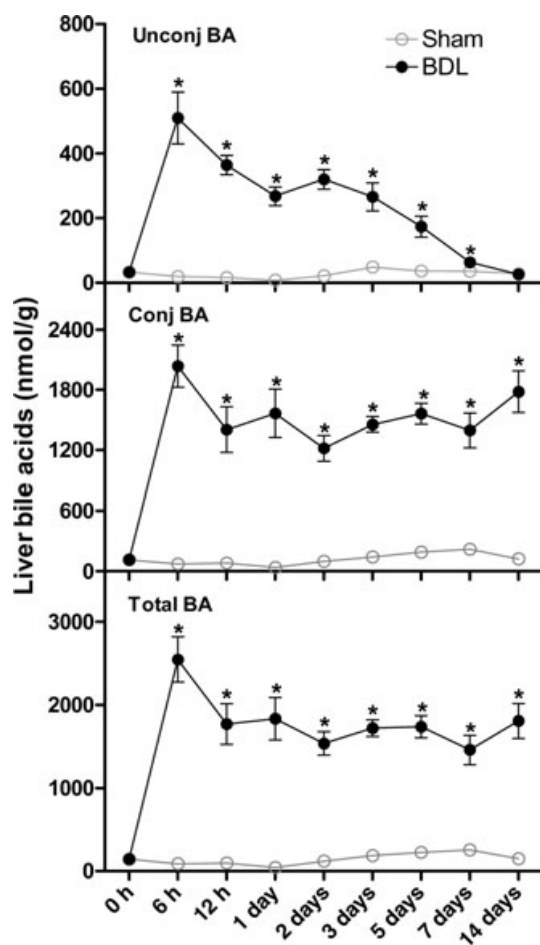


Fig. 3. Unconjugated -, conjugated - and total bile acid (BA) concentrations in livers of sham-operated controls and bile duct-ligated (BDL) mice. All BA data are expressed as mean \pm SE for six mice in each group. * $P < 0.05$ [sham-operated controls (open circles) vs. BDL mice (closed circles)].

Cyp8b1, which is the critical enzyme (12 α -hydroxylase) for CA biosynthesis and Cyp7b1, which is a critical enzyme for the alternative pathway of BA biosynthesis, were down-regulated at all times after BDL. However, Cyp27a1, which is involved in both the classic and alternative pathways of BA biosynthesis, was largely unaffected by BDL (Fig. S3).

Liver inflammation

The mRNA expression of ten pro-inflammatory markers TNF α , Spp1, IL-1 α , IL-1 β , MIP-2, VCAM-1, ICAM-1, Selectin, Serpin1 and HO-1 are shown in Figure 5. After BDL, TNF α (2.5-fold) and Spp1 (six-fold) increased from 1 to 5 days, and remained at higher levels than sham controls thereafter. Similarly, IL-1 α and IL-1 β in livers of BDL mice increased from 3 to 5 days, and remained at higher levels than sham controls thereafter. In contrast, MIP-2 in livers of BDL mice was about 25-fold higher than sham controls 6 h after sur-

gery. VCAM-1 in BDL mice increased about 150% at 5 days and remained at higher levels than sham controls thereafter. In contrast, ICAM-1, Selectin, Serpin1 and HO-1 in BDL mice underwent an initial increase at 6 h and a second increase at 5 days. Taken together, BDL resulted in an early and sustained increase of inflammation markers in mouse livers (Fig. 5).

Proinflammatory effects of bile acids

Although our previous study demonstrated that 200 μ M TCA, DCA or CDCA induced chemokines in cultured mouse hepatocytes without causing cell death (11), our current studies indicate that TCA, T β MCA and β MCA increase to levels between 300 and 1000 μ M in serum at 6 h after BDL (Fig. 2). Therefore, we tested if a mixture of the three bile acids with the highest concentrations were able to cause cell injury or induce pro-inflammatory genes (Fig. 6). Compared with cells treated with vehicle (saline), cells exposed to a mixture of TCA (1 mM), T β MCA (1 mM) and β MCA (341 μ M) did not show morphological evidence of injury (Fig. 6A) and did not increase caspase-3 activity (Fig. 6B) or release ALT (Fig. 6C). Treatment with Gal/murine TNF- α , a positive control for apoptosis, resulted in an 11-fold increase of caspase-3 activity (Fig. 6B). In contrast, the bile acid mixture induced the pro-inflammatory genes MIP-2 (21-fold), KC (>three-fold) and ICAM-1 (>three-fold) in hepatocytes (Figs 6D–F).

