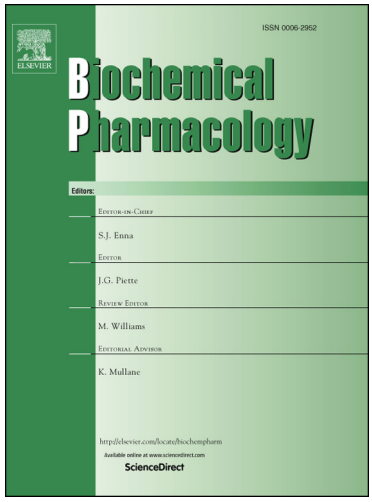

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Anticholestatic mechanisms of ursodeoxycholic acid in lipopolysaccharide-induced cholestasis

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Abstract

Lipopolysaccharide (LPS) from Gram (-) bacteria induces inflammatory cholestasis by impairing the expression/localization of transporters involved in bile formation (*e.g.*, Bsep, Mrp2). Therapeutic options for this disease are lacking. Ursodeoxycholic acid (UDCA) is the first choice therapy in cholestasis, but its anticholestatic efficacy in this hepatopathy remains to be evaluated. To asses it, male Wistar rats received UDCA for 5 days (25 mg/Kg/day, i.p.) with or without LPS, administered at 8 a.m. of the last 2 days (4 mg/Kg/day, i.p.), plus half of this dose at 8 p.m. of the last day. Then, plasma alkaline phosphatase (ALP), bile flow, basal and taurocholate-stimulated bile acid output, total glutathione output, and total/plasma membrane liver protein expression of Bsep and Mrp2 by confocal microscopy were assessed. mRNA levels of both transporters were assessed by Real-time PCR. Plasma pro- inflammatory cytokines (IL-6 and TNF-α) were measured by ELISA. Our results showed that, UDCA attenuated LPS-induced ALP plasma release and the impairment in the excretion of the Bsep substrate, taurocholate. This was associated with an improved Bsep expression at both mRNA and protein levels, and by an improved localization of Bsep in plasma membrane. UDCA failed to reduce the increase in plasma pro-inflammatory cytokines induced by LPS and Mrp2 expression/function. In conclusion, UDCA protects the hepatocyte against the damaging effect of bile acids accumulated by the LPS-induced secretory failure. This involved an enhanced synthesis of Bsep and an improved membrane stability of the newly synthesized transporters.

Keywords. Ursodeoxycholic acid; hepatocellular transporters; bile acids; bile acid export pump; lipopolysaccharide-induced cholestasis.

Abbreviations: ALP, alkaline phosphatase; Bsep, bile salt export pump; BSP, bromosulfophthalein; DAMP, danger-associated molecular pattern; FXR, farsenoid X receptor; GSH, glutathione; JNK, c-jun-NH2-terminal kinase; LPS, lipopolysaccharide; Mrp2, multidrug resistance-associated protein 2*;* Ntcp, Na^+/taurocholate cotransporting polypeptide; Oatp, organic anion-transporting polypeptide; PAMP, pathogen-associated molecular pattern; PXR, pregnane X receptor; ROI, region of interest; TC, taurocholate; RT-PCR, reverse transcriptase polymerase chain reaction; RXRα, 9-*cis*-retinoic acid receptor-α; UDCA, ursodeoxycholic acid.

1. Introduction

Inflammatory cholestasis is a frequent, often severe clinical condition, characterized by the impairment in bile production induced by pro-inflammatory cytokines [1]. Sepsis-induced cholestasis is prototypical of this kind of cholestasis [2]. It is an early, common complication found in more than 20% of hospitalized, icteric septic patients [3], and a main cause for admission and pediatric mortality in intensive care units [4].

Sepsis-induced cholestasis is causally associated with endotoxin, a lipopolysaccharide (LPS) from the Gram (-) bacterial wall. LPS is a pathogen-associated molecular pattern (PAMP) that is sensed by pattern-recognition receptors in macrophages, mainly Kupffer cells in liver [5]; LPS binding is followed by NLRP3 inflammasome activation, and the further release of pro-inflammatory cytokines [6]. These cytokines induce transcriptional and post-transcriptional impairment in the expression of hepatocellular carriers of bile acids and glutathione (GSH), the main osmotically active solutes driving bile formation [1, 2, 7]. LPS injection to rats induces downregulation of the bile acid basolateral importers Na^+/taurocholate cotransporting polypeptide (Ntcp) and organic anion-transporting polypeptide (Oatp) 1, 2, and 4 [8]. In the canalicular pole, the expressions of multidrug resistance-associated protein 2 (Mrp2, aka Abcc2), the bilirubin glucuronide/GSH transporter, and bile salt export pump (Bsep, aka Abcb11), the main bile acid carrier, are also impaired [9], via transcriptional and postranscriptional mechanisms, [10]. Bsep and Mrp2 also suffer endocytic internalization in endotoxemia [11, 12], which may lead to their delivery to degradation compartments [11].

At present, there is no specific treatment for sepsis-induced cholestasis. However, it is imperative to find it, since cholestasis potentiates the development of the final septic

shock [13]. Actually, the increased levels of bile acids occurring in this condition are predictive of sepsis-associated mortality [14], since bile acids act as danger-associated molecular patterns (DAMPs) which, like PAMPs, activate the NLRP3 inflammasome, and synergize with LPS the cytokine release [13].

Ursodeoxycholic acid (UDCA) treatment is the only disease-modifying therapy in primary biliary cholangitis [15, 16], and the “first choice” therapeutic approach for most cholestatic hepatopathies [17]. UDCA may counteract both inflammation and bile acid-induced liver damage via multiple mechanisms, including *i*) protection against bile acid-induced necrotic and apoptotic cell death, *ii*) induction of hepatocellular metabolic changes that reduces bile acid levels and toxicity, and *iii*) anti-inflammatory and immunomodulatory properties [17-19]. Some of these effects seem to involve its role as an activating ligand of the nuclear receptors pregnane X receptor (PXR) [20] and farsenoid X receptor (FXR) [21], two transcription factors that regulate genes involved in synthesis and disposal of bile acids [22, 23]. However, the most relevant UDCA effects *in vivo* are FXR-independent and postranscriptional in nature [24]. For example, UDCA favors reinsertion of canalicular transporters that have been endocytosed after the cholestatic insult [25, 26].

Despite all these alleged protective mechanisms may be of benefit to preserve biliary secretory function in sepsis-induced cholestasis, the anticholestatic efficacy and action mechanisms of UDCA in this hepatopathy have not been evaluated yet. This study sought to address this issue, by using LPS-induced cholestasis in the rat as an experimental model.

2. Materials and methods

2.1 Chemicals. LPS from *Salmonella typhimurium*, UDCA, bromosulfophthalein (BSP), 3α-hydroxysteroid dehydrogenase, NADPH, β-NAD, and GSH reductase were purchased from Sigma-Aldrich (St. Louis, MO). Sodium taurocholate (TC) was purchased from Santa Cruz Biotechnology (Dallas, Texas, U.S.A.). All the other reagents were of the highest analytical grade available from commercial sources.

2.2 Animals and treatments. Animals were obtained from our bioterium and received humane care according to the "Guide for the Care and Use of Laboratory Animals" published by the NIH (publication 25-28, revised in 1996). Animal experiments were approved by the Bioethical Committee for the Care and Use of Laboratory Animals of the Faculty of Biochemical and Pharmaceutical Sciences of the National University of Rosario (Resolution N° 489/2015). Adult (90 days), male Wistar rats weighing 300-350 g were used throughout. Animals were maintained on a standard diet and water *ad libitum*, under a constant 12 hour-light/12 hour-dark cycle. During the experiments, animal welfare was warranted by monitoring vital signs (heart and respiratory rates) and pain sensitivity (paw-withdrawal latency). All the treatments and the experiments with animals were performed in The Institute of Experimental Physiology (IFISE).

Rats were randomized in 4 experimental groups:

LPS group. The animals received UDCA vehicle (propylene glycol) for 5 days. At 8:00 am of the last two day, the animals were given a single, i.p. injection of LPS (4 mg/Kg/day, i.p., dissolved in saline) and, at 8:00 pm of the last day, a third dose of LPS of 2 mg/Kg was injected.

UDCA+LPS group. The animals received a daily dose of UDCA (25 mg/Kg/day, i.p., dissolved in propylene glycol) for 5 days [27-29]; this dosage is within the therapeutic range used in humans to treat cholestatic diseases [30]. Over the last two days, LPS was given as stated in the previous group.

Control group. The animals received UDCA and LPS vehicles throughout.

Experiments were carried out 12 hours after the last LPS (or LPS vehicle) administration.

2.3 Surgical procedures. Rats were anesthetized with Ketamina and Xylazina (100 mg/kg p.c. and 3,5 mg/kg p.c., respectively) and thus maintained throughout. Body temperature was measured with a rectal probe, and maintained at 37ºC with a heating lamp. The femoral vein and the common bile duct were cannulated with PE50 and PE10 polyethylene tubing, respectively (Intramedic, Clay Adams, Parsippany, NJ). At the end of each experiment, animals were sacrificed by exsanguination, and the liver was removed and weighed.

2.4. Experimental and analytical procedures. A sample of basal bile was collected for 10 min to monitor basal bile flow, which was determined gravimetrically, assuming a density of 1 g/mL. Basal bile was assayed for both total bile acids and glutathione, the two main biliary solutes acting as driving force for bile formation [31]. Total bile acids were measured by using the method of the *α*-hydroxysteroid dehydrogenase [32], whereas total glutathione content was assessed by using the recycling method of Tietze [33], as modified by Griffith [34]. Once finished the bile collection period, animals were sacrificed by exsanguination, and the liver removed. The activity of the cholestasis

enzyme alkaline phosphatase (ALP; EC 3.1.3.1) was assessed in plasma by using a commercial kit (Wiener Lab., Rosario, Argentina). A blood sample from the tail vein was also obtained 2 hs after the second LPS injection using heparin as anticoagulant, to measure plasma levels of the inflammatory cytokines IL-6 and TNF-α, by using commercial kits (Rat IL-6 ELISA Kit from Life Technologies and Rat TNF alpha ELISA Ready-SET-Go!® from eBioscience, respectively).

2.5. Bsep/Mrp2 localization and protein expression. Liver samples were sectioned and frozen in isopentane precooled at -70°C in freezer, and stored at this temperature for further immunofluorescence and confocal microscopy analysis (LSM880, Carl Zeiss LLC, Thornwood, NY, USA). Liver sections were fixed and stained as described [35, 36], followed by overnight incubation with a rabbit anti-rat Bsep (1:200; Kamiya Biomedical Co., Seattle, WA, USA), or with a mouse anti-rat Mrp2 (1:200; [M2III-6], Alexis Biochemicals, San Diego, CA, USA), followed by incubation with a Cy2-conjugated donkey anti-rabbit IgG or a Cy2-conjugated donkey anti-mouse IgG, respectively (1:200, 1 h; Jackson ImmunoResearch Laboratory, West Grove, PA). The tight junctional-associated protein Occludin was used to delimitate the border of the bile canaliculi. Occludin was labeled with a mouse anti-rat Occludin (when Bsep immunostaining was carried out, 1:200, overnight; Invitrogen) or with a rabbit anti-rat Occludin (when Mrp2 immunostaining was carried out, 1:200, overnight; Zymed, San Francisco, USA), followed by incubation with a Cy3-conjugated donkey anti-mouse or a Cy3-conjugated donkey anti-rabbit (1:200, 1 h, Jackson ImmunoResearch Laboratory, West Grove, PA.), respectively. To ensure comparable staining and image capture performance for the different groups belonging to the same experimental protocol, liver slices were prepared

on the same day, mounted on the same glass slide, and subjected to the staining procedure and confocal microscopy analysis simultaneously. Quantification of the degree of Bsep and Mrp2 endocytic internalization was performed on confocal images using ImageJ 1.34m (National Institutes of Health), as described elsewhere [37]. For this purpose, intensity of fluorescence associated with the transporters along an 8-µm line perpendicular to the canaliculus (from -4 µm to 4 µm of the canalicular center). For each section, data from at least 15 different canaliculi were collected, and used for statistical comparison. At least two animals per group were used to assess the localization profile of the transporters, and the number of photographs analyzed per animal was 4-7. Each measurement was normalized to the sum of all intensities of the respective measurement. For quantification of total Bsep- and Mrp2-associated fluorescence intensity as a measure of protein expression, all sections were stained in a serial manner, and the same settings of confocal parameters were applied for each slide. The images were then loaded into ImageJ, the region of interest (ROI) traced, grey level intensities analyzed, and background subtracted systematically [38]. The images were then analyzed with ImageJ/FIJI image processing software, and the total fluorescence intensity of Bsep and Mrp2 were calculated. The results were then referred to control values.

