J. Cell. Mol. Med. Vol 15, No 5, 2011 pp. 1156-1165

Bile acids decrease intracellular bilirubin levels in the cholestatic liver: implications for bile acid-mediated oxidative stress

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Received: December 11, 2009; Accepted: April 8, 2010

Abstract

High plasma concentrations of bile acids (BA) and bilirubin are hallmarks of cholestasis. BA are implicated in the pathogenesis of cholestatic liver damage through mechanisms involving oxidative stress, whereas bilirubin is a strong antioxidant. We evaluated the roles of bilirubin and BA on mediating oxidative stress in rats following bile duct ligation (BDL). Adult female Wistar and Gunn rats intraperitoneally anaesthetized with ketamine and xylazine underwent BDL or sham operation. Cholestatic markers, antioxidant capacity, lipid peroxidation and heme oxygenase (HO) activity were determined in plasma and/or liver tissue 5 days after surgery. HepG2-rNtcp cells were used for *in vitro* experiments. Plasma bilirubin levels in control and BDL animals positively correlated with plasma antioxidant capacity. Peroxyl radical scavenging capacity was significantly higher in the plasma of BDL Wistar rats (210 $\pm$ 12%, $P < 0.0001$) compared to controls, but not in the liver tissues. Furthermore after BDL, lipid peroxidation in the livers increased (179 $\pm$ 37%, $P < 0.01$), whereas liver HO activity significantly decreased to 61% of control levels ($P < 0.001$). Addition of taurocholic acid (TCA, $\geq$50 μmol/l) to liver homogenates increased lipid peroxidation ($P < 0.01$) in Wistar, but not in Gunn rats or after the addition of bilirubin. In HepG2-rNtcp cells, TCA decreased both HO activity and intracellular bilirubin levels. We conclude that even though plasma bilirubin is a marker of cholestasis and hepatocyte dysfunction, it is also an endogenous antioxidant, which may counteract the pro-oxidative effects of BA in circulation. However, in an animal model of obstructive cholestasis, we found that BA compromise intracellular bilirubin levels making hepatocytes more susceptible to oxidative damage.

Keywords: taurocholic acid • heme oxygenase • carbon monoxide • lipid peroxidation

Introduction

Obstructive cholestasis, characterized by a failure to secrete bile into the bile duct and intestine, results in the accumulation of bile acids (BA) and bilirubin in circulation. Elevated activities of cholestatic enzymes and plasma levels of bilirubin and BA are used as laboratory markers of cholestasis.

The accumulation of BA inside hepatocytes is the major cause of cholestatic liver damage [1], including structural and functional injuries of hepatocyte membranes [2], cell death [3] and activation of inflammatory and fibrogenic signalling pathways [4]. Several studies have suggested an important role of increased oxidative stress in the pathogenesis of cholestatic injury [5, 6]. Accumulated BA within hepatocytes impair mitochondrial respiration and electron transport and stimulate the generation of reactive oxygen species (ROS) in hepatic mitochondria [7]. Accordingly, mitochondrial free radicals may then modify nucleic acids, proteins and lipids. In fact, an increase in lipid peroxidative products has been observed in cholestatic livers [8].

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doi:10.1111/j.1582-4934.2010.01098.x

The liver possesses a complex defence system including antioxidant enzymes and substrates to control the formation of ROS and repair oxidative damage [9]. Bilirubin, a product of heme catabolism, is a potent antioxidant substance both *in vitro* [10] and *in vivo* [11]. *In vitro* studies with liposomes have shown that both unconjugated (UCB) and conjugated bilirubin (CB) are protective against lipid peroxidation, surpassing that of α-tocopherol, an important lipid-soluble antioxidant [10]. Antioxidant properties of bilirubin were further confirmed by a number of animal and clinical studies demonstrating the protective effects of bilirubin on the development of atherosclerosis [12–14], cancer [15, 16] and other oxidative stress-mediated diseases [17].

The objective of this study was to address the seemingly dichotomous effects of high levels of the antioxidant bilirubin and the pro-oxidant BA in obstructive cholestasis using an animal model.

Materials and methods

Animals

Female Wistar rats obtained from Anlab (Prague, Czech Republic) and hyperbilirubinemic Gunn rats (RHA/jj, in-house colony from 1st Faculty of Medicine, Charles University in Prague) with a congenital deficiency of bilirubin uridine 5′-diphospho (UDP)-glucuronosyltransferase, both weighing from 200 to 280 g, were provided water and food *ad libitum*. All aspects of the animal studies met the accepted criteria for the care and experimental use of laboratory animals, and all protocols were approved by the Animal Research Committee of the 1st Faculty of Medicine, Charles University in Prague.

Reagents

L-Ascorbic acid, 2,6-di-*tert*-butyl-4-methylphenol (BHT), bovine serum albumin (BSA) 98%, UCB, chloroform (high-performance liquid chromatography [HPLC] grade), hemin, nicotinamide adenine dinucleotide phosphate (NADPH), sulfosalicylic acid, taurocholic acid (TCA) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade purchased from Penta (Prague, Czech Republic). UCB was purified and recrystallized according to McDonagh and Assisi [18]. Purified UCB was dissolved in 0.1 M NaOH and immediately neutralized with phosphoric acid. The mixture was subsequently diluted with BSA solution to reach a final concentration of 480 μM UCB and 500 μM BSA in phosphate buffer (25 mM, pH 7.0).