Discussion

The objective of this study was to analyse the BA composition in serum and liver after BDL, with the major goal to establish which BAs actually increase during BDL, and to what concentrations of these BAs hepatocytes are exposed to during obstructive cholestasis. Applying this information, murine hepatocytes were exposed to a mixture of BAs with the highest levels in serum and liver, and cell viability and inflammatory gene expressions were assessed. The data should provide an important reference point for past and future studies on mechanisms of hepatocellular injury during cholestasis.

The major BAs in serum and livers of sham-operated mice are TCA, β MCA and T β MCA. These particular BAs also show the highest increase after BDL. Previous studies on BA analysis using GC or GC-MS also identified CA and β MCA (including both conjugated and unconjugated) as the most abundant BAs in BDL rats (19, 20) and mice (21, 22). Semi-quantitative analysis indicated the most exclusive abundance of taurine conjugates in BDL mice with negligible amounts of glycine conjugates (21, 22). Interestingly, TUDCA, which is thought to be a secondary BA produced by intestinal bacteria (23), was present in livers of BDL mice at appreciable concentrations, suggesting that TUDCA may be synthesized by livers of BDL mice. The BA chromatograph suggests that livers of BDL

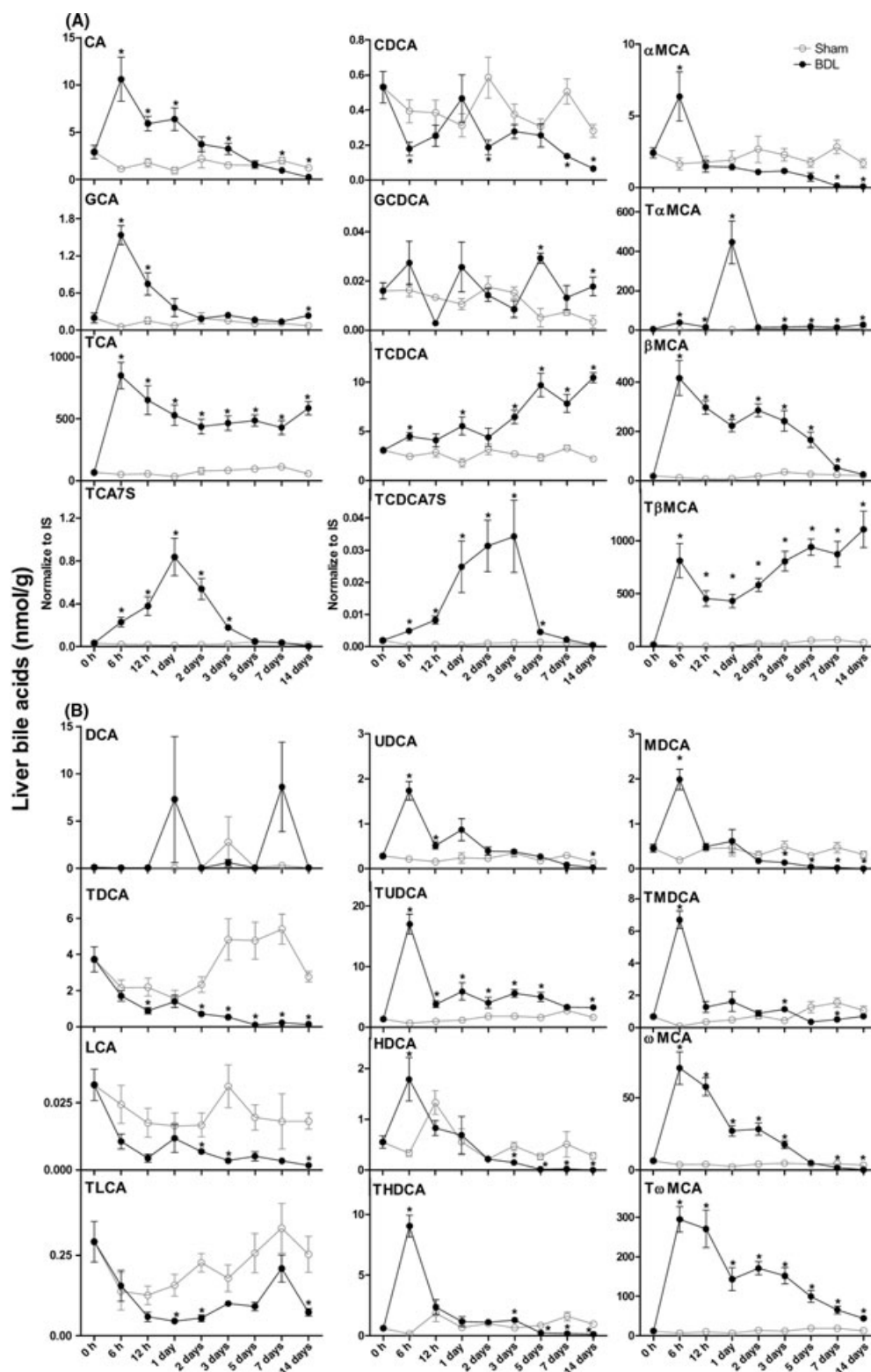


Fig. 4. Individual primary bile acids (BAs) (A) and secondary BAs (B) in livers of sham-operated controls and bile duct-ligated (BDL) mice. All BA data are expressed as mean \pm SE for six mice in each group. * $P < 0.05$ [sham-operated controls (open circles) vs. BDL mice (closed circles)].

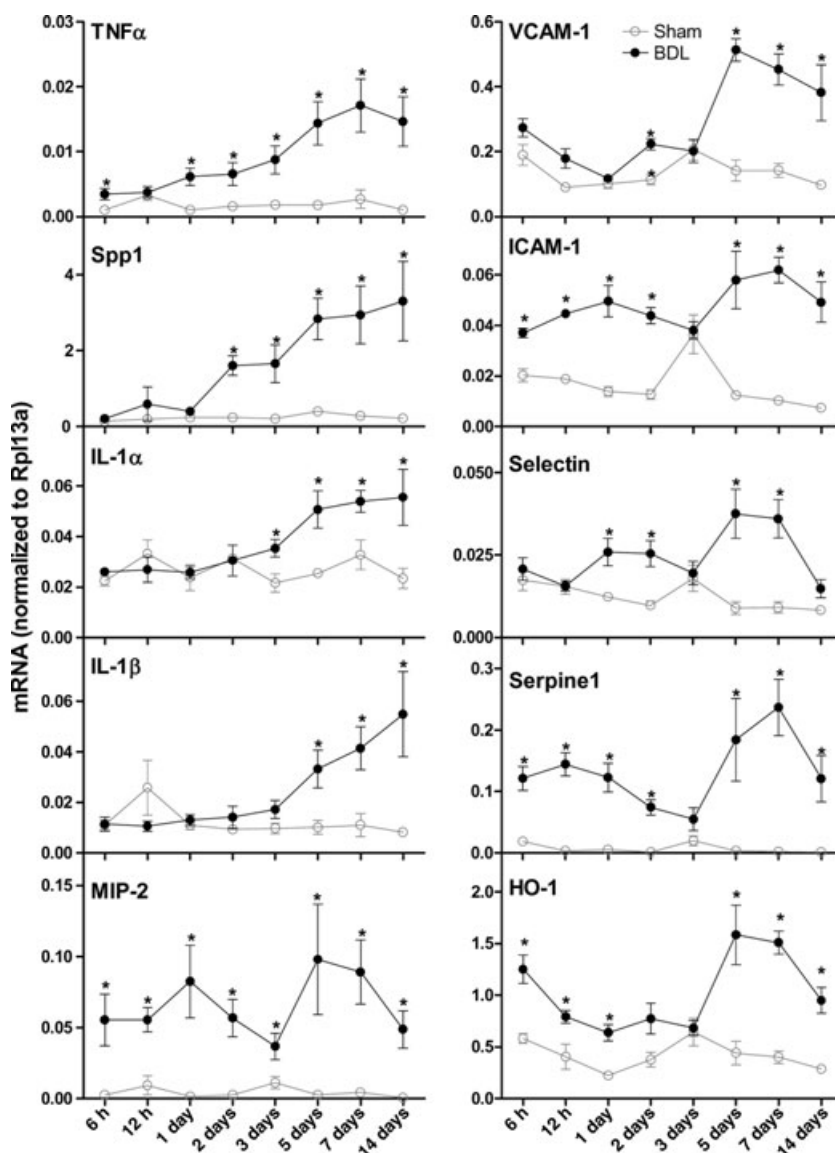


Fig. 5. The mRNA expression of inflammatory genes in livers of sham-operated controls and bile duct-ligated (BDL) mice. Total RNA from livers of sham-operated (open circles) and BDL (closed circles) mice were analysed by multiplex suspension array. The mRNA level of each gene was normalized to Rpl13a. All data are expressed as mean \pm SE for six mice in each group. * P < 0.05 [sham-operated controls (open circles) vs. BDL mice (closed circles)].