2.6. Bsep/Mrp2 transport function. Transport activity of Bsep and Mrp2 was assessed by using sodium TC and BSP as model substrates. Based upon its relative affinity for Bsep as compared to other naturally occurring bile acids, TC is considered one of the best substrate to assess Bsep-mediated transport [39]. Regarding BSP, this cholephilic dye is taken up by Oatps and excreted with high affinity via Mrp2 after GSH conjugation

by glutathione-*S*-transferases [40, 41]; its Mrp2-mediated transfer but not the conjugation step is the rate-limiting one of its plasma-to-bile transport [42].

TC was administered in single, i.v. doses of 8 μmol/100 g of b.w., and bile samples were then collected in 2-minute intervals over the next 10 minutes; after this time the exogenously administered TC was completely cleared in control animals. Bile salts were measured in bile with the *α*-hydroxysteroid dehydrogenase method [32], and TC excretion was calculated by assuming that all the excess in biliary excretion rate of bile acids over the basal one after TC injection is fully due to TC excretion. This assumption was confirmed by ancillary experiments in which biliary bile salt composition was analyzed by HPLC (Waters, Mildford, MA), as reported previously [43]; only increments in TC but not in other biliary bile salts in the rat were recorded after TC administration (data not shown).

BSP was administered in single, i.v. doses of 3 mg/100 g of b.w. Bile samples were then collected in 5-minute intervals over the next 60 minutes, a time period sufficient to virtually fully depurate the dye in control animals. BSP in bile was determined spectrophotometry at wavelength of 580 nm, after appropriate dilution with 0.1 N NaOH [44].

2.7 RNA isolation and real time reverse transcriptase polymerase chain reaction (RT-PCR). After treatment, total RNA was isolated from liver tissue using TRIzol® reagent (Invitrogen). Yield of extracted RNA was evaluated spectrophotometrically at 260 nm, through the following conversion: 1 OD = 40 μg/μL of RNA. Purity and integrity of RNA was assessed by the A260/A280 ratio and 1,2 % agarose gel electrophoresis under denaturing conditions (110 V, 40 min), and using SYBER-SAFE as marker. The

gel was then observed in a UV transilluminator. A260/A280 ratios should be between 1.8 and 2.2, whereas the 28S rRNA band should be about twice the intensity of the 18S band, and higher molecular weight bands indicative of DNA contamination should be absent. The first-strand cDNA was synthesized from 1 μg of total RNA using Superscript III Reverse Transcriptase (Invitrogen) and random primers according to the manufacturer's suggested protocol. Primers for *Abcb11* were designed using the "Primer-BLAST" program [45], and sequences of primer pairs for *Abcc2* were as previously described [46] (Table 1). Real time PCR reactions were performed on a Mx3000P System (Stratagene, La Jolla, CA) with Platinum Taq DNA Polymerase (Invitrogen) and SYBR Green quantification. The thermocycling regime was 2 min at 95 °C for initial denaturation; and then 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s; and the final step was 1 min at 95 °C, 30 s at 55°C and 30 s at 95 °C. Results for *Abcb11* and *Abcc2* mRNA were normalized to the expression of 18S rRNA as the housekeeping gene by using the $2^{-\Delta\Delta Ct}$ method 47]. The primers for 18S rRNA were those previously designed by Ruiz et. al. [46] (Table 1). The specificity of each reaction was verified with a dissociation curve using a temperature range from 55 ºC to 95 ºC, with continuous fluorescence measurements.

2.8. Statistical analysis. Data are expressed as means ± SEM. Statistical analysis was carried out by using ANOVA or Student *t* test, unless another statistical test has been determined to be more appropriate. The variances of the densitometric profiles of Bsep and Mrp2 localization were compared with the Mann-Whitney U test [48]. Values of $P<0.05$ were considered to be statistically significant.

3. Results

3.1. Blood and biliary parameters of inflammation and cholestasis. As shown in Table 2, LPS significantly increased ALP plasmatic levels, a plasma membrane enzyme that is induced in both hepatocytes and cholangiocytes, and released from the cell surface into blood by detergent bile acids that are retained in cholestasis [49] UDCA pretreatment reduced partially this increase, suggesting protection against intrahepatic bile acid building and/or bile acid-induced plasma membrane damage.

As expected, plasma levels of the pro-inflammatory cytokines TNF-α and IL-6 were both increased by LPS. UDCA pretreatment was unable to significantly reduce these increments; only a tendency was observed for TNF-α, but it did not reach statistical significance (Table 2). This suggests that the beneficial effects of UDCA are not mainly due to an attenuation of the inflammatory response evoked by LPS.

As shown in Table 3, LPS significantly reduced bile flow, and coadministration of UDCA did not attenuate this decrease. UDCA did not significantly increase the basal biliary excretion rate of both bile acids and glutathione when coadministered with LPS. However, when a bolus of TC was administered so that to acutely challenge the Bsep-mediated secretory pathway, the decrease in both bile flow and bile acid output induced by LPS was partially counteracted by UDCA coadministration; this suggests that Bsep transport function, and hence bile acid-dependent bile flow, are indeed improved by UDCA, but it is required to force the transport system with higher levels of intracellular bile acids for this recovery to become apparent.

3.2 Bsep and Mrp2 transport function. Bsep and Mrp2 transport function was assessed by recording the changes over time in the biliary excretion of TC and BSP, respectively, following a bolus injection of these compounds

As shown in Fig. 1, TC was transported less efficiently by LPS-treated rats, as indicated by the 45% lower value of accumulated biliary excretion of this compound, as compared to controls after 4 min of TC injection. UDCA prevented partially this decrease, reaching a 40% higher value of cumulative TC excretion as compared to LPS group.

As depicted in Fig. 2, BSP was also transported less efficiently by LPS-treated rats, as suggested by the 30% lower value of accumulated biliary excretion of the dye as compared to controls after 60 min of its injection. This impairment was however not counteracted by UDCA, since cumulative BSP excretion in the co-treated rats with UDCA was similar to that of LPS alone (Fig. 2, upper panel).

3.3. Protein expression and localization of Bsep and Mrp2. As shown in Table 4, total Bsep and Mrp2 protein content, as assessed by measuring the total Mrp2- and Bsep-associated green fluorescence in the fields, was significantly impaired by LPS, with reductions of 59% and 31%, respectively.

Figure 3 *A* shows confocal images of Bsep (green) and the canaliculus border marker, occludin (red). In control livers, transporter-associated fluorescence was confined to the canalicular space. In LPS-treated rats, the carrier relocalized from the canalicular space to the pericanalicular, as indicated by the increased fluorescence at a greater distance from the bile canaliculus, and co-treatment with UDCA counteracted this redistribution. Figure 3 *B* shows the analysis of the distribution profiles of transporter-associated

fluorescence along a line perpendicular to the canaliculus. LPS-treated livers showed a statistically significant (P<0.05) wider profile with increased fluorescence at a greater distance from the canalicular membrane, a finding consistent with endocytic retrieval of these transporters into the intracellular compartment (Table 5) On the other hand, in livers from the LPS+UDCA co-treated group, the distribution of both Bsep and Mrp2 was almost identical to that in control livers (P>0.05). Analysis of the distribution profiles of the tight junctional protein occludin as a marker of the canalicular edge demonstrates a conserved width of the canaliculi in all the experimental groups, thus ruling out the influence of changes in this parameter on the Bsep distribution profiles. These data demonstrate that UDCA protects against LPS-induced retrieval of Bsep. Essentially the same results were obtained when the distribution pattern of Mrp2-associated fluorescence was assessed, with re-localization of Mrp2 outside the limits of the canaliculus that is reversed by UDCA co-treatment (Fig. 4).

3.4. Bsep and Mrp2 mRNA expression. RT-PCR analysis shows that both Bsep and Mrp2 mRNA abundance in rat livers from LPS-treated rats was markedly decreased by 85% and 75%, respectively (Table 6). UDCA co-treatment to LPS-treated rats normalized mRNA expressions of both transporters.

4. Discussion

UDCA has been suggested as a rationale alternative to treat sepsis-induced cholestasis due to its multiple anticholestatic mechanisms [50], and because it is often

successful as the first-choice therapy for most cholestatic disorders [17]. However, several authors agree that the available experimental and clinical evidence is still insufficient [50-52]. Therefore, we have provided here for the first time experimental evidence that UDCA bears multiple anticholestatic effects in LPS-induced cholestasis.

First, UDCA helped to preserve hepatocyte integrity against detergent bile acids accumulated in cholestasis, as suggested by the reduction in plasma ALP levels compared to that in LPS-treated rats (see Table 2). ALP is a membrane-bound enzyme that is induced and released into the bloodstream by detergent, endogenous bile acids [53], and represents an early indication of membrane damage leading to hepatocellular lysis [54]. The counteraction of this pathomechanim by UDCA most likely reflects its membrane-protective effects [55] as well as its lack of capability to induce ALP expression [56] and to remove ALP from the plasma membrane in cholestatic livers [57].

In agreement with previous works [7, 58], we show here that LPS represses the gene expression of the main canalicular bile acid transporter Bsep, leading to downregulation of the transporter at a protein level (see Table 4). UDCA upregulated at a transcriptional level these otherwise downregulated bile acid transporter (see Table 6), which resulted in an ameliorated capability of the hepatocyte to excrete an i.v. load of the model Bsep substrate, TC (see Fig. 1).

Improvement of Bsep gene expression by UDCA administration has been reported to occur under other cholestatic conditions, as for example in mice exposed to α-naphtylisothiocyanate [59], but not in normal animals [60] or healthy humans [61]. Therefore, the cholestatic context seems to be important for UDCA to improve transcriptionally Bsep. Transcriptional Bsep downregulation in LPS-induced cholestasis is caused by the cytokines released during the acute-phase response rather than by

accumulated bile acids [7]. Cytokine-mediated repression of FXR, the main nuclear receptor involved in the maintenance of Bsep levels, has been suggested as a chief causal factor [62]. The possibility that UDCA improves Bsep levels by enhancing the remnant Bsep transactivating activity of FXR by acting as a FXR ligand is however unlikely, because it has a very weak (if any) FXR agonist activity [21]. Apart from reducing FXR levels, cytokines impair the DNA binding of FXR to the Bsep IR-1 responsive element [63, 64]. FXR binds to DNA as a heterodimer with the 9-*cis*-retinoic acid receptor-α (RXRα), and this binding is impaired by cytokines via a c-jun-NH2-terminal kinase (JNK)-dependent RXR phosphorylation [63, 65]. Although RXRα levels are reduced in LPS-induced cholestasis [62, 63], this mechanism may be still operating, since a partial replacement of RXRα by other RXR isoforms not affected by cytokines, such as RXRβ and RXRγ, occurs [62]. In line with this, UDCA can inhibit JNK activity in hepatocytes exposed to different stressor, such as hydrophobic bile acids [66] and lipotoxic fatty acids [67].