Bile duct ligation (BDL)

Rats were anaesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) intraperitoneally, and biliary trees were exposed through midline abdominal incisions. Microsurgical ligation of bile ducts and resections of extrahepatic biliary tracts were performed as previously described ($n = 7$ in each group) [19]. Sham-operated (SH) rats underwent the same procedure without bile duct resection and ligation ($n = 6$ in each group).

Tissue preparation

After 5 days, all animals were killed and blood (5 ml) was collected from superior vena cava, transferred to tubes containing EDTA, mixed, and placed on ice. An aliquot was centrifuged to separate plasma. Livers were then harvested, thoroughly washed with 10 ml heparinized saline, and rinsed in ice-cold reaction buffer (0.1 M phosphate buffer, pH 7.4). For RNA analysis, 100 mg of tissue was immediately placed in 1.5 ml microfuge tubes containing RNAlater (Qiagen, Valencia, CA, USA). Tubes were stored at −20°C until total RNA isolation.

For HO activity, HO-1 protein, and lipid peroxidation measurements, 100–150 mg tissue was diluted 1:9 (by weight) in reaction buffer, diced, and sonicated with an ultrasonic cell disruptor (Model XL2000, Misonics, Farmingdale, NY, USA). Sonicates were kept on ice and assayed for HO activity or lipid peroxidation within 1 hr or frozen in liquid nitrogen and stored at −80°C until analysis of HO-1 protein.

For liver carbon monoxide (CO) measurements, 150–200 mg tissue was diluted 1:4 in reaction buffer and then sonicated as described above.

For malondialdehyde (MDA) and 4-hydroxyalkenal analysis, 200 mg of tissue was placed in the Eppendorf tube containing 0.1 M PBS, pH 7.4 with 1% BHT, diced and sonicated. Sonicates were stored at −80°C until analysis.

Markers of cholestasis

Plasma biochemical markers (alkaline phosphatase [ALP], albumin) were determined in an automatic analyser (Hitachi, Model 717, Tokyo, Japan), using standard assays. Total plasma BA levels were determined spectrophotometrically using a Bile Acids kit (Trinity Biotech, Jamestown, NY, USA).

Liver histology

For histological examination, left lateral lobes of livers were fixed overnight in 10% buffered formalin (pH 7.4) at 4°C followed by a standard procedure for paraffin embedding. Serial sections (6 μm thick) were cut and stained with haematoxylin and eosin, Shikata's orcein method, or elastic-van Gieson stain. Each slide was viewed using standard light microscopy.

Peroxyl radical scavenging capacity

Peroxyl radical scavenging capacity was measured fluorometrically as a proportion of chain-breaking antioxidant consumption present in a biologic sample (plasma, liver homogenate) relative to that of Trolox (a reference and calibration antioxidant compound) as previously described [20].

Bilirubin determination

Plasma and liver CB and UCB levels were determined using an HPLC method as previously described [21]. Briefly, pigments were extracted into chloroform-hexane and subsequently delipidated by second extraction into a minute volume of alkaline aqueous solution. The resulting droplet was separated on HPLC.

Heme oxygenase (HO) activity

Twenty microlitres of 10% liver sonicate (2 mg fresh weight [FW]) was incubated for 15 min. at 37°C in CO-free septum-sealed vials containing 20 μl of 150 μM methemalbumin and 20 μl of 4.5 mM NADPH as previously described [22]. Blank reaction vials contained 0.1 M phosphate buffer, pH 7.4, in place of NADPH. Reactions were terminated by adding 5 μl of 30% (w/v) sulfosalicylic acid. The amount of CO generated by the reaction and released into the vial headspace was quantitated by gas chromatography (GC) with a reduction gas analyser (Trace Analytical, Menlo Park, CA, USA). HO activity was calculated as pmol CO/hr/mg FW.

Real-time RT-PCR

Total liver RNA was isolated using phenol : chloroform extraction and cDNA was generated using random hexamer primers and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA). Real-time PCR was performed with TaqMan® Gene Expression Assay Kit for HO-1 (Applera, Alameda, CA, USA). Data were normalized to glyceraldehyde 3-phosphate dehydrogenase and hypoxanthine phosphoribosyl transferase levels, and then expressed as fold change from control.

Western blots

One hundred micrograms of liver sonicates were mixed with equal volume of loading buffer. Samples were separated on 12% polyacrylamide gel and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking in Tween-PBS with 5% milk for at least 1 hr, membranes were incubated with HO-1 antibody (1:666; Stressgen, Victoria, BC, USA), or β-actin (1:8000; Sigma-Aldrich) for 1 hr. After washing, membranes were incubated with anti-mouse IgG-HRP (1:2000; Sigma-Aldrich) for 30 min. After washing, immunocomplexes on the membranes were visualized with ECL Western Blotting Detection Reagents (Amersham Biosciences, Buckinghamshire, UK). HO-1 protein bands were quantified by densitometry, normalized to β-actin, and then expressed as fold change from control.

Liver tissue CO

Forty microlitres of liver sonicate was added to CO-free, septum-sealed vials containing 5 μl of 60% (w/v) sulfosalicylic acid. After 30 min. incubation on ice, CO released into the vial headspace was quantitated by GC as previously described [23].

Carbonylhaemoglobin (COHb) determination

Total haemoglobin was estimated to be 15 g/dl for all the animals. COHb was measured by GC as previously described [24] and expressed as percentage of total haemoglobin.