mice had a significant increase in polyhydroxy BAs, mainly taurine-conjugated tetrahydroxy BAs (data not shown). The accurate concentrations of polyhydroxy BAs were not given here because of lack of reference BAs, but the chromatograph peak area of one taurine-conjugated tetrahydroxy BA was increased about 300-fold in livers of BDL mice (data not shown). Marshall *et al.* (22) reported that polyhydroxy BAs were markedly elevated and consisted of about 6–10% of total BAs in serum and livers of wild-type mice 7 days after BDL. This suggests that hydroxylation is a pathway to detoxify high concentrations of trihydroxy BAs, such

as TCA and TMCAs in livers of BDL mice. Glucuronidation and sulphation are thought to be important to detoxify high concentrations of BAs in liver (24, 25). Consistent with a previous study (22), no significant differences were observed for the glucuronidated BAs between sham and BDL mice. Hofmann (26) has discussed the reasons why glucuronidation is not a major metabolic pathway for BAs. In the present study, sulphates of TCA and TCDCA in livers of BDL mice increased about 17-fold during the first 3 days, but thereafter decreased rapidly to similar concentrations as in the livers of sham mice. Thus, sulphation dose

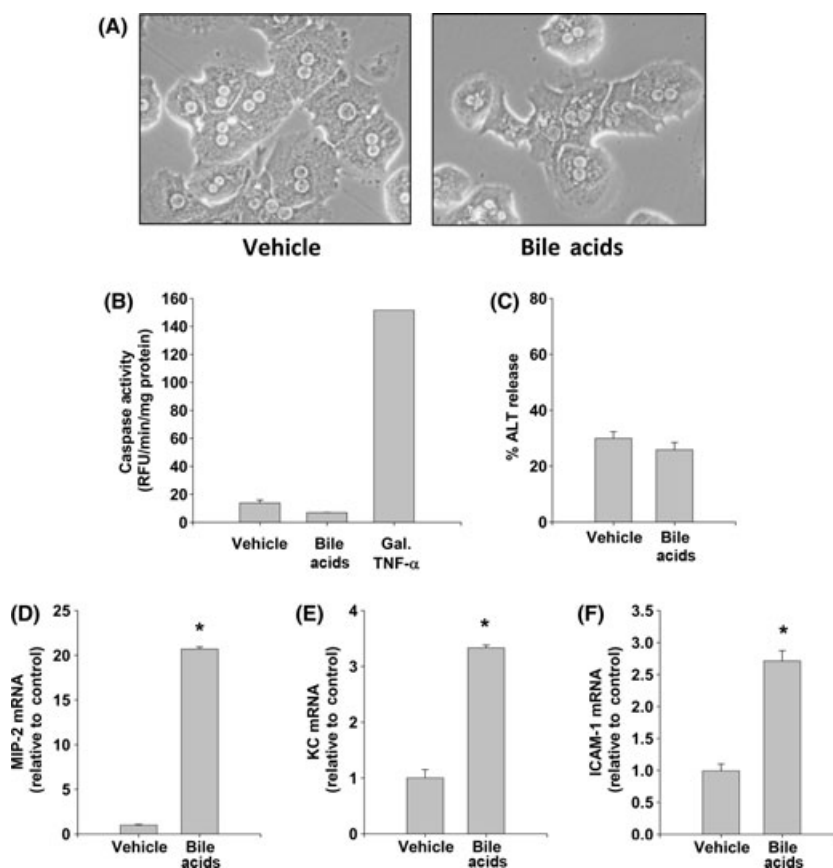


Fig. 6. Effect of a bile acid mixture on hepatocyte viability and inflammatory gene expression. Murine hepatocytes were isolated and treated with a mixture of 341 μ M β MCA, 1 mM T β MCA and 1 mM TCA (Bile Acids). As positive control for caspase-dependent apoptosis, cells were treated with 5 mM galactosamine (Gal) and 100 ng/ml recombinant murine TNF- α . Photomicrographs (200 \times) of cells treated with vehicle (saline) or bile acids (A) were taken 6 h after treatment. Activities of caspase 3 (B) and ALT (C) were measured at 6 h. mRNA levels of the CXC chemokines MIP-2 (D) and KC (E) and of the adhesion molecule ICAM-1 (F) were measured at 6 h. Data are expressed as mean \pm SE of $n = 3$ experiments. * $P < 0.05$ (compared to vehicle-treated hepatocytes).

not appear to be an important pathway of BA detoxification in livers of BDL mice. This is consistent with the previous studies in BDL rats (19, 27) and mice (22). Taken together, TCA, β MCA and T β MCA are the predominant BAs in serum and livers of BDL mice, and hydroxylation is the major pathway of BA detoxification in BDL mice.