Similarly to Bsep, Mrp2 gene transcription was enhanced by UDCA in LPS cholestatic rats (see Table 6); this may reflect, again, the capability of UDCA to counteract cytokine-mediated effects on Mrp2 transcription mechanisms, which is also JNK-dependent in nature [63, 65]. In addition, the well characterized inductive effect that UDCA has *per se* on Mrp2 expression [60] may be another contributing factor. Surprisingly, an enhanced Mrp2 mRNA levels in the co-treated group did not lead to an improved expression of Mrp2 either at the protein level (see Table 4) or at a functional levels, as evaluated by assessing the biliary excretion of its exogenous substrate, BSP (see Fig. 2). Similarly, biliary excretion of GSH, an endogenous Mrp2 substrate [68], was not ameliorated either (see Table 3). Differences in post-transcriptional events (*e.g.*, mRNA processing,

steady-state mRNA stability, translational efficacy), as well as in post-translational events (*e.g.*, transporter sorting and targeting to the canalicular membrane, protein modifications that affect its degradation rate) may be causal factors. Actually, Mrp2 and Bsep have marked differences in their post-transcriptional processing, including intracellular location at different vesicular pools after LPS-induced endocytic internalization [69] and differential intracellular degradation after internalization; Mrp2 and Bsep both suffer ubiquitination, but then Mrp2 traffics from early endosomes into the lysosomal degradation pathway [70], whereas Bsep more likely suffers proteosomal degradation [71] Interestingly, higher rates of lysosomal protein degradation occur in endotoxemic rats [72], which may have selectively enhanced Mrp2 degradation after internalization. The lack of beneficial effect of UDCA on Mrp2 protein expression and function should not be necessarily regarded as a drawback, since it helps to retain both GSH and bilirubin, two endogenous anti-oxidant compounds that have been shown to counteract oxidative stress-induced cholestasis 73, 74], a factor that has been causally linked to LPS-induced cholestasis [75, 76].

The improvement in Bsep activity induced by UDCA in LPS-treated rats was apparent under conditions of increased bile acid load but not under basal conditions, where neither bile flow nor total bile acid excretion was modified (see Table 3). It should be born in mind that basal bile acid excretion depends not only on the intrinsic activity of Bsep but also on the size of the bile acid pool [77] and the extrusion rate via alternative, bile acid basolateral export pumps, such as Mrp3 and Mrp4 [78]; reduction and increments in these two last parameters, respectively, decreases canalicular bile acid output. UDCA may reduce bile acid pool size, since it decreases absorption of endogenous bile acids at the intestinal levels [79] and inhibits Cyp7a1, the rate-limiting

enzyme of bile acid synthesis [80]. As for basolateral bile acid extrusion, LPS increases the expression of Mrp3 [8, 81], as the result of an adaptive response in cholestasis aimed to redirect bile acid flux back to bloodstream for urinary elimination [82]. UDCA can further upregulate Mrp3 in the endotoxemic liver [24], and to upregulate Mrp4 as well [83]. As a corollary, the lack of improvement in bile acid biliary output by UDCA in LPS cholestatic rats under basal conditions should not be regarded as absence of anticholestatic effect of UDCA, but as a consequence of other beneficial effects aimed to limit bile acid accumulation via both reduced bile acid synthesis and enhanced bile acid basolateral efflux [17].

UDCA improved localization of the canalicular transporters Bsep and Mrp2 in their membrane domain by counteracting LPS-induced endocytic internalization (see Figs. 3 and 4, respectively). In line with this, UDCA stabilizes Bsep in the apical membrane of MDCK II cells, by preventing its endocytic internalization [84]. Bsep endocytic internalization occurs via a clathrin-mediated mechanism from clathrin-rich "non-raft" membrane domains under both normal [85] and cholestatic conditions [86], and the same holds true for Mrp2, at least under cholestatic conditions [86]. However, both Bsep and Mrp2 mainly reside in caveolin/cholesterol-rich "raft" domain, where they are fully functional due to its cholesterol dependency [87, 88]. Therefore, they need to be transferred to "non-raft" microdomains to be endocytosed [86]. Since UDCA is a membranotropic agents that was show to expand and stabilize "raft" structures by mimicking cholesterol [89], perhaps it stabilizes Bsep and Mrp2 in these enlarged "raft" microdomains, thus preventing their transfer to "non-raft" structures. UDCA may have also favored the vesicular trafficking of these transporters back to the canalicular membrane after endocytosis. Indeed, UDCA has been shown to stimulate the apical

vesicular targeting of both newly-synthesized [90] and pre-existing canalicular transporters localized in subapical endosomes [91]. Actually, anticholestatic effects of UDCA associated with counteraction of canalicular transporter endocytosis have been shown in other experimental models of cholestasis [26, 92].

UDCA attenuated the effects of LPS without reducing expression of TNF-α and IL-6, two critical, sentinel cytokines involved in hepatic acute phase response (see Table 2). In line with our results, UDCA treatment was not instrumental in reducing the hepatic expression of the cytokines TNF-α and IL-1β in cholestatic rats with bile-duct ligation [93], a cholestatic model largely associated causally with endotoxemia [94]. Lack of effect on cytokine production might, overall, be a rather beneficial UDCA property though, since cytokines are involved in the hepatic regenerative response [95], which, in the setting of liver cell damage, should be conserved to favor healing.

In conclusion, UDCA can protect the hepatocyte against the damaging effect of bile acids accumulated by LPS-induced bile acid secretory failure. This beneficial effect involves both transcriptional and posttranscriptional mechanisms, such as improved synthesis and function of the main bile acid canalicular transporter, Bsep, and an enhanced membrane stability of the carrier. Our results may be of relevance not only for sepsis-induced cholestasis, but also for the treatment of other cholestatic liver diseases in which LPS [96] or inflammatory cytokines [97, 98] also play a key role.

Conflict of interest

The authors have no conflict of interest.

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References

1. Trauner M, Fickert P, Stauber RE. (1999) Inflammation-induced cholestasis. J Gastroenterol Hepatol. 14, 946-959. DOI: 10.1046/j.1440-1746.1999.01982.x
2. Bhogal HK, Sanyal AJ. (2013) The molecular pathogenesis of cholestasis in sepsis. Front Biosci (Elite Ed). 5:87-96. DOI: 10.2741/E598
3. Whitehead MW, Hainsworth I, Kingham JG. (2001) The causes of obvious jaundice in South West Wales: perceptions versus reality. Gut. 48:409-413. DOI: 10.1136/gut.48.3.409
4. Mayr FB, Yende S, Angus DC. (2014) Epidemiology of severe sepsis. Virulence. 5:4-11. DOI: 10.4161/viru.27372
5. Sturm E, Havinga R, Baller JF, Wolters H, van Rooijen N, Kamps JAAM, et al. (2005) Kupffer cell depletion with liposomal clodronate prevents suppression of Ntcp expression in endotoxin-treated rats. J Hepatol. 42:102-109. DOI: 10.1016/j.jhep.2004.09.019
6. Akira S, Uematsu S, Takeuchi O. (2006) Pathogen recognition and innate immunity. Cell. 124:783-801. DOI: 10.1016/j.cell.2006.02.015
7. Hartmann G, Cheung AK, Piquette-Miller M. (2002) Inflammatory cytokines, but not bile acids, regulate expression of murine hepatic anion transporters in endotoxemia. J Pharmacol Exp Ther. 303:273-281. DOI: 10.1124/jpet.102.039404
8. Cherrington NJ, Slitt AL, Li N, Klaassen CD. (2004) Lipopolysaccharide-mediated regulation of hepatic transporter mRNA levels in rats. Drug Metab Dispos. 32:734-741. DOI: 10.1124/dmd.32.7.734

9. Lee JM, Trauner M, Soroka CJ, Stieger B, Meier PJ, Boyer JL. (2000) Expression of the bile salt export pump is maintained after chronic cholestasis in the rat. Gastroenterology. 118:163-172. DOI: 10.1016/S0016-5085(00)70425-2

10. Vos TA, Hooiveld GJEJ, Koning H, Childs S, Meijer DKF, Moshage H, et al. (1998) Up-regulation of the multidrug resistance genes, Mrp1 and Mdr1b, and down-regulation of the organic anion transporter, Mrp2, and the bile salt transporter, Spgp, in endotoxemic rat liver. Hepatology. 28:1637-1644. DOI: 10.1002/hep.510280625

11. Kubitz R, Wettstein M, Warskulat U, Häussinger D. (1999) Regulation of the multidrug resistance protein 2 in the rat liver by lipopolysaccharide and dexamethasone. Gastroenterology. 116:401-410. DOI: 10.1016/S0016-5085(99)70138-1

12. Bolder U, Jeschke MG, Landmann L, Wolf F, de Sousa C, Schlitt HJ, et al. (2006) Heat stress enhances recovery of hepatocyte bile acid and organic anion transporters in endotoxemic rats by multiple mechanisms. Cell Stress Chaperones. 11:89-100. DOI: 10.1379/CSC-143R.1

13. Hao H, Cao L, Jiang C, Che Y, Zhang S, Takahashi S, et al. (2017) Farnesoid X receptor regulation of the NLRP3 inflammasome underlies cholestasis-associated sepsis. Cell Metab. 25:856-867. DOI: 10.1016/j.cmet.2017.03.007

14. Recknagel P, Gonnert FA, Westermann M, Lambeck S, Lupp A, Rudiger A, et al. (2012) Liver dysfunction and phosphatidylinositol-3-kinase signalling in early sepsis: experimental studies in rodent models of peritonitis. PLoS Med. 9:e1001338. DOI: 10.1371/journal.pmed.1001338

15. Corpechot C, Carrat F, Bahr A, Chretien Y, Poupon RE, Poupon R. (2005) The effect of ursodeoxycholic acid therapy on the natural course of primary biliary cirrhosis. Gastroenterology. 128:297-303. DOI: 10.1053/j.gastro.2004.11.009

16. Corpechot C, Abenavoli L, Rabahi N, Chrétien Y, Andréani T, Johanet C, et al. (2008) Biochemical response to ursodeoxycholic acid and long-term prognosis in primary biliary cirrhosis. Hepatology. 48:871-877. DOI: 10.1002/hep.22428

17. Roma MG, Toledo FD, Boaglio AC, Basiglio CL, Crocenzi FA, Sanchez Pozzi EJ. (2011) Ursodeoxycholic acid in cholestasis: linking action mechanisms to therapeutic applications. Clin Sci (Lond). 121:523-544. DOI: 10.1042/CS20110184

18. Beuers U. (2006) Drug insight: Mechanisms and sites of action of ursodeoxycholic acid in cholestasis. Nat Clin Pract Gastroenterol Hepatol. 3:318-328. DOI: 10.1038/ncpgasthep0521

19. Paumgartner G, Beuers U. (2004) Mechanisms of action and therapeutic efficacy of ursodeoxycholic acid in cholestatic liver disease. Clin Liver Dis. 8:67-81, vi. DOI: 10.1016/S1089-3261(03)00135-1

20. Fickert P, Zollner G, Fuchsbichler A, Stumptner C, Weiglein AH, Lammert F, et al. (2002) Ursodeoxycholic acid aggravates bile infarcts in bile duct-ligated and Mdr2 knockout mice via disruption of cholangioles. Gastroenterology. 123:1238-1251. DOI: 10.1053/gast.2002.35948

21. Schuetz EG, Strom S, Yasuda K, Lecureur V, Assem M, Brimer C, et al. (2001) Disrupted bile acid homeostasis reveals an unexpected interaction among nuclear hormone receptors, transporters, and cytochrome P450. J Biol Chem. 276:39411-39418. DOI: 10.1074/jbc.M106340200

22. Guo GL, Lambert G, Negishi M, Ward JM, Brewer HB, Kliewer SA ,et al. (2003) Complementary roles of farnesoid X receptor, pregnane X receptor, and constitutive androstane receptor in protection against bile acid toxicity. J Biol Chem. 278:45062-45071. DOI: 0.1074/jbc.M307145200

23. Jonker JW, Liddle C, Downes M. (2012) FXR and PXR: potential therapeutic targets in cholestasis. J Steroid Biochem Mol Biol. 130:147-158. DOI: 10.1016/j.jsbmb.2011.06.012

24. Zollner G, Fickert P, Fuchsbichler A, Silbert D, Wagner M, Arbeiter S, et al. (2003) Role of nuclear bile acid receptor, FXR, in adaptive ABC transporter regulation by cholic and ursodeoxycholic acid in mouse liver, kidney and intestine. J Hepatol. 39:480-488. DOI: 10.1016/S0168-8278(03)00228-9

25. Dombrowski F, Stieger B, Beuers U. (2006) Tauroursodeoxycholic acid inserts the bile salt export pump into canalicular membranes of cholestatic rat liver. Lab Invest. 86:166-174. DOI: 10.1038/labinvest.3700371