Lipid peroxidation

Twenty microlitres of liver sonicate was incubated for 30 min. at 37°C with 100 μM ascorbate (80 μl) and 6 μM Fe^{2+} (0.5 μl). BHT (100 μM) was added for the blank reaction. CO produced into vial was quantitated by GC as previously described [25]. The amount of CO produced serves as an index of lipid peroxidation and was expressed as pmol CO/hr/mg FW. Total amounts of lipid peroxidation end-products, MDA and 4-hydroxyalkenals were determined using Bioxytech® LPO-586 Assay (Oxis International, Beverly Hills, CA, USA).

Cell culture

The human hepatoblastoma cell line (HepG2) (purchased from American Type Culture Collection, Manassas, VA, USA) and HepG2 cell line stably transfected with Ntcp transporter (HepG2-rNtcp), kindly provided by Professor Ulrich Beuers (University of Munich, Germany; currently University of Amsterdam, Netherlands), were cultured as described previously [26]. Cells were grown on 10 cm Petri dishes (Orange Scientific, Braine-l'Alleud, Belgium), incubated with TCA for 24 hrs for HO activity and intracellular bilirubin or 4 hrs for mRNA determination. After incubation, cells were quickly washed three times with 10 ml PBS, harvested, centrifuged and pellet was dispersed in 300 μl of 0.1 M phosphate buffer, pH 7.4. For mRNA determination, cell pellets were snap frozen in liquid nitrogen and stored at −80°C until analysis. An aliquot of the cell sonicate was used for protein determination (Bio-Rad DC protein assay, Hercules, CA, USA).

Statistical analyses

Normally distributed data are presented as means ± S.D. and analysed by Student t-test. Non-normally distributed datasets are expressed as medians (25%–75%) and analysed by Mann-Whitney rank sum test. The association between plasma bilirubin levels and antioxidant capacity was tested using Spearman rank-order correlation analysis. Differences were deemed statistically significant when $P < 0.05$.

Results

Markers of cholestasis and liver histology

Significant increases in total BA and ALP were observed in all BDL rats (Table 1). As expected, plasma bilirubin levels were significantly elevated in Wistar rats after BDL. In Gunn rats, which are deficient in bilirubin UDP-glucuronosyltransferase, plasma UCB levels remained unchanged after BDL (Table 2), as expected because most of the bilirubin does not enter the intestinal lumen *via* biliary excretion, but rather *via* direct diffusion across the intestinal mucosa [27].

Histological analysis of liver specimens from BDL rats revealed signs of impaired bile flow, such as large bile duct obstruction with intralobular bilirubinostasis (predominantly in perivenular localisation) and biliary interface activity with portal tract oedema, swelling of periportal hepatocytes and marked ductular proliferation with a disruption of the parenchymal limiting plates, accompanied by polymorphonuclear infiltration. Bile plugs

Table 1 Cholestatic markers and liver and body weights

	Wistar (SH) ($n = 6$)	Wistar (BDL) ($n = 7$)	Gunn (SH) ($n = 6$)	Gunn (BDL) ($n = 7$)
Body weight (g)	235 (233–237)	218 (212–240)	253 (206–280)	220 (200–264)
Liver weight (g)	10.4 (9.8–11.0)	14.0 (13.6–14.6)**	11.0 (10.5–11.5)	12.9 (11.5–13.6)*
TBA (μmol/l)	14.5 (10.3–23.3)	326 (290–404)**	8.5 (8.0–12.8)	288 (248–416)**
ALP (μkat/l)	2.1 (1.9–2.2)	4.4 (4.2–4.6)**	0.9 (0.8–1.0)	5.1 (3.9–5.6)**

Cholestatic markers and liver and body weights in SH and BDL Wistar and Gunn rats 5 days after surgery. Data are presented as median (25–75%).
*$P < 0.05$,**$P < 0.001$ compared to corresponding SH group.
TBA: total plasma bile acids, ALP: alkaline phosphatase.

Table 2 Plasma and liver bilirubin

	SH-plasma (μmol/l) ($n = 6$)	BDL-plasma (μmol/l) ($n = 7$)	SH-liver (nmol/g) ($n = 6$)	BDL-liver (nmol/g) ($n = 7$)
Wistar				
TB	0.32 (0.16–0.37)	193.8 (176.4–195.5)**	2.65 (2.33–3.50)	40.7 (31.5–57.1)**
CB	0	181.4 (164.8–190.2)**	1.73 (1.18–2.39)	39.2 (30.1–54.8)**
UCB	0.32 (0.16–0.37)	8.67 (3.75–13.03)**	1.07 (0.88–1.28)	2.03 (1.75–2.37)*
Gunn				
UCB	137.8 (130.0–145.4)	149.1 (135.4–212.6)	45.2 (40.7–46.1)	24.6 (19.6–28.5)*

Plasma and liver bilirubin in SH and BDL Wistar and Gunn rats 5 days after surgery. Data are presented as medians (25–75%).
*$P < 0.05$, **$P < 0.001$, compared to corresponding SH group.
TB: total bilirubin, UCB: unconjugated bilirubin, CB: conjugated bilirubin.

were present in a few cholangioles and bile infarcts were found in periportal zones.