A large number of studies suggest that during cholestasis BAs produce hepatocellular injury in part by inducing apoptosis (3). It is proposed that this occurs through exposure of hepatocytes to pro-apoptotic BAs, including TLCA, DCA, GDCA and GCDCA (3, 5). Exposure of hepatocytes to concentrations of 50 μ M or above of these BAs is typically needed to stimulate apoptosis in cultured rodent hepatocytes (28–32). The present study demonstrates, however, that maximal concentrations of these BAs in serum of BDL mice are approximately 1000-fold less (i.e. TLCA: 24 nM; DCA: 130 nM; GDCA: 13.8 nM; GCDCA: 27 nM) than that needed to stimulate apoptosis *in vitro*. Even the intracellular levels of DCA or TDCA never exceeded 10 nmol/g liver (roughly

10 μ M) and generally, the serum concentrations correlate closely with the tissue levels of all BAs. We showed previously that exposure to 200 μ M DCA did not cause cell injury in hepatocytes (11). Furthermore, serum concentrations of anti-apoptotic BAs (31–35) (i.e. TCDCAs: 83.5 μ M; TUDCA: 26 μ M) are approximately 1000-fold higher than pro-apoptotic BAs in serum of BDL mice. Accordingly in BDL mice, concentrations of pro-apoptotic BAs do not reach concentrations sufficient to stimulate hepatocyte apoptosis, indicating that prior *in vitro* studies do not accurately recapitulate what occurs in mice *in vivo*. In direct support of this conclusion, a mixture of the bile acids (TCA, T β MCA, β MCA) that reached the highest levels after BDL *in vivo* did not cause apoptosis or necrosis in isolated hepatocytes. Consistent with this, there is no evidence for caspase activation and relevant apoptotic cell death during BDL (7–10). Consequently, pan-caspase inhibitors do not affect hepatocellular injury after BDL (7).

Studies in BDL mice have demonstrated an important role for inflammation, especially neutrophils, in the

mechanism of liver injury during cholestasis (12, 13, 18, 36). It has been proposed that exposure of hepatocytes to pathological concentrations of BAs increases production of pro-inflammatory mediators that initiate an inflammatory response (11, 18). Consistent with this, treatment of primary mouse hepatocytes with 200 μ M TCA increased expression of cytokines, chemokines, adhesion molecules and other proteins that influence immune cell levels and function in the liver (11). Our present studies demonstrated that concentrations of TCA, T β MCA and β MCA were increased to >300–1000 μ M within 6 h after BDL. Exposure of isolated hepatocytes to a mixture of these *in vivo* relevant bile acids substantially induced pro-inflammatory genes including the CXC chemokines MIP-2 and KC and the adhesion molecule ICAM-1. Furthermore, mRNA levels of MIP-2 and ICAM-1 were significantly increased as early as 6 h after BDL *in vivo* consistent with previous findings (7). Both MIP-2 and ICAM-1 contribute to liver injury after BDL, and both are upregulated in primary mouse hepatocytes treated with 200 μ M TCA (11, 13, 37). Collectively, these results support the hypothesis that during cholestasis, exposure of hepatocytes to pathological concentrations of BAs increases expression of pro-inflammatory mediators that initiate an inflammatory injury process.

The assessment of BA-uptake and efflux transporter mRNA expression indicated that, in general, uptake transporters, Oatp1a1, Oatp1b2 and Nctp are down-regulated after 1 day of BDL compared to sham-operated animals, and alternative efflux transporters Mrp4 and Ost β are up-regulated. In addition, key enzymes regulating BA synthesis, Cyp8b1 and Cyp 7b1, are consistently down-regulated after BDL. These changes indicate an adaptive response to BDL, which includes reduced BA synthesis, reduced uptake into the liver and enhanced export into the blood. These data are consistent with previous observations showing similar changes in mRNA and protein expression in various models of cholestasis (21, 22, 38–44). Interestingly, the modulation of transporters appears to be independent of the inflammatory response, suggesting that the accumulating BAs may be mainly responsible for these effects (44). Although we were not able to quantify polyhydroxy BAs, which can make up to 6–10% of total BAs in serum and liver after BDL (22), the fact that these BAs are hydrophilic and represent detoxification products of TCA and TMCA makes it highly unlikely that these BAs could have a cytotoxic effect.