26. Beuers U, Bilzer M, Chittattu A, Kullak-Ublick GA, Keppler D, Paumgartner G, et al. (2001) Tauroursodeoxycholic acid inserts the apical conjugate export pump, Mrp2, into canalicular membranes and stimulates organic anion secretion by protein kinase C-dependent mechanisms in cholestatic rat liver. Hepatology. 33:1206-1216. DOI: 10.1053/jhep.2001.24034

27. Okan A, Astarcioglu H, Tankurt E, Sagol O, Altekin E, Astarcioglu I, et al. (2002) Effect of ursodeoxycholic acid on hepatic steatosis in rats. Dig Dis Sci. 47:2389-2397. DOI: 10.1023/A:1020523017873

28. Sánchez Pozzi EJ, Crocenzi FA, Pellegrino JM, Catania VA, Luquita MG, Roma MG, et al. (2003) Ursodeoxycholate reduces ethinylestradiol glucuronidation in the

rat: role in prevention of estrogen-induced cholestasis. J Pharmacol Exp Ther. 306:279-286. DOI: 10.1124/jpet.103.049940

29. Crocenzi FA, D'Andrea V, Catania VA, Luquita MG, Pellegrino JM, Ochoa JE, et al. (2005) Prevention of Mrp2 activity impairment in ethinylestradiol-induced cholestasis by ursodeoxycholate in the rat. Drug Metab Dispos. 33:888-891. DOI: 10.1124/dmd.104.003533
30. Lindor KD, Kowdley KV, Luketic VAC, Harrison ME, McCashland T, Befeler AS, et al. (2009) High-dose ursodeoxycholic acid for the treatment of primary sclerosing cholangitis. Hepatology. 50:808-814. DOI: 10.1002/hep.23082
31. Esteller A. (2008) Physiology of bile secretion. World J Gastroenterol. 14:5641-5649. DOI: 10.3748/wjg.14.5641
32. Talalay P. (1960) Enzymatic analysis of steroid hormones. Methods Biochem Anal. 8:119-143. DOI: 10.1002/9780470110249.ch3
33. Tietze F. (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem. 27:502-522. DOI: 10.1016/0003-2697(69)90064-5
34. Griffith OW. (1980) Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem. 106:207-212. DOI: 10.1016/0003-2697(80)90139-6
35. Mottino AD, Cao J, Veggi LM, Crocenzi FA, Roma MG, Vore M. (2002) Altered localization and activity of canalicular Mrp2 in estradiol-17b-D-glucuronide-induced cholestasis. Hepatology. 35:1409-1419. DOI: 10.1053/jhep.2002.33327
36. Mottino AD, Crocenzi FA, Pozzi EJ, Veggi LM, Roma MG, Vore M. (2005) Role of microtubules in estradiol-17β-D-glucuronide-induced alteration of canalicular Mrp2

localization and activity. Am J Physiol Gastrointest Liver Physiol. 288:G327-G336. DOI: 10.1152/ajpgi.00227.2004

37. Crocenzi FA, Mottino AD, Cao J, Veggi LM, Sánchez Pozzi EJ, Vore M, et al. (2003) Estradiol-17β-D-glucuronide induces endocytic internalization of Bsep in rats. Am J Physiol Gastrointest Liver Physiol. 285:G449-G459. DOI: 10.1152/ajpgi.00508.2002
38. Cregger M, Berger AJ, Rimm DL. (2006) Immunohistochemistry and quantitative analysis of protein expression. Arch Pathol Lab Med. 130:1026-1030. DOI: 10.1043/1543-2165(2006)130[1026:IAQAOP]2.0.CO;2
39. Gerloff T, Stieger B, Hagenbuch B, Mandon J, Landmann L, Roth J, et al. (1998) The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. J Biol Chem. 273:10046-10050. DOI: 10.1074/jbc.273.16.10046
40. Cui Y, Konig J, Keppler D. (2001) Vectorial transport by double-transfected cells expressing the human uptake transporter SLC21A8 and the apical export pump ABCC2. Mol Pharmacol. 60:934-943. DOI: 10.1124/mol.60.5.934
41. Tanaka H, Sano N, Takikawa H. (2003) Biliary excretion of phenolphthalein sulfate in rats. Pharmacology. 68:177-182. DOI: 10.1159/000070456
42. Yokota M, Iga T, Sugiyama Y, Suyama A, Awazu S, Hanano M. (1981) Comparative hepatic transport of sulfobromophthalein and its glutathione conjugate in rats. J Pharmacobiodyn. 4:287-293. DOI: 10.1248/bpb1978.4.287
43 Tietz, PS, Thiestle, JL, Miller, LJ, LaRusso NF. (1984) Development and validation of a method for measuring the glycine and taurine conjugates of bile acids in bile by high-performance liquid chromatography. J Chromatogr. 336:249-257. DOI: 10.1016/S0378-4347(00)85148-8

44. Jansen PLM, Groothuis GMM, Peters WHM, Meijer DFM. (1987) Selective hepatobiliary transport defect for organic anions and neutral steroids in mutant rats with hereditary-conjugated hyperbilirubinemia. Hepatology. 7:71-76. DOI: 10.1002/hep.1840070116

45. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics. 13:134. DOI: 10.1186/1471-2105-13-134

46. Ruiz ML, Villanueva SS, Luquita MG, Ikushiro S, Mottino AD, Catania VA. (2007) Beneficial effect of spironolactone administration on ethynylestradiol-induced cholestasis in the rat: involvement of up-regulation of multidrug resistance-associated protein 2. Drug Metab Dispos. 35:2060-2066. DOI: 10.1124/dmd.107.016519

47. Pfaffl MW. (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29:e45. DOI: 10.1093/nar/29.9.e45

48. Boaglio AC, Zucchetti AE, Sanchez Pozzi EJ, Pellegrino JM, Ochoa JE, Mottino AD, et al. (2010) Phosphoinositide 3-kinase/protein kinase B signaling pathway is involved in estradiol 17beta-D-glucuronide-induced cholestasis: complementarity with classical protein kinase C. Hepatology. 52:1465-1476. DOI: 10.1002/hep.23846

49.. Moss DW. (1997) Physicochemical and pathophysiological factors in the release of membrane-bound alkaline phosphatase from cells. Clin Chim Acta. 257:133-140. DOI: 10.1016/S0009-8981(96)06438-8

50. Geier A, Fickert P, Trauner M. (2006) Mechanisms of disease: mechanisms and clinical implications of cholestasis in sepsis. Nat Clin Pract Gastroenterol Hepatol. 3:574-585. DOI: 10.1038/ncpgasthep0602

51. Chand N, Sanyal AJ. (2007) Sepsis-induced cholestasis. Hepatology. 45:230-241. DOI: 10.1002/hep.21480

52. Gilroy RK, Mailliard ME, Gollan JL. (2003) Gastrointestinal disorders of the critically ill. Cholestasis of sepsis. Best Pract Res Clin Gastroenterol. 17:357-367. DOI: 10.1016/S1521-6918(03)00027-1

53. Kaplan MM, Righetti A. (1970) Induction of rat liver alkaline phosphatase: the mechanism of the serum elevation in bile duct obstruction. J Clin Invest. 49:508-516. DOI: 10.1172/JCI106260

54. Billington D, Evans CE, Godfrey PP, Coleman R. (1980) Effects of bile salts on the plasma membranes of isolated rat hepatocytes. Biochem J. 188:321-327. DOI: 10.1042/bj1880321

55. Basiglio CL, Mottino AD, Roma MG. (2010) Tauroursodeoxycholate counteracts hepatocellular lysis induced by tensioactive bile salts by preventing plasma membrane-micelle transition. Chem Biol Interact. 188:386-392. DOI: 10.1016/j.cbi.2010.08.004

56. Carulli N, Loria P, Bertolotti M, Ponz de Leon M, Menozzi D, Medici G, et al. (1984) Effects of acute changes of bile acid pool composition on biliary lipid secretion. J Clin Invest. 74:614-624. DOI: 10.1172/JCI111459

57. Accatino L, Figueroa C, Pizarro M, Solis N. (1995) Enhanced biliary excretion of canalicular membrane enzymes in estrogen-induced and obstructive cholestasis,

and effects of different bile acids in the isolated perfused rat liver. J Hepatol. 22:658-670. DOI: 10.1016/0168-8278(95)80221-5

58. Teng S, Piquette-Miller M. (2005) The involvement of the pregnane X receptor in hepatic gene regulation during inflammation in mice. J Pharmacol Exp Ther. 312:841-848. DOI: 10.1124/jpet.104.076141
59. Zhang L, Su H, Li Y, Fan Y, Wang Q, Jaing J, et al. (2018) Different effects of ursodeoxycholic acid on intrahepatic cholestasis in acute and recovery stages induced by alpha-naphthylisothiocyanate in mice. Toxicol Appl Pharmacol. 342:69-78. DOI: 10.1016/j.taap.2018.01.019
60. Fickert P, Zollner G, Fuchsbichler A, Stumptner C, Pojer C, Zenz R, et al. (2001) Effects of ursodeoxycholic and cholic acid feeding on hepatocellular transporter expression in mouse liver. Gastroenterology. 121:170-183. DOI: 10.1053/gast.2001.25542
61. Marschall HU, Wagner M, Zollner G, Fickert P, Diczfalusy U, Gumhold J, et al. (2005) Complementary stimulation of hepatobiliary transport and detoxification systems by rifampicin and ursodeoxycholic acid in humans. Gastroenterology. 129:476-485. DOI: 10.1016/j.gastro.2005.05.009
62. Beigneux AP, Moser AH, Shigenaga JK, Grunfeld C, Feingold KR. (2000) The acute phase response is associated with retinoid X receptor repression in rodent liver. J Biol Chem. 275:16390-16399. DOI: 10.1074/jbc.M000953200
63. Geier A, Dietrich CG, Voigt S, Ananthanarayanan M, Lammert F, Schmitz A, et al. (2005) Cytokine-dependent regulation of hepatic organic anion transporter gene transactivators in mouse liver. Am J Physiol Gastrointest Liver Physiol. 289:G831-G841. DOI: 10.1152/ajpgi.00307.2004

64. Kim MS, Shigenaga J, Moser A, Feingold K, Grunfeld C. (2003) Repression of farnesoid X receptor during the acute phase response. J Biol Chem. 278:8988-8995. DOI: 10.1074/jbc.M212633200

65. Li D, Zimmerman TL, Thevananther S, Lee HY, Kurie JM, Karpen SJ. (2002) Interleukin-1 β-mediated suppression of RXR:RAR transactivation of the Ntcp promoter is JNK-dependent. J Biol Chem. 277:31416-31422. DOI: 10.1074/jbc.M204818200

66. Sommerfeld A, Reinehr R, Haussinger D. (2015) Tauroursodeoxycholate protects rat hepatocytes from bile acid-induced apoptosis via β1-Integrin- and protein kinase A-dependent mechanisms. Cell Physiol Biochem. 36:866-883. DOI: 10.1159/000430262

67. Pfaffenbach KT, Gentile CL, Nivala AM, Wang D, Wei Y, Pagliassotti MJ. (2010) Linking endoplasmic reticulum stress to cell death in hepatocytes: roles of C/EBP homologous protein and chemical chaperones in palmitate-mediated cell death. Am J Physiol Endocrinol Metab. 298:E1027-E1035. DOI: 10.1152/ajpendo.00642.2009

68. Yang B, Hill CE. (2001) Nifedipine modulation of biliary GSH and GSSG/conjugate efflux in normal and regenerating rat liver. Am J Physiol. 281:G85-G94. DOI: 10.1152/ajpgi.2001.281.1.G85

69. Zinchuk V, Zinchuk O, Okada T. (2005) Experimental LPS-induced cholestasis alters subcellular distribution and affects colocalization of Mrp2 and Bsep proteins: a quantitative colocalization study. Microsc Res Tech. 67:65-70. DOI: 10.1002/jemt.20184

70. Aida K, Hayashi H, Inamura K, Mizuno T, Sugiyama Y. (2014) Differential roles of ubiquitination in the degradation mechanism of cell surface-resident bile salt export

pump and multidrug resistance-associated protein 2. Mol Pharmacol. 85:482-491. DOI: 10.1124/mol.113.091090