Bilirubin increases antioxidant capacity in plasma, but not in liver homogenates of BDL rats

Peroxyl radical scavenging capacity was significantly higher in BDL compared to SH Wistar rats (210 ± 13 and 100 ± 30%, respectively $P < 0.001$) (Fig. 1A). We suggest that this increase could be attributed to elevated plasma bilirubin levels. In fact, plasma antioxidant capacity correlated positively with plasma total bilirubin levels (Spearman correlation coefficient = 0.45, $P = 0.027$). Unlike in plasma, we did not find any differences in peroxyl radical scavenging capacity in liver homogenates of BDL and SH Wistar rats (113 ± 17 and 100 ± 17%, respectively, $P = 0.21$) (Fig. 1A).

To further confirm our hypothesis, we investigated the effect of bilirubin and that of TCA on peroxyl radical scavenging capacity in normal rat plasma. Addition of bilirubin resulted in a dose-dependent increase in peroxyl radical scavenging capacity, whereas no effect was observed with TCA (Fig. 1B).

Liver bilirubin levels are relatively decreased compared to plasma in BDL animals

Markedly different antioxidant capacities of plasma and liver homogenates of BDL Wistar rats prompted us to measure bilirubin concentrations in those two compartments. Compared to SH rats, plasma bilirubin levels were 606 times higher in BDL Wistar rats. Surprisingly, in liver sonicates, only a 15-fold increase of bilirubin was observed in BDL rats. In Gunn rat livers, we found a significant decrease of 46% in the liver bilirubin levels in BDL rats compared to SH rats, whereas no significant differences were found in plasma (Table 2).

Bilirubin production is decreased and lipid peroxidation is increased following BDL

To identify the possible underlying mechanism for the relative lack of bilirubin in cholestatic hepatocytes, we investigated the rate of bilirubin production in the liver of Wistar rats. Activity of HO, the rate-limiting enzyme of bilirubin synthetic pathway, was

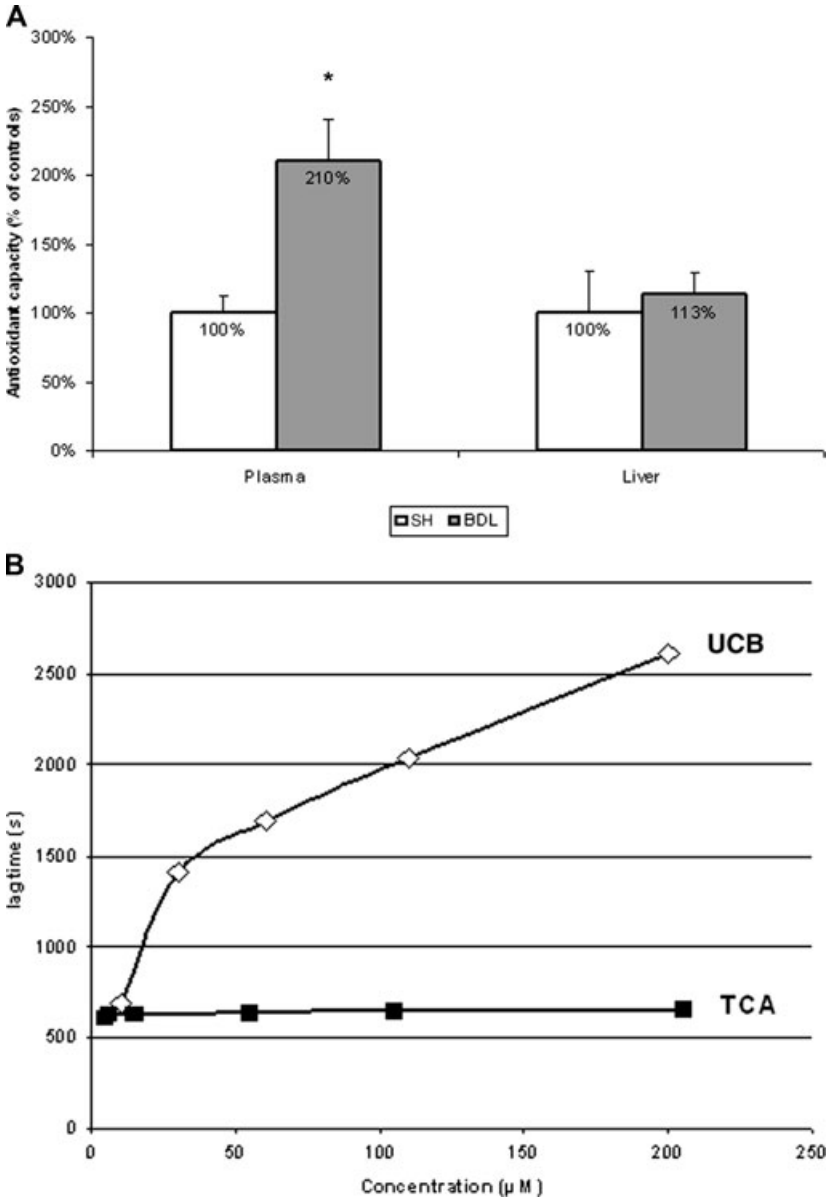

Fig. 1 Antioxidant capacity in plasma and liver homogenates of control (SH) and BDL rats. Effect of bilirubin and TCA. (**A**) Peroxyl radical scavenging capacity of plasma and liver homogenates from SH ($n = 6$) and BDL Wistar rats ($n = 7$). (**B**) Effect of UCB and TCA on peroxyl radical scavenging capacity (lag time) of normal rat plasma.

significantly decreased (54%) in the livers of BDL rats compared to controls (199 ± 36 *versus* 327 ± 48 pmol CO/hr/mg FW, $P = 0.003$). Similarly, HO-1 mRNA expression and HO-1 protein were also significantly down-regulated in BDL livers (Fig. 2A).

Surprisingly, we found significant increases in liver tissue CO and blood COHb levels of cholestatic rats compared to those of controls (7.9 ± 2.4 *versus* 4.0 ± 1.1 pmol CO/mg FW and 0.36 ± 0.04 *versus* 0.18 ± 0.02% total haemoglobin, respectively, $P < 0.05$, Fig. 2B). Because lipid peroxidation represents another source of CO *in vivo* [28], besides HO activity, we analysed the potential for lipid peroxidation. Livers of BDL animals were more susceptible to lipid peroxidation than control livers (85 ± 18 *versus* 47 ± 17 pmol CO/mg FW, respectively, $P = 0.005$). These results were confirmed by direct measurements of liver MDA and 4-hydroxyalkenals, which were significantly higher in BDL compared to SH rats (122 ± 15 and 99 ± 3 μmol/g, respectively, $P = 0.004$).

TCA increases lipid peroxidation in the liver homogenates

To investigate the role of BA in lipid peroxidation, we analysed the effect of increasing concentrations of TCA in normal Wistar

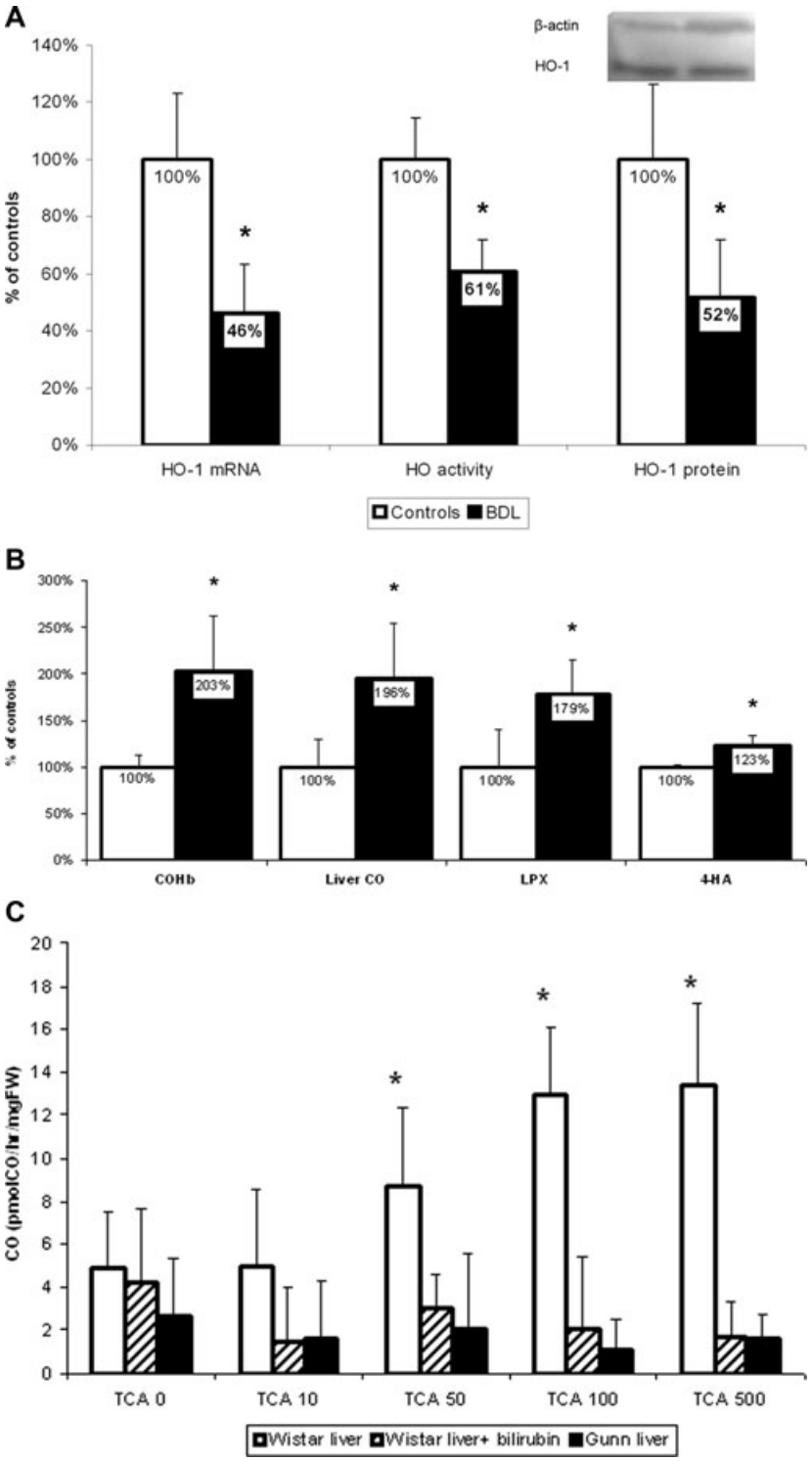

Fig. 2 Bilirubin production and lipid peroxidation following BDL in Wistar rats. Effect of TCA and bilirubin on lipid peroxidation in Wistar and Gunn rat liver homogenates. (**A**) Activity and expression of HO in liver tissue of sham-operated and BDL Wistar rats. Densitometric values of HO-1 protein were normalized to β-actin and all data are expressed as percentage of controls. $^{*}P < 0.05$. (**B**) CO in liver tissue and in the blood (COHb), lipid peroxidation and 4-hydroxyalkenals of cholestatic Wistar rats compared to control animals. Data are expressed as percentage of controls. $^{*}P < 0.05$. (**C**) TCA was added to normal liver homogenates of Wistar and Gunn rats or Wistar rat liver homogenates with 40 μM bilirubin in concentrations of 0, 10, 50, 100 and 500 μM and lipid peroxidation was measured. $^{*}P < 0.05$.