An important issue is the comparison of our data in mice to BA levels in humans (45). In mice TCA, β MCA and T β MCA are the most abundant BAs in serum. However, in human control serum, CA, CDCA and its glycoconjugate GCDCA and DCA and its glycoconjugate GDCA are found at similar concentrations of 0.3–0.8 μ M (45). Although the data for patients with cholestatic liver disease are not as detailed, total CDCA and CA (conjugated and unconjugated) show

the highest increase in liver tissue after obstructive cholestasis (46). The levels reported in this study (70–400 nmol CDCA/g liver and 200–600 nmol CA/g liver) (46) are similar to the total CA levels, which are mostly TCA, quantified after BDL in mice (Fig. 4). The major difference between humans and mice is the presence of high levels of MCA and very low concentrations of CDCA in mice. In patients with primary biliary cirrhosis, elevated serum levels of CA and CDCA have been reported (47, 48). Levels of 30–40 μ M of both CA and CDCA were measured in patients with the most severe disease status; the glycoconjugate representing 50–55% and the taurine conjugate 40–45% of the total CA and CDCA levels in serum (48). Although these clinical data provide the rationale for the use of 50 μ M GCDCA in many *in vitro* studies (28–32), the mechanism of BA toxicity was investigated in rodent, but not human hepatocytes. It takes even higher concentrations (500 μ M) of GCDCA to cause significant cell injury in human hepatocytes (49). Thus, serum GCDCA concentrations measured under severe cholestatic conditions in humans may approach concentrations used in cell culture studies, and may cause some direct toxicity. It is quite obvious from our studies that mouse hepatocytes are never exposed to μ M concentrations of GCDCA and other potentially toxic BAs. It is therefore justified to conclude that a direct cytotoxic effect of BAs in the mouse BDL model is of limited relevance.

In summary, our detailed analysis of BA composition changes in liver and serum between 6 h and 14 days after BDL indicates that the concentration of only a limited number of mainly conjugated BAs such as TCA, β MCA and T β MCA are substantially elevated. These BAs consistently are the predominant BAs in serum and liver over the entire time course of observation. Although GCDCA and other conjugated BAs can increase in BDL animals compared with sham-operated controls, the changes are several orders of magnitude lower compared with TCA and T β MCA. These findings suggest that during BDL, the concentrations of cytotoxic BAs are insufficient to cause hepatocellular injury. In contrast, concentrations of TCA and T β MCA quantified in BDL animals are able to induce pro-inflammatory mediators in hepatocytes. Thus, based on this detailed BA composition analysis and the exposure of hepatocytes to pathophysiologically relevant BAs, we conclude that BAs act mainly as inflammagens and not as cytotoxic mediators, in this murine model of obstructive cholestasis.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The mRNA levels of bile acid uptake transporters in livers of sham-operated controls and bile duct-ligated (BDL) mice. Total RNA from livers of sham-operated (open circles) and BDL (closed circles) mice were analysed by multiplex suspension array. The mRNA level of each gene was normalized to Rpl13a. All data are expressed as mean \pm SE for six mice in each group. * $P < 0.05$ [sham-operated controls (open circles) vs. BDL mice (closed circles)].

Fig. S2. The mRNA expression of canalicular (A) and basolateral (B) bile acid efflux transporters in livers of sham-operated controls and bile duct-ligated (BDL) mice. Total RNA from livers of sham-operated (open circles) and BDL (closed circles) mice were analysed by multiplex suspension array. The mRNA level of each gene was normalized to Rpl13a. All data are expressed as mean \pm SE for six mice in each group. * $P < 0.05$ [sham-operated controls (open circles) vs. BDL mice (closed circles)].

Fig. S3. The mRNA expression of enzymes involved in bile acid (BA) synthesis in livers of sham-operated controls and bile duct-ligated (BDL) mice. Total RNA from livers of sham-operated (open circles) and BDL (closed circles) mice were analysed by multiplex suspension array. The mRNA level of each gene was normalized to Rpl13a. All data are expressed as mean \pm SE for six mice in each group. * $P < 0.05$ [sham-operated controls (open circles) vs. BDL mice (closed circles)].

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