71. Hayashi H, Sugiyama Y. (2007) 4-phenylbutyrate enhances the cell surface expression and the transport capacity of wild-type and mutated bile salt export pumps. Hepatology. 45:1506-1516. DOI: 10.1002/hep.21630
72. Saadane A, Delautier D, Leboucher J, Kharbajou M, Feldmann G, Lardeux B, et al. (1999) Stimulation of liver RNA and protein breakdown in endotoxemic rats: role of glucocorticoids. Shock. 11:429-435. DOI: Not found.
73. Basiglio CL, Toledo FD, Boaglio AC, Arriaga SM, Ochoa JE, Sánchez Pozzi EJ, et al. (2014) Physiological concentrations of unconjugated bilirubin prevent oxidative stress-induced hepatocanalicular dysfunction and cholestasis. Arch Toxicol. 88:501-514. DOI: 10.1007/s00204-013-1143-0
74. Ji B, Ito K, Sekine S, Tajima A, Horie T. (2004) Ethacrynic-acid-induced glutathione depletion and oxidative stress in normal and Mrp2-deficient rat liver. Free Radic Biol Med. 37:1718-1729. DOI: 10.1016/j.freeradbiomed.2004.08.020
75. Sekine S, Yano K, Saeki J, Hashimoto N, Fuwa T, Horie T. (2010) Oxidative stress is a triggering factor for LPS-induced Mrp2 internalization in the cryopreserved rat and human liver slices. Biochem Biophys Res Commun. 399:279-285. DOI: 10.1016/j.bbrc.2010.07.069
76. Yano K, Sekine S, Nemoto K, Fuwa T, Horie T. (2010) The effect of dimerumic acid on LPS-induced downregulation of Mrp2 in the rat. Biochem Pharmacol. 80:533-539. DOI: 10.1016/j.bcp.2010.04.036

77. Carey MC, Duane WC (1994). Enterohepatic circulation; in Arias IM, Boyer JL, Fausto N, Jakoby WB, Schachter DA, Shafritz DA (eds): *The liver: Biology and Pathobiology.* New York, Raven Press, pp 719-767.

78. Halilbasic E, Claudel T, Trauner M. (2013) Bile acid transporters and regulatory nuclear receptors in the liver and beyond. J Hepatol. 58:155-168. DOI: 10.1016/j.jhep.2012.08.002

79. Stiehl A, Raedsch R, Rudolph G. (1990) Acute effects of ursodeoxycholic and chenodeoxycholic acid on the small intestinal absorption of bile acids. Gastroenterology. 98:424-428. DOI: 10.1016/0016-5085(90)90834-N

80. Fukushima K, Ichimiya H, Higashijima H, Yamashita H, Kuroki S, Chijiiwa K, et al. (1995) Regulation of bile acid synthesis in the rat: relationship between hepatic cholesterol 7 α-hydroxylase activity and portal bile acids. J Lipid Res. 36:315-321. DOI: Not found

81. Donner MG, Warskulat U, Saha N, Haussinger D. (2004) Enhanced expression of basolateral multidrug resistance protein isoforms Mrp3 and Mrp5 in rat liver by LPS. Biol Chem. 385:331-339. DOI: 10.1515/BC.2004.029

82. Rius M, Nies AT, Hummel-Eisenbeiss J, Jedlitschky G, Keppler D. (2003) Cotransport of reduced glutathione with bile salts by MRP4 (ABCC4) localized to the basolateral hepatocyte membrane. Hepatology. 38:374-384. DOI: 10.1053/jhep.2003.50331

83. Zollner G, Wagner M, Moustafa T, Fickert P, Silbert D, Gumhold J, et al. (2006) Coordinated induction of bile acid detoxification and alternative elimination in mice: role of FXR-regulated organic solute transporter-alpha/beta in the adaptive

response to bile acids. Am J Physiol Gastrointest Liver Physiol. 290:G923-G932. DOI: 10.1152/ajpgi.00490.2005

84. Kagawa T, Orii R, Hirose S, Arase Y, Shiraishi K, Mizutani A, et al. (2014) Ursodeoxycholic acid stabilizes the bile salt export pump in the apical membrane in MDCK II cells. J Gastroenterol. 49:890-899. DOI: 10.1007/s00535-013-0833-y
85. Hayashi H, Inamura K, Aida K, Naoi S, Horikawa R, Nagasaka H, et al. (2012) AP2 adaptor complex mediates bile salt export pump internalization and modulates its hepatocanalicular expression and transport function. Hepatology. 55:1889-1900. DOI: 10.1002/hep.25591
86. Miszczuk GS, Barosso IR, Larocca MC, Marrone J, Marinelli RA, Boaglio AC, et al. (2018) Mechanisms of canalicular transporter endocytosis in the cholestatic rat liver. Biochim Biophys Acta Mol Basis Dis. 1864:1072-1085. DOI: 10.1016/j.bbadis.2018.01.015
87. Paulusma CC, de Waart DR, Kunne C, Mok KS, Elferink RP. (2009) Activity of the bile salt export pump (ABCB11) is critically dependent on canalicular membrane cholesterol content. J Biol Chem. 284:9947-9954. DOI: 10.1074/jbc.M808667200
88. Guyot C, Hofstetter L, Stieger B. (2014) Differential effects of membrane cholesterol content on the transport activity of multidrug resistance-associated protein 2 (ABCC2) and of the bile salt export pump (ABCB11). Mol Pharmacol. 85:909-920. DOI: 10.1124/mol.114.092262
89. Lim SC, Duong HQ, Choi JE, Lee TB, Kang JH, Oh SH, et al. (2011) Lipid raft-dependent death receptor 5 (DR5) expression and activation are critical for ursodeoxycholic acid-induced apoptosis in gastric cancer cells. Carcinogenesis. 32:723-731. DOI: 10.1093/carcin/bgr038

90. Kubitz R, Sutfels G, Kuhlkamp T, Kolling R, Haussinger D. (2004) Trafficking of the bile salt export pump from the Golgi to the canalicular membrane is regulated by the p38 MAP kinase. Gastroenterology. 126:541-553. DOI: 10.1053/j.gastro.2003.11.003

91. Kurz AK, Graf D, Schmitt M, vom DS, Haussinger D. (2001) Tauroursodeoxycholate-induced choleresis involves p38(MAPK) activation and translocation of the bile salt export pump in rats. Gastroenterology. 121:407-419. DOI: 10.1053/gast.2001.26262

92. Boaglio AC, Miszczuk GS, Barosso IR, Toledo FD, Crocenzi FA, Roma MG. (2014) Tauroursodeoxycholate (TUDC) prevents activation of the pro-cholestatic signalling pathways, cPKC and PI3K/Akt, in estradiol 17ß-d-glucuronide ($E_2$17G)-induced cholestasis. J.Hepatol. 60 (Suppl. 1), S182. DOI: Not found.

93. He H, Mennone A, Boyer JL, Cai SY. (2011) Combination of retinoic acid and ursodeoxycholic acid attenuates liver injury in bile duct-ligated rats and human hepatic cells. Hepatology. 53:548-557. DOI: 10.1002/hep.24047

94. Diamond T, Dolan S, Thompson RL, Rowlands BJ. (1990) Development and reversal of endotoxemia and endotoxin-related death in obstructive jaundice. Surgery. 108:370-374. DOI: Not found.

95. Fausto N, Campbell JS, Riehle KJ. (2006) Liver regeneration. Hepatology. 43:S45-S53. DOI: 10.1002/hep.20969

96. Nolan JP. (1989) Intestinal endotoxins as mediators of hepatic injury--an idea whose time has come again. Hepatology. 10:887-891. DOI: 10.1002/hep.1840100523

97. Kosters A, Karpen SJ. (2010) The role of inflammation in cholestasis: clinical and basic aspects. Semin Liver Dis. 30:186-194. DOI: 10.1055/s-0030-1253227

98. Geier A, Wagner M, Dietrich CG, Trauner M. (2007) Principles of hepatic organic anion transporter regulation during cholestasis, inflammation and liver regeneration. Biochim Biophys Acta. 1773:283-308. DOI: 10.1016/j.bbamcr.2006.04.014

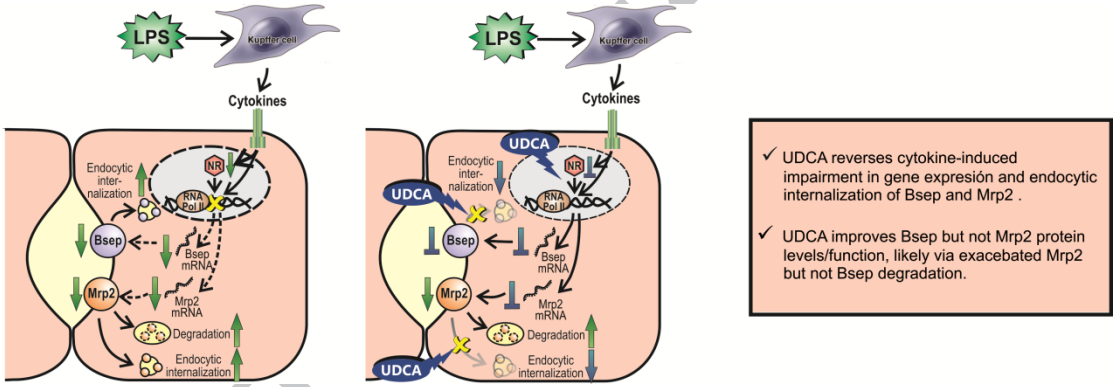
Figure legends

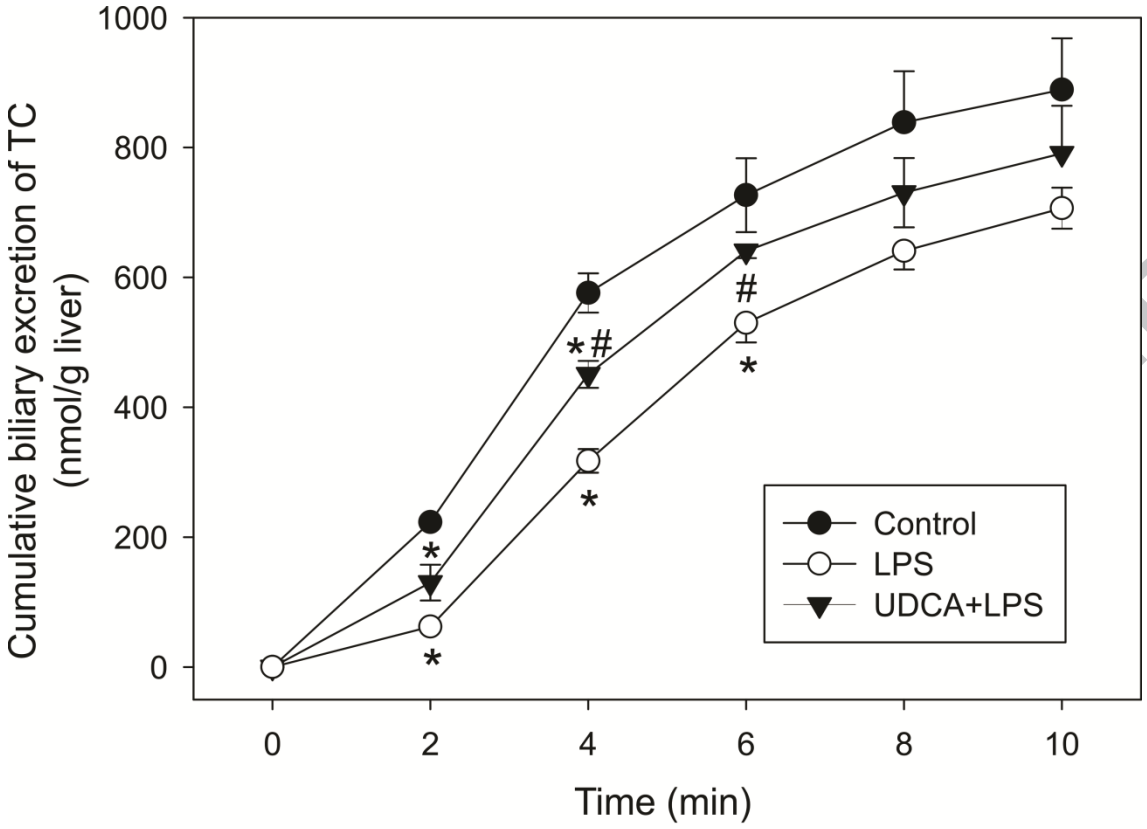
Figure 1. *Upper panel.* Cumulative biliary excretion of TC; bile samples were collected every 2 min for 10 min, after a single, i.v. dose of 8 μmol/100 g of b.w. of the bile salt. *Lower panel.* Area under the curve (AUC) of cumulative biliary excretion of TC graphs. Data are mean value ± SEM for 3-4 individual experiments. *$P<0.05$ *vs.* control; #$P<0.05$ *vs.* LPS.