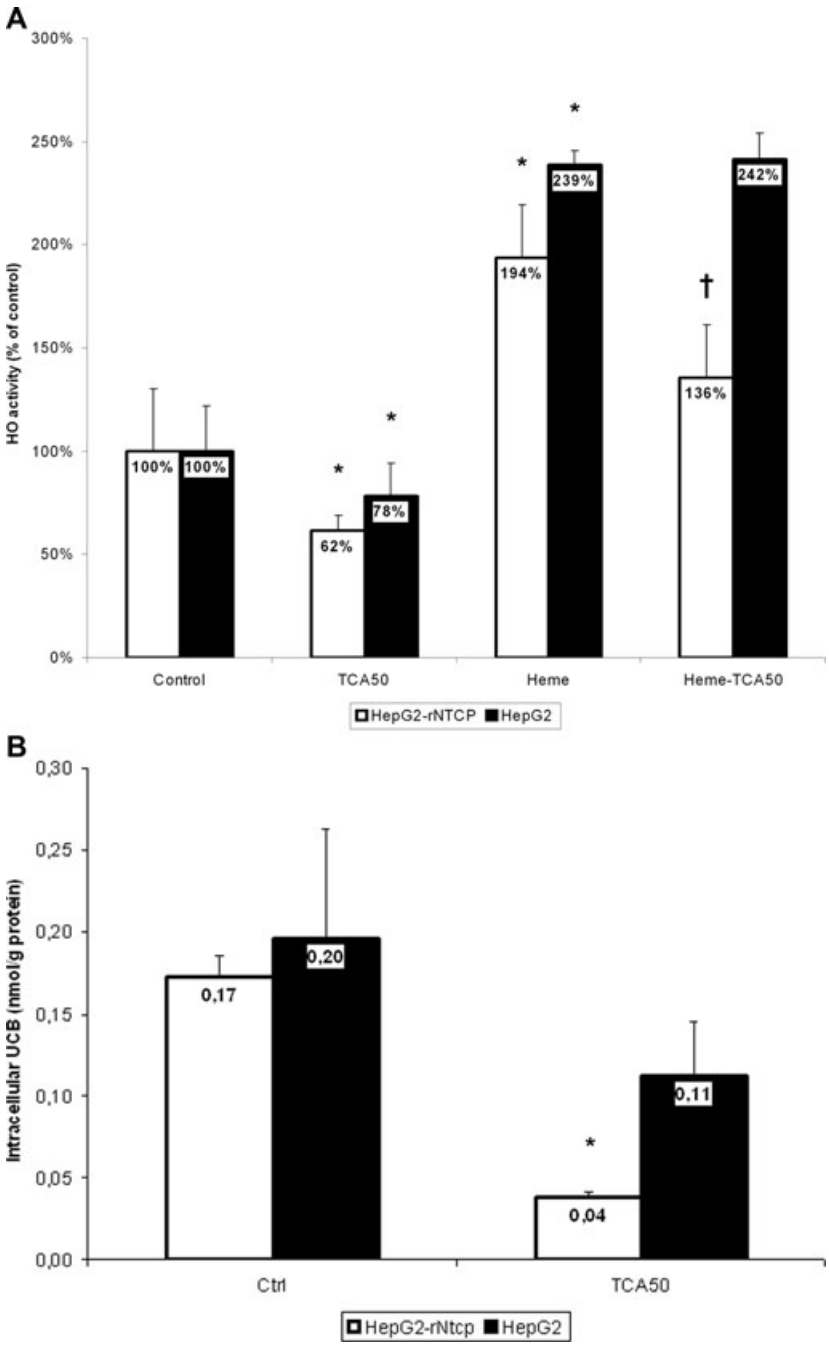

Fig. 3 Effect of TCA on HO activity and intracellular bilirubin *in vitro*. (**A**) HepG2 and HepG2-rNtcp cells (stably transfected with Ntcp transporter) were incubated for 24 hrs with 50 μM TCA, 30 μM heme (HO-1 inducer) or co-incubated with 50 μM TCA and 30 μM heme and HO activity was determined. $^{*}P < 0.05$ compared to controls, $^{\dagger}P < 0.05$ compared to heme-treated cells. (**B**) Intracellular bilirubin levels were measured in HepG2 and HepG2-rNtcp cells 24 hrs after incubation with 50 μM TCA. $^{*}P < 0.05$.

rat and Gunn rat liver homogenates. TCA at concentrations of 50, 100 and 500 μM significantly increased lipid peroxidation in Wistar rat liver homogenates. This effect was completely abolished by addition of 40 μM UCB. In Gunn rats, TCA had no effect on lipid peroxidation within mentioned concentration range (Fig. 2C).

TCA decreases intracellular bilirubin in HepG2-rNtcp cells

Treatment of HepG2 cells stably transfected with the Ntcp transporter with 50 μM TCA resulted in a significant decrease in HO activity (2.43 ± 0.73 nmol CO/hr/mg protein in controls

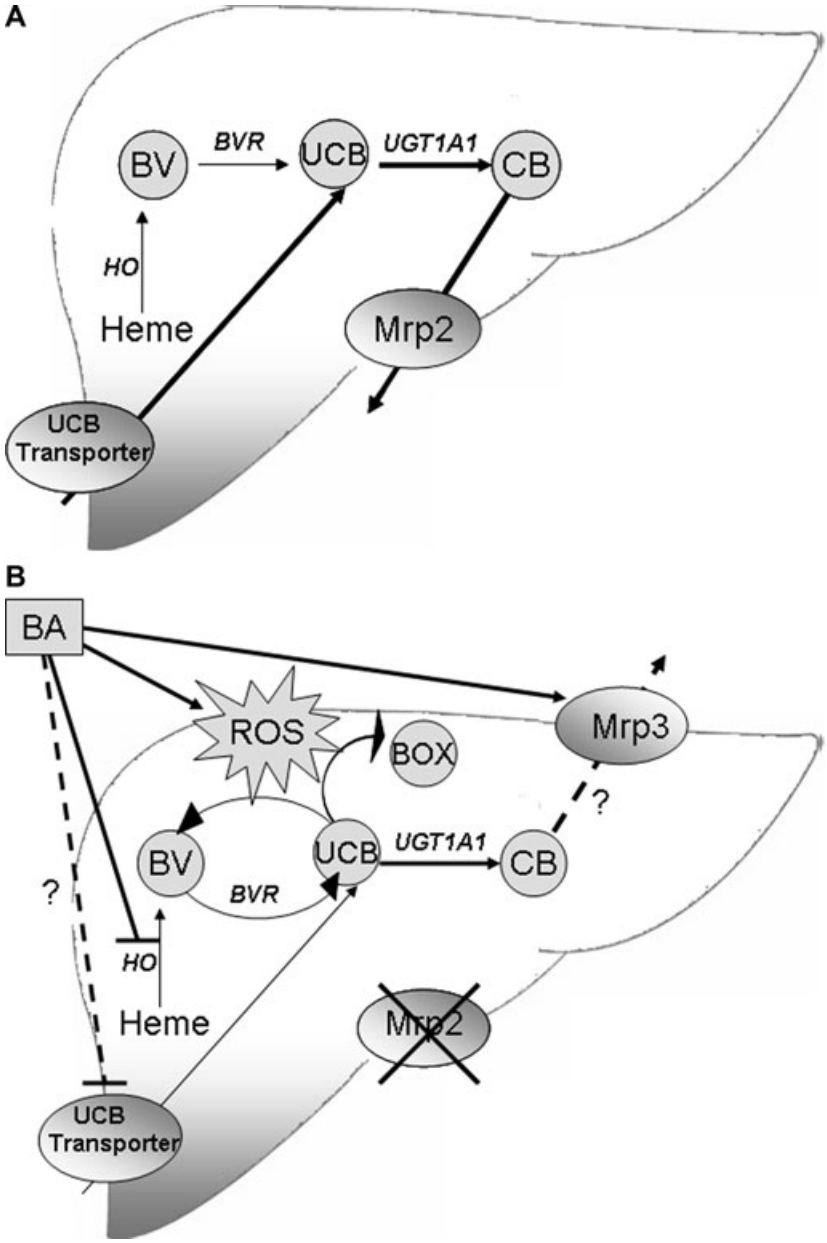

Fig. 4 Proposed bilirubin metabolism in normal (**A**) and cholestatic (**B**) rat liver. (**A**) Under normal conditions, UCB enters hepatocyte *via* carrier mediated mechanism (though the transporter involved still remains to be identified) [36, 37] or is produced intracellulary by oxidative degradation of heme. Intracellular UCB undergoes conjugation catalysed by bilirubin UDP-glucuronosyltransferase (UGT1A1) and CB is eliminated into bile *via* Mrp2 transporter. (**B**) High concentrations of bile acids in cholestatic liver lower bilirubin concentration by (1) triggering oxidative stress which leads to bilirubin consumption through biliverdin reductase (BVR) catalytic cycle [38] and *via* bilirubin oxidation products (BOX) formation (reviewed in [39]), (2) down-regulation of HO resulting in lower bilirubin production and (3) possibly by altering the expression of the basolateral transporters [31]. CB enters systemic circulation across the sinusoidal membrane possibly *via* up-regulated sinusoidal Mrp3 transporter.

versus 1.50 ± 0.11 nmol CO/hr/mg protein in TCA-treated cells, $P = 0.01$, Fig. 3A) and HO-1 mRNA (100% ± 15% *versus* 57% ± 23%, $P = 0.03$). As expected, treatment with 30 μM heme resulted in increase in HO activity though this effect was significantly reduced by co-treatment with TCA. Treatment of HepG2 cells (lacking Ntcp transporter for conjugated BA) with 50 μM TCA resulted in milder decrease in HO activity compared to HepG2-rNtcp cells (1.56 ± 0.34 nmol CO/hr/mg protein in controls *versus* 1.22 ± 0.19 nmol CO/hr/mg protein in TCA-treated cells, $P = 0.03$) and no decrease in HO-1 mRNA (100% ± 10% *versus* 89% ± 13%, $P = 1$). Interestingly, no decrease in HO activity has been observed upon co-treatment with heme plus TCA compared to heme-treated HepG2 cells.

Following treatment with 50 μM TCA, intracellular bilirubin decreased 78% in HepG2-rNtcp cells and only 43% decrease in HepG2 cells compared to control (untreated) cells (Fig. 3B). The decrease of intracellular bilirubin by 78% corresponded to a 31% decrease in HO activity in HepG2-rNtcp cells, suggesting that intracellular bilirubin might be also influenced by other mechanisms (Fig. 4).

Discussion

In this study, we demonstrated that bilirubin is not only a marker of cholestasis and hepatocyte dysfunction; but also, it is an endogenous antioxidant, counteracting the pro-oxidative effects of BA. In addition, we showed that BA lower intrahepatic bilirubin levels and bilirubin production presumably through an interaction between BA and bilirubin.

We found that BDL significantly increases the antioxidative capacity of plasma. Because of the significant positive correlation of plasma antioxidant capacity with bilirubin levels, it appears that bilirubin is the major antioxidant factor. This is supported by the finding that additions of UCB to normal rat plasma increased its antioxidative properties in dose-dependent fashion. These results agree with the data of Granato *et al.* [29] who demonstrated that bilirubin effectively suppresses ROS generation in freshly isolated hepatocytes.

However, a completely different circumstance may exist within liver tissue. BDL did not increase the antioxidant capacity in liver homogenates. We propose that this finding could, at least in part, be explained by the markedly different increases of bilirubin levels in plasma and liver compartments. After BDL, plasma levels of bilirubin increased more than 600-fold; whereas, in the liver only a 15-fold increase was observed. Furthermore, considering the high plasma bilirubin levels, the contamination of the liver with trace amounts of blood could artifactually actually increase liver bilirubin levels. Therefore, liver tissue bilirubin might be even lower in BDL animals compared to controls. Importantly, in Gunn rats, where BDL does not significantly affect plasma bilirubin levels (due to bilirubin elimination across the intestinal mucosa rather than the biliary tract), we observed a marked drop (54%) of liver bilirubin in BDL animals. These findings are of particular importance showing, for the first time, that intracellular bilirubin is actually consumed during cholestasis and that plasma bilirubin concentrations do not necessarily reflect tissue bilirubin metabolism.

To identify possible mechanisms responsible for this lowering of liver bilirubin levels following BDL, we treated HepG2 and HepG2-rNtcp cells with TCA. We found, that TCA down-regulates both the expression and activity of HO (the key enzyme in bilirubin production) and this down-regulation is more pronounced in cells expressing the Ntcp transporter. Accordingly, TCA lowered intracellular bilirubin levels. The markedly higher decrease in intracellular bilirubin compared to that of HO activity suggests that other mechanisms might also be involved. We have previously demonstrated an increased consumption of intracellular bilirubin during oxidative stress [30]. These effects could, together with BA-mediated alteration of bilirubin transport mechanisms [31], account for the relatively low hepatocyte bilirubin levels (Fig. 4).

The present study shows that in obstructive cholestasis, high concentrations of BA are responsible for increased lipid peroxidation in the liver as measured by the accumulation of MDA and 4-hydroxyalkenals, the products of lipid peroxidation. These findings agree with published data showing that MDA levels are increased in the livers of BDL rats [32, 33]. We have also observed increases in liver tissue CO and blood COHb in cholestatic animals. Because HO activity and expression (the main source of CO) are decreased in the livers of BDL animals, our observed elevations of CO concentration could be due to lipid peroxidation [25]. This is supported by our experiments where the addition of TCA to normal liver homogenates increased lipid peroxidation in a dose-dependent manner. However, addition of 40 μM bilirubin to liver homogenate completely abolished this effect. Additionally, no increase in lipid peroxidation was observed following the addition of TCA to liver homogenates from hyperbilirubinemic Gunn rats. All these data further confirm the opposing roles of BA and bilirubin in the development of oxidative stress and support the hypothesis that the higher BA/bilirubin ratio in cholestatic livers could lead to an increased susceptibility of the BDL livers to lipid peroxidation. These observations support also our previous data, showing that treatment of mice with HO inducer, rosuvastatin, led to simultaneous increase in heart HO activity and bilirubin content, but decrease in lipid peroxidation. Pre-treatment with a potent HO inhibitor, tin mesoporphyrin, completely abolished this effect [34].

There are several limitations of our study. We did not measure total BA in cholestatic liver homogenates, however, based on the previous work by Naito *et al.* [35] we can assume that similar concentrations of BA exist both in plasma and the liver. Secondly, only a short-term BDL was performed in our study, therefore, we cannot speculate about the course of chronic cholestasis. To clarify the exact role of HO expression in cholestasis, further studies with HO-1 knockout animals should be performed.

We conclude that high concentrations of BA in cholestasis are responsible for increased lipid peroxidation in the liver. In contrast, bilirubin has an antioxidative effect and is responsible for increased antioxidant capacity of cholestatic plasma. However, in the liver, BA maintain relatively low intracellular bilirubin levels. Therefore, the increase in BA/bilirubin ratio might be implicated in the pathogenesis of oxidative stress-mediated cholestatic liver injury.

Acknowledgements

We thank Professor Ulrich Beuers from University of Amsterdam for providing us with HepG2-rNtcp cells, Iva Subhanova for determination of mRNA in cell cultures, Olga Svejdova for excellent technical assistance and Iveta Mrazova for microsurgical ligation of bile ducts in rats. This work was supported by grant IGA MZ NR/9366–3 from the Czech Ministry of Health.

Conflict of interest

The authors confirm that there are no conflicts of interest.

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