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Hepatic and extrahepatic synthesis and disposition of dinitrophenyl-S-glutathione in bile duct ligated rats.

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Running title: Extrahepatic elimination of DNP-SG in BDL rats.

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Abbreviations:

CDNB - 1-chloro-2,4-dinitrobenzene, DNP-SG - dinitrophenyl-S-glutathione, DNP-CG - dinitrophenyl cysteinyl glycine, Mrp1, Mrp2 and Mrp3 - multidrug resistance-associated proteins 1, 2 and 3, γGT - γ-glutamyl-transferase, GST - glutathione-S-transferase, GSH – reduced glutathione, GSSG - oxidized glutathione.

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SUMMARY

The ability of the kidney and small intestine to synthesize and subsequently eliminate dinitrophenyl-S-glutathione (DNP-SG), a substrate for the multidrug resistance-associated proteins (Mrps), was assessed in bile duct ligated rats 1, 7 and 14 days after surgery, using an *in vivo* perfused jejunum model with simultaneous urine collection. A single i.v. dose of 30 μmol/Kg b.w. of 1-chloro-2,4-dinitrobenzene (CDNB) was administered and its glutathione conjugate DNP-SG and dinitrophenyl cysteinyl glycine (DNP-CG) derivative, which is the result of γ-glutamyl-transferase action on DNP-SG, were determined in urine and intestinal perfusate by HPLC. Intestinal excretion of these metabolites was unchanged at day 1, and decreased at days 7 and 14 (-39% and -33%, respectively) after surgery with respect to Shams. In contrast, renal excretion was increased by 114%, 150% and 128% at days 1, 7 and 14. Western blot studies revealed decreased levels of apical Mrp2 in liver and jejunum but increased levels in renal cortex from BDL animals, these changes being maximal between days 7 and 14. Assessment of expression of basolateral Mrp3 at day 14 post-surgery indicated preserved levels in renal cortex, duodenum, jejunum, distal ileum and colon. Analysis of expression of glutathione-S-transferases alpha, mu and pi, as well as activity towards CDNB, indicates that formation of DNP-SG was impaired in liver, preserved in intestine and increased in renal cortex. In conclusion, increased renal tubular conversion of CDNB to DNP-SG followed by subsequent Mrp2-mediated secretion into urine partially compensates for altered liver function in experimental obstructive cholestasis.

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INTRODUCTION

The liver plays a major role in the elimination of a wide variety of potentially toxic endo and xenobiotics including bilirubin, drugs and carcinogens, e.g. in the form of amphiphilic anionic conjugates (Nathanson and Boyer, 1991). Biliary excretion of these anionic compounds is mediated by a primary-active adenosine triphosphate (ATP)-dependent pump, identified as multidrug resistance-associated protein 2 (Mrp2 or Abcc2) (Paulusma et al., 1996; Buchler et al., 1996). As in hepatocytes, renal proximal tubular cells and jejunal cells also exhibit constitutive expression of Mrp2 at the apical membrane (Schaub et al., 1997; Mottino et al., 2000). Only few papers are available reporting the role of extrahepatic tissues in Mrp2 substrate disposition under conditions of deficient biliary secretory function. We have recently demonstrated increased intestinal secretion of dinitrophenyl-S-glutathione (DNP-SG), a prototypical substrate of Mrp2, in 70-75% hepatectomized rats receiving a single i.v. injection of its unconjugated parent compound, 1-chloro-2,4-dinitrobenzene (CDNB) (Villanueva et al., 2005). Although Mrp2 expression was preserved in regenerating rat liver, the substantial loss of liver mass led to a significant decrease in the overall capability for biliary secretion of the conjugated derivate. Interestingly, we observed that increased DNP-SG intestinal secretion occurred in spite of unchanged Mrp2 expression, likely as a result of increased intracellular glutathione conjugation of CDNB followed by subsequent Mrp2-mediated excretion. In contrast, intrahepatic conjugation of CDNB was substantially impaired in regenerating rat liver, mainly as a result of *in situ* inhibition of glutathione-S-transferase (GST). Impaired synthesis of DNP-SG was consistent with decreased capability for subsequent excretion, therefore avoiding intracellular accumulation of the Mrp2 substrate. Thus, in addition to changes in activity of Mrps, changes in the activity of phase II biotransformation reactions in hepatic and extrahepatic tissues condition the final disposition of conjugated amphiphilic endo- and xenobiotics.

Some forms of experimental cholestasis result in downregulation of expression of hepatic Mrp2, as demonstrated in rat models (Trauner et al., 1997, Vos et al., 1998).

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Extrahepatic Mrp2 expression was specifically evaluated in rats with obstructive cholestasis produced by bile duct ligation (BDL). Mrp2 expression is decreased in intestine (Dietrich et al., 2004) but increased in kidney (Lee et al, 2001, Tanaka et al., 2002) in BDL rats. Decreased Mrp2 expression in intestine is accompanied by decreased transport activity for amphiphilic compounds, although the status of intestinal phase II enzymes involved in conversion of hydrophobic xenobiotics into amphiphilic, Mrp2 substrates, remains unknown. Taken together, these studies suggest that the kidney plays a major role in elimination of Mrp2 substrates following experimental BDL. Up-regulation of alternative Mrps such as Mrp3 (or Abcc3) and Mrp4 (or Abcc4) at the basolateral membrane of the liver (Soroka et al., 2001, Donner and Keppler et al., 2001, Denk et al., 2004), with concomitant vectorial change in liver transport of their substrates from bile to blood followed by urinary elimination, would also support a major role for the kidney in this model of cholestasis. Whether renal elimination of Mrp2 substrates in BDL rats is conditioned by availability of conjugated compounds originated in the liver cell or by its own conjugating capability remains unknown. To what extent renal metabolism and disposition of Mrp2 substrates compensate for impaired liver function in BDL rats *in vivo* has not been studied.