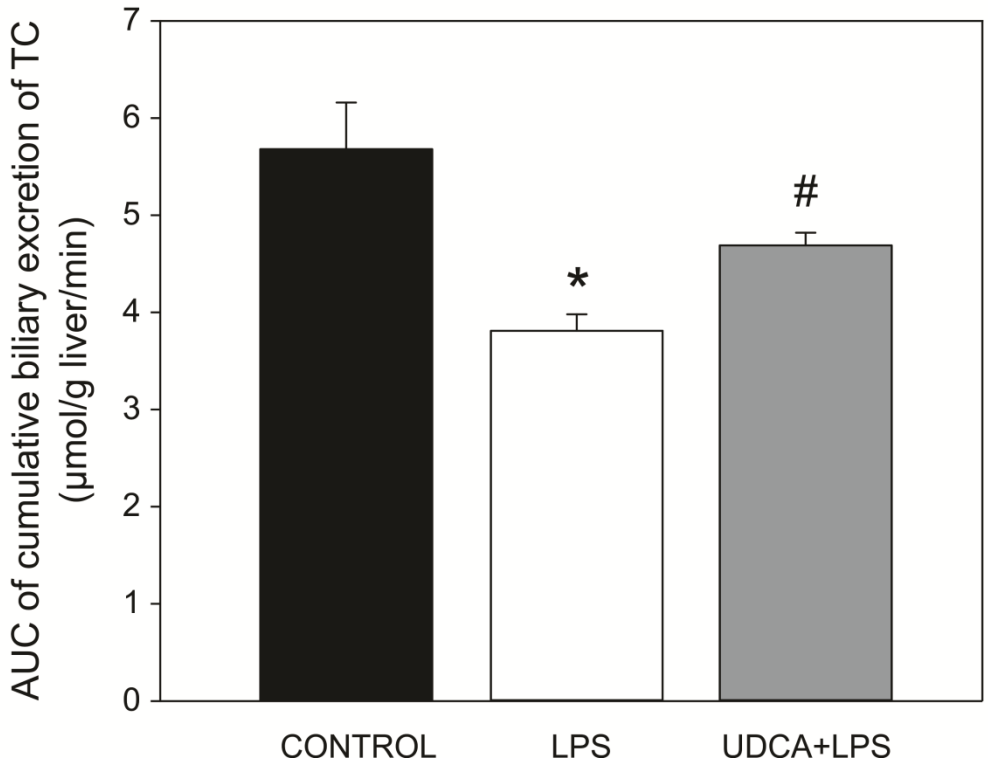
Figure 2. *Upper panel.* Cumulative biliary excretion of BSP; bile samples were collected every 5 min for 60 min, after a single, i.v. dose of 3 mg/100 g of b.w. *Lower panel.* Area under the curve (AUC) of cumulative biliary excretion of BSP graphs. Data are mean value ± SEM for 3-4 individual experiments. *$P<0.05$ *vs.* control; #$P<0.05$ *vs.* LPS.

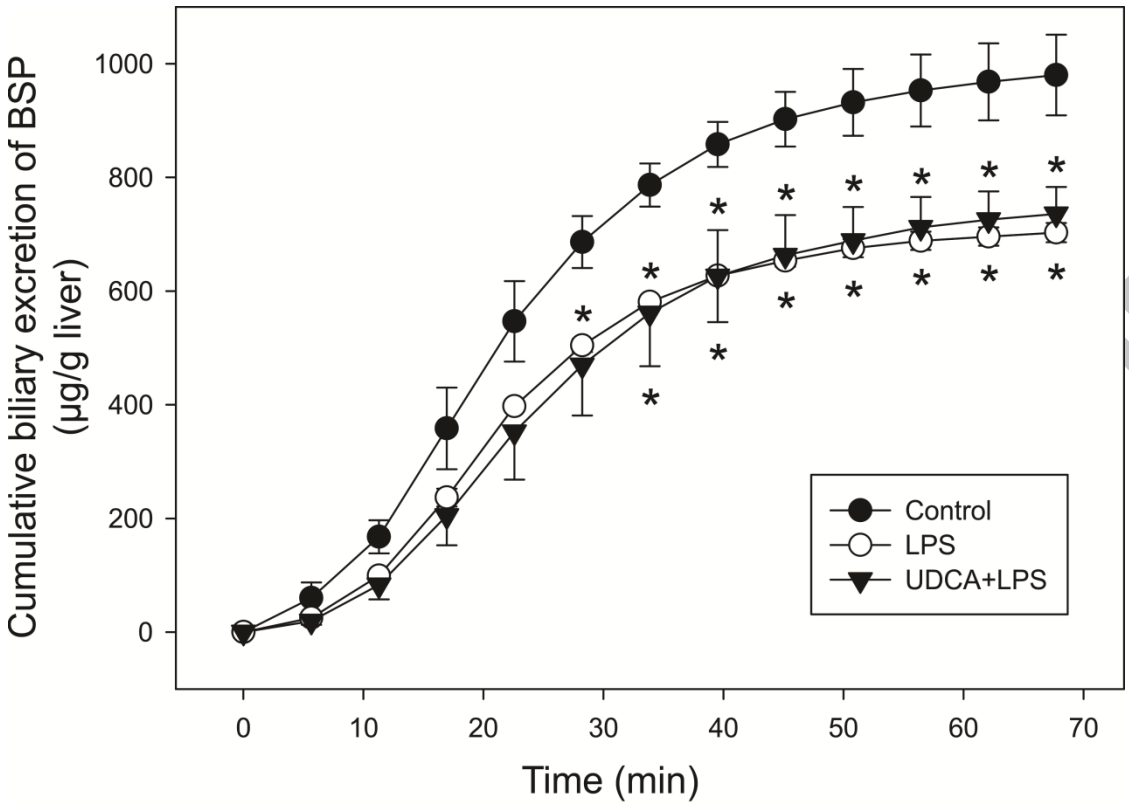
Figure 3. *Effect of UDCA co-treatment on LPS-induced endocytic internalization of Bsep. A.* Representative confocal images showing co-staining of Bsep (green) with occludin (red; used to visualize the bile canaliculus limits), illustrative of the endocytic internalization of Bsep induced by LPS (arrows), and its protection by UDCA. The lower panels show densitometric analysis of the fluorescence intensity associated with Bsep or occludin along a 8 μm line perpendicular to the canaliculus (from -4 μm to +4 μm from the canalicular centre), corresponding to the confocal images shown in the upper panels. Data are mean values for at least 15 different canaliculi. Data are shown as mean ± SEM.

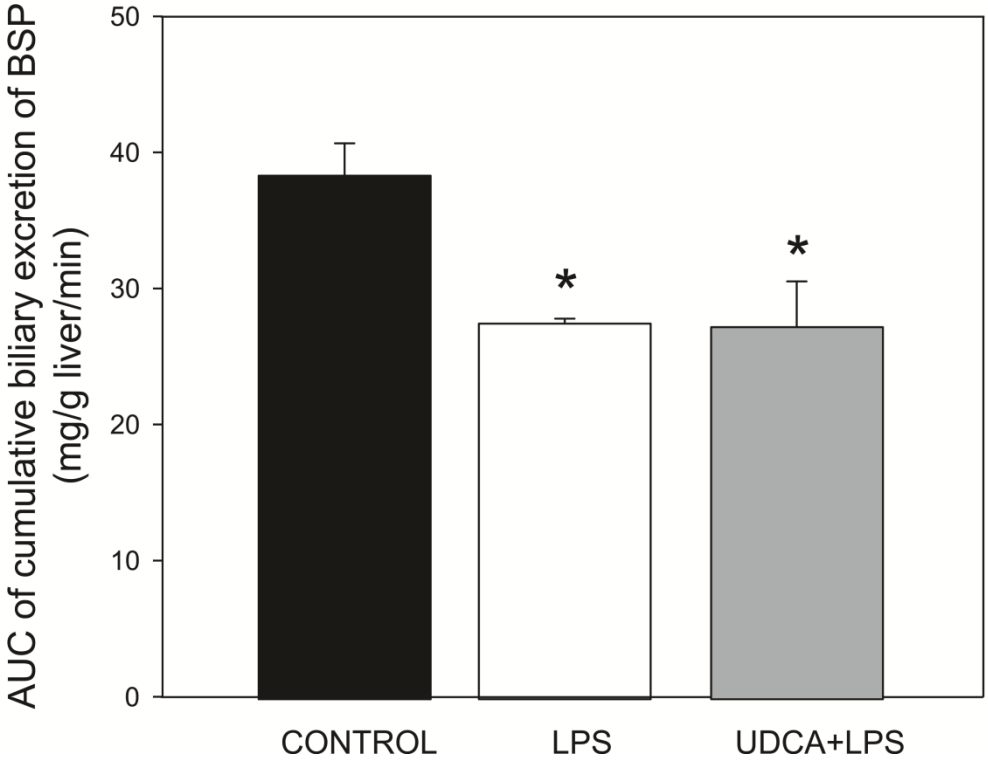
Figure 4. *Effect of UDCA co-treatment on LPS-induced endocytic internalization of Mrp2. A.* Representative confocal images showing co-staining of Mrp2 (green) with occludin (red; used to visualize the bile canaliculus limits), illustrative of the endocytic internalization of Mrp2 induced by LPS (arrows), and its protection by UDCA. The lower panels show densitometric analysis of the fluorescence intensity associated with Mrp2 or occludin along a 8 µm line perpendicular to the canaliculus (from -4 µm to +4 µm from the canalicular center), corresponding to the confocal images shown in the upper panels. Data are mean values for at least 15 different canaliculi. Data are shown as mean ± SEM.

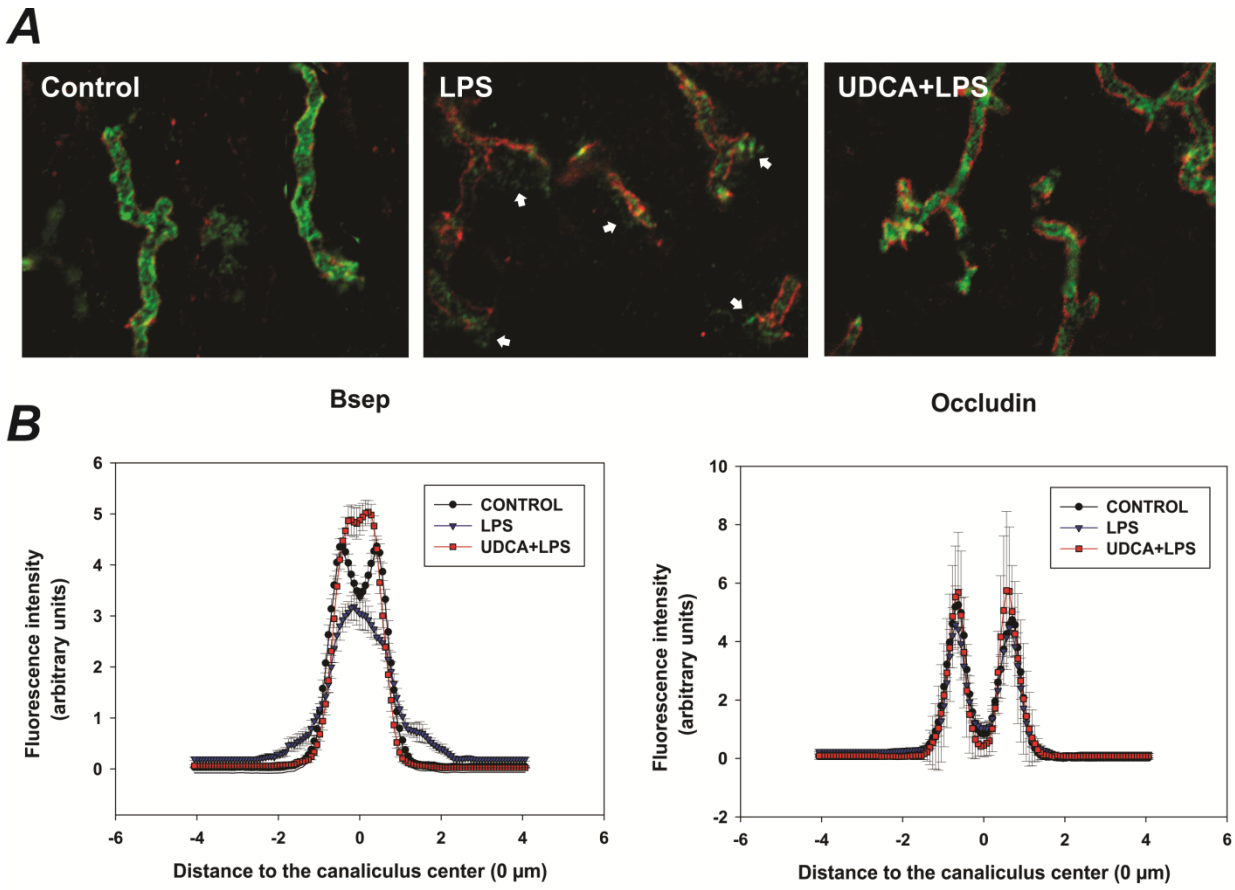


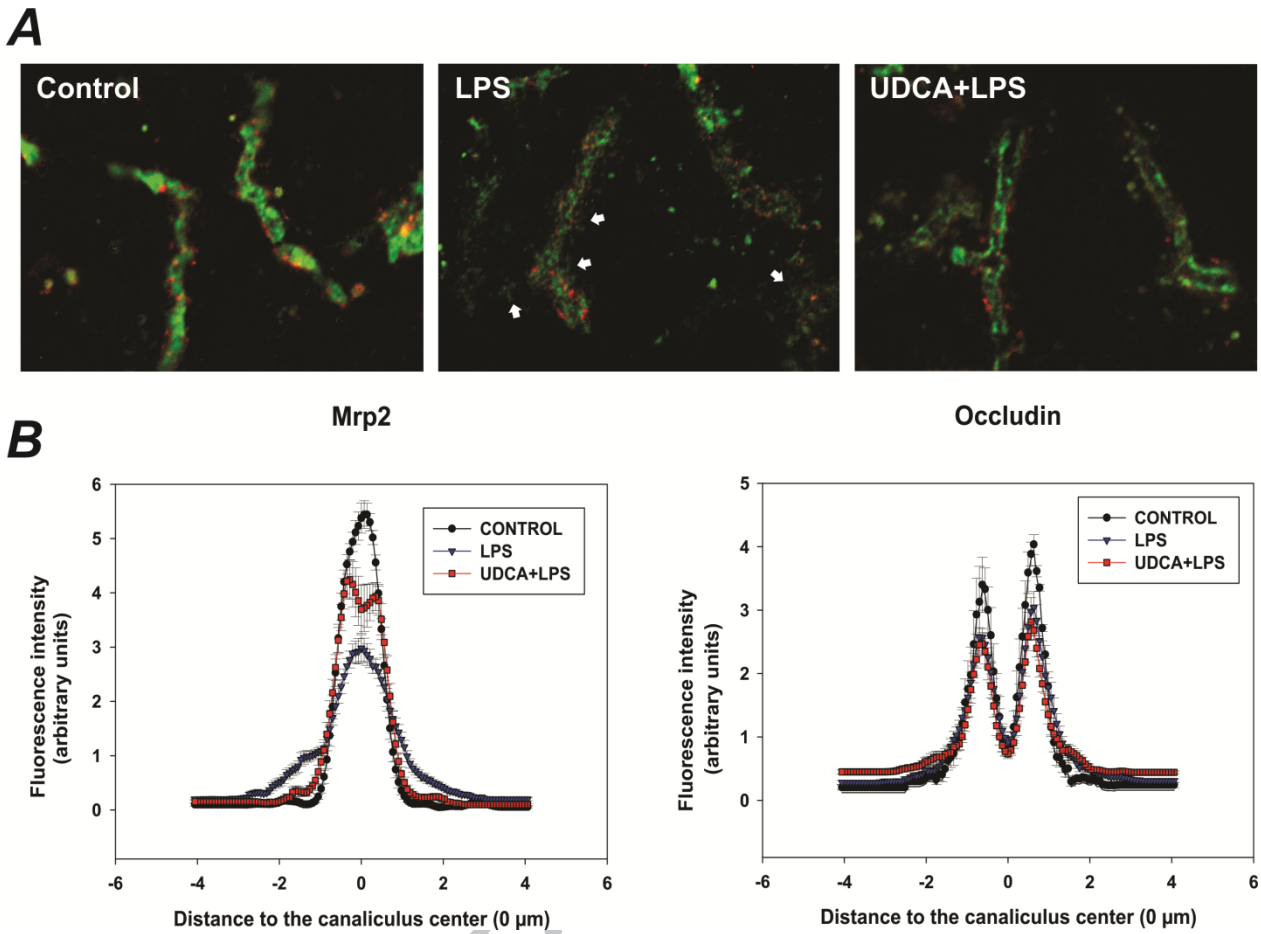

Cumulative biliary excretion of TC
(nmol/g liver)
1000
800
600
400
200
0
0
2
4
6
8
10
Time (min)
Control
LPS
UDCA+LPS
*
*#
*
#
*
*

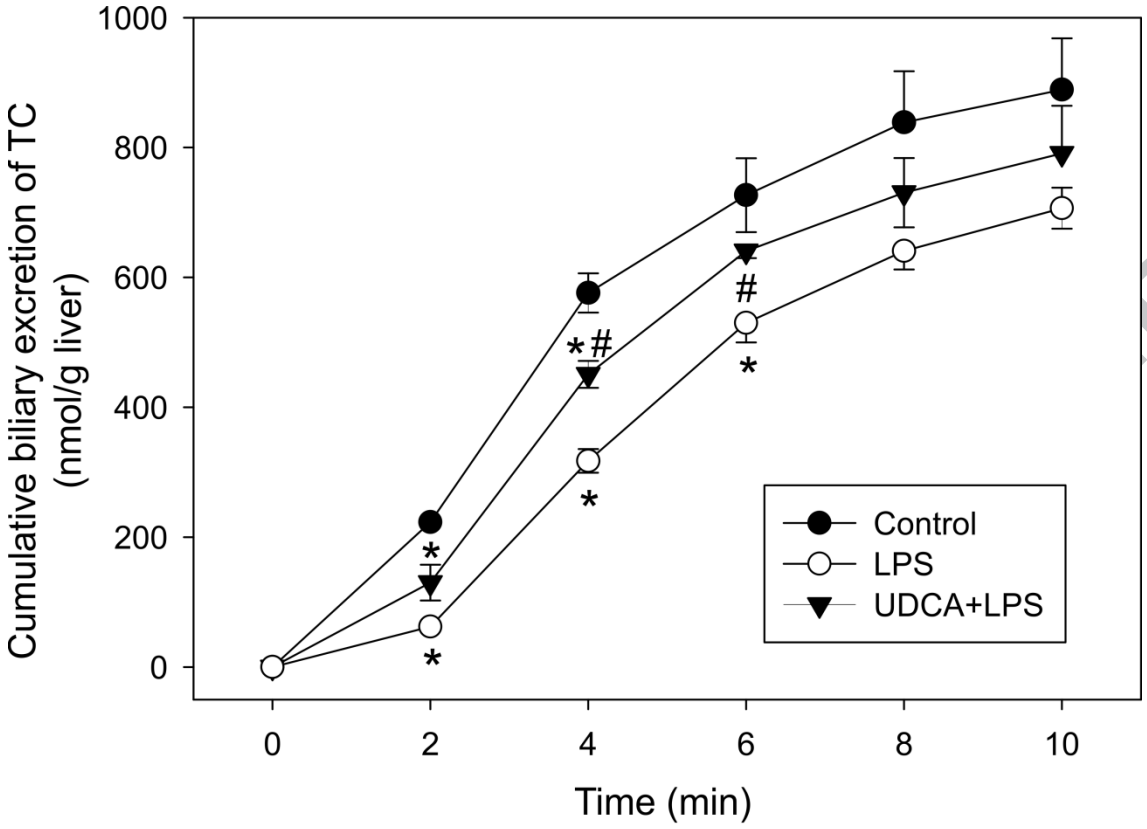

AUC of cumulative biliary excretion of TC
(µmol/g liver/min)
7
6
5
4
3
2
1
0
*
#
CONTROL
LPS
UDCA+LPS

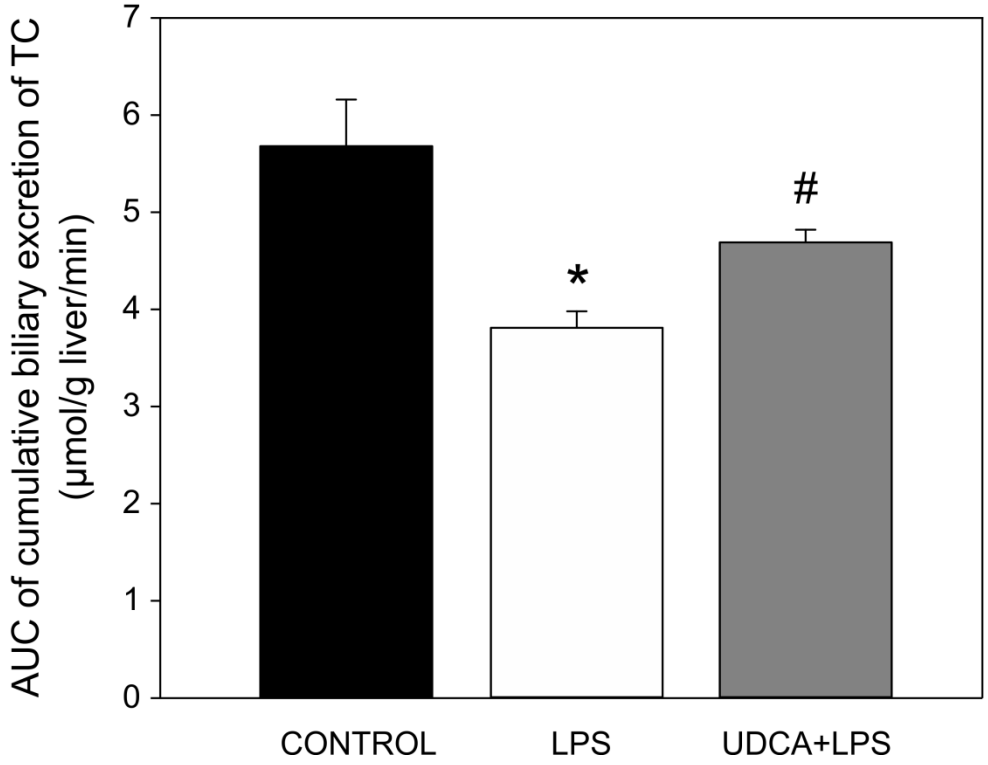

Cumulative biliary excretion of BSP
(μg/g liver)
1000
800
600
400
200
0
0
10
20
30
40
50
60
70
Time (min)
Control
LPS
UDCA+LPS

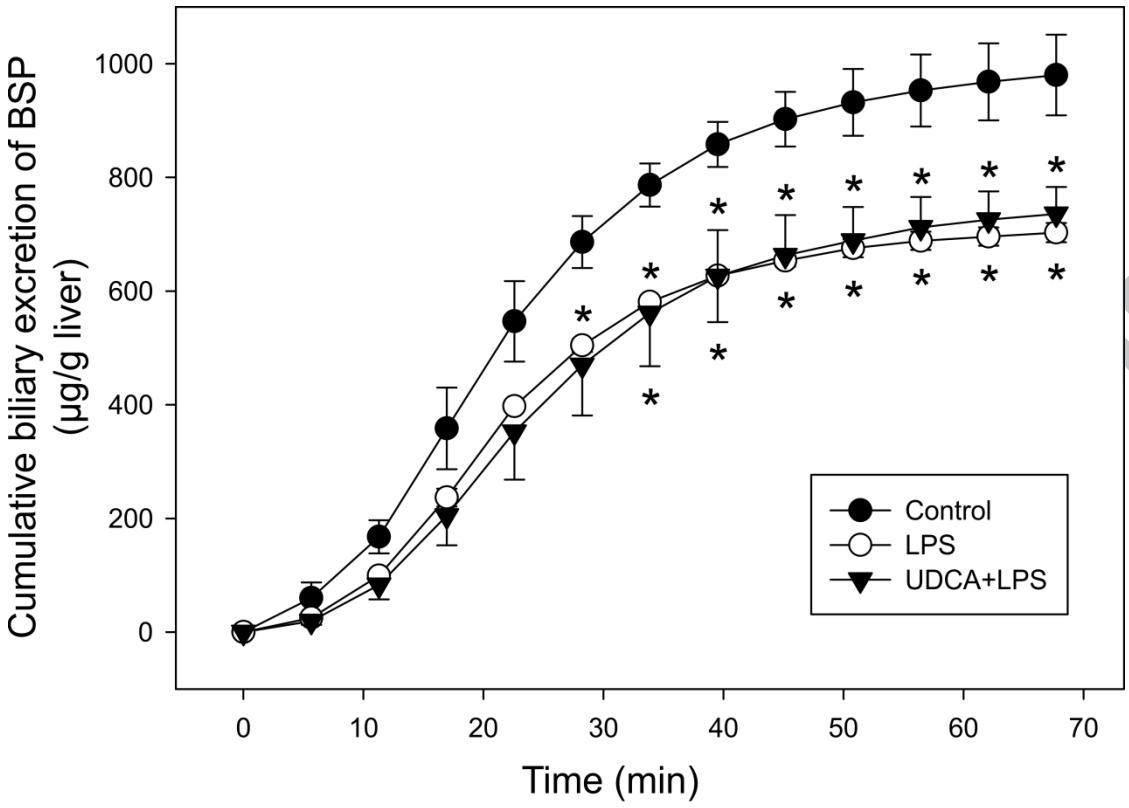

AUC of cumulative biliary excretion of BSP
(mg/g liver/min)
50
40
30
20
10
0
CONTROL
LPS
UDCA+LPS

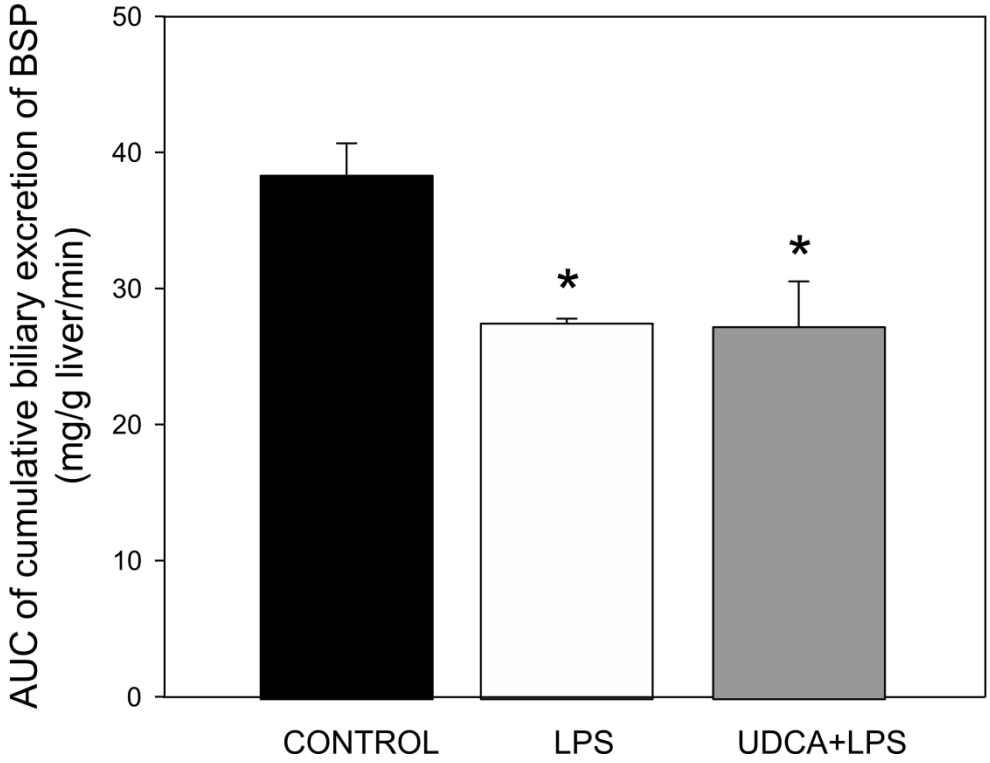





Cumulative biliary excretion of TC
(nmol/g liver)
1000
800
600
400
200
0
0
2
4
6
8
10
Time (min)
Control
LPS
UDCA+LPS
*
*#
*
#
*
*


AUC of cumulative biliary excretion of TC
(µmol/g liver/min)
7
6
5
4
3
2
1
0
*
#
CONTROL
LPS
UDCA+LPS





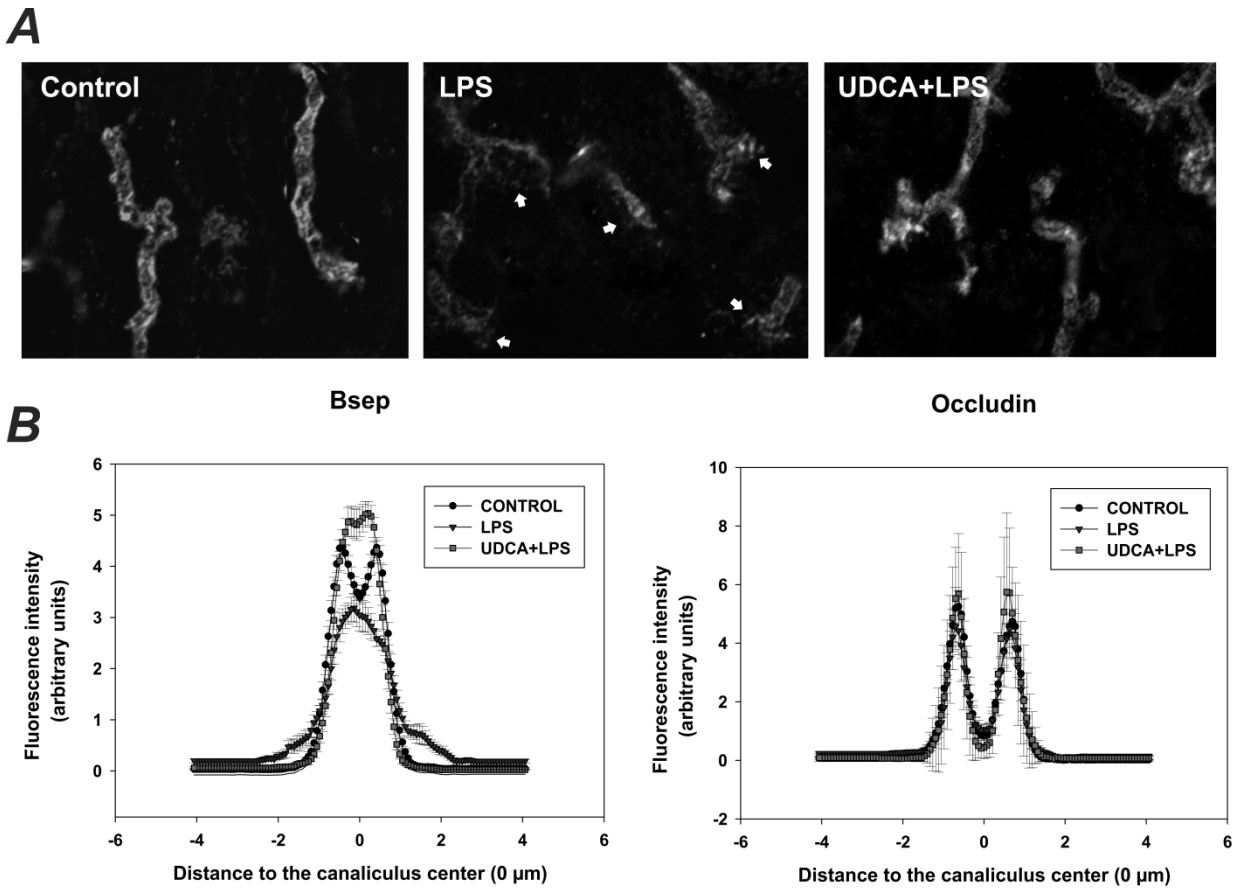


Table 1. Sequence of primers for RT-PCR analysis of rat Abcb11 and Abcc2 mRNAs, and 18s rRNA.

	Primer	Primer sequence (5' → 3')
Abcb11 (Bsep)	Forward	CCGAAGGCTCAGGGTATTGG
	Reverse	ATCAGGTGACATGGTGGCAG
Abcc2 (Mrp2)	Forward	ACCTTCCACGTAGTGATCCT
	Reverse	ACCTGCTAAGATGGACGGTC
18s rRNA	Forward	GTAACCCGTTGAACCCCATT
	Reverse	CCATCCAATCGGTAGTAGCG

Table 2. Plasma levels of ALP and the pro-inflammatory cytokines, TNF-α and IL-6.

	Control	LPS	UDCA+LPS
ALP (U/l)	207±23	404±21*	316±24*#
TNF-α (pg/ml)	49±6	133±33*	100±22*
IL6 (pg/mL)	N.D.	3604 ±603*	3312 ±558*

*P<0.05 *vs.* control; #P<0.05 *vs.* LPS; N.D. = No detectable. n = 6-22.

Table 3. Bile flow and biliary excretion of total bile acids and total GSH.

	Control	LPS	UDCA+LPS
Bile flow (µl/min per g liver)			
Basal	3.4 ± 0.1	2.7 ± 0.1*	2.6 ± 0.1*
TC-stimulated†	5.3 ± 0.2	4.1 ± 0.2*	4.7 ± 0.1#
Bile acid output (nmol/min per g liver)			
Basal	128 ± 16	94 ± 13	86 ± 13
TC-stimulated†	226 ± 16	152 ± 7*	216 ± 5#
Total GSH output (nmol/min per g liver)	3.7 ± 0.7	1.3 ± 0.2*	0.8 ± 0.1*

†TC-stimulated values correspond to the maximum value obtained after a TC i.v. injection of 8 µmol/100 g of b.w. *P<0.05 *vs.* control; #P<0.05 *vs.* LPS. n= 3-26.

mRNA levels Total fluorescence intensity (% of mean control values)	Control	LPS	UDCA+LPS
Mrp2	100 ± 7 100 ± 7	25 ± 10* 41 ± 2*	190 ± 49# 31 ± 2*
Bsep	100 ± 29 100 ± 5	15 ± 3* 69 ± 3*	79 ± 3# 163 ± 7*#

Table 4. Mrp2 and Bsep expression by measuring total fluorescence intensity.

*P<0.05 *vs.* control; #P<0.05 *vs.* LPS

Table 5. Variance ranges of the fluorescence intensity profiles.

	Control	LPS	UDCA+LPS
Bsep	0.25 - 1.00	0.66 - 2.29*	0.12 - 1.24#
Mrp2	0.12 - 1.56	0.78 - 2.83*	0.25 - 2.15#

*P<0.05 *vs.* control; #P<0.05 *vs.* LPS; n= 15-22.

Table 6. Mrp2 and Bsep mRNA expression by RT-PCR.

*P<0.05 vs. control; #P<0.05 vs. LPS; n = 3-4.