In the current study we evaluated the effect of BDL in rats on *in vivo* formation of DNP-SG, a model substrate of Mrp2, and on its urinary and intestinal disposition, following systemic administration of a single dose of CDNB 1, 7, and 14 days after surgery. The expression of GST and Mrp2 in liver, jejunum and renal cortex and the expression of basolateral Mrp3 in kidney and intestine were also assessed. Major findings indicate that both renal conjugation of CDNB and subsequent urinary elimination of DNP-SG were increased in BDL rats, with maximal increases being detected from 7 to 14 days after surgery. This was consistent with increased expression of GST and Mrp2 and preserved expression of Mrp3 in renal cortex. Intestinal secretion of DNP-SG contributed minimally to overall CDNB clearance irrespective of the treatment.

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MATERIALS AND METHODS

Chemicals. Leupeptin, phenylmethylsulfonyl fluoride, pepstatin A, 1-chloro-2,4-dinitrobenzene (CDNB), glutathione, glutathione reductase, and NADPH were obtained from Sigma Chemical Co (St. Louis, MO). All other chemicals and reagents used in the current study were commercial products of analytical grade purity.

***Animals and surgical procedures*.** Adult Male Wistar rats weighing 300-350 g (National University of Rosario) were used. Animals had free access to food and water and received humane care as outlined in the NIH guidelines for the Care and Use of Laboratory Animals. Bile duct ligation (BDL) was performed as described previously (Gartung et al., 1996) under ether anesthesia. Experiments were performed 1, 7 or 14 days after surgery (BDL1, BDL7, and BDL14 groups, respectively). Controls underwent a sham operation that consisted of exposure, but not ligation, of the common bile duct, and were studied 1, 7 or 14 days later.

Distribution of the different experimental groups was as follows:

i- Sham (n=9), BDL1 (n=3), BDL7 (n=3) and BDL14 (n=3) were used for detection of Mrp2 content in plasma membranes from liver, intestine and kidney.

ii- Sham (n=9), BDL1 (n=3), BDL7 (n=3) and BDL14 (n=3) were used in *in vivo* experiments for determination of biliary, urinary and intestinal excretion of DNP-SG and its serum level, and for determination of liver, intestine and kidney mass.

iii- Sham (n=3) and BDL14 (n=3) were used for detection of Mrp3 relative to Mrp2 in homogenates and total membranes from intestine and kidney.

iv- Sham (n=6), BDL1 (n=3) and BDL7 (n=3) were used for determination of GST expression and activity in cytosol from liver, intestine and kidney and for determination of glutathione content in homogenate.

In vivo assessment of Mrp2 activity. Mrp2 activity was evaluated in urine and intestinal perfusate through determination of DNP-SG, a model substrate of Mrp2,

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and its derivative dinitrophenyl cysteinyl glycine (DNP-CG). DNP-CG is the result of γGT (γ-glutamyl-transferase) action on DNP-SG at the luminal side of secretory epithelia (Hinchman et al., 1991). Studies were performed using the *in situ* single-pass intestinal perfusion technique (Gotoh et al., 2000) with simultaneous urine collection. After a 30-min stabilization period, a single bolus of CDNB (30 μmol/Kg b.w. in 1:19 dimethylsulfoxide:saline, i.v.) was administered. Urine and intestinal perfusate were collected for 90 min at 30- and 15-min intervals, respectively. Bile from Sham rats was collected for the same period. A blood sample was taken 5 min after CDNB injection from the tail vein. DNP-SG and DNP-CG content was assessed in all samples by HPLC, as previously described (Mottino et al., 2001), using authentic standards. Saline was administered intravenously throughout the experiment to replenish body fluids.

Western blot studies of Mrp2, Mrp3 and GST. The liver was perfused *in situ* with ice-cold saline through the portal vein and used for preparation of crude plasma membranes by differential centrifugation, as described (Meier et al., 1984). The proximal jejunum (~ 30 cm length) was removed and carefully rinsed with ice-cold saline. Brush border membranes from the intestinal mucosa were prepared as described (Mottino et al., 2000). Renal cortex was isolated and brush border membranes were obtained by Mg/EGTA precipitation as previously described (Ohoka et al. 1993), with some modifications (Torres et al., 2003). Cytosolic fractions from these same tissues were obtained by ultracentrifugation as previously described (Siekevitz, 1962) in a different set of animals. Protein concentration in plasma membrane preparations and cytosols was measured using bovine serum albumin as a standard (Lowry et al., 1951). Western blot detection of Mrp2 content in plasma membranes was performed as previously described by using a monoclonal antibody to human Mrp2 (M_2 III-6, Alexis Biochemicals, Carlsbad, CA) (Mottino et al., 2000). Western blot studies of the different GST classes were performed in cytosolic fractions by using goat antisera against rat alpha and mu (GS9 and GS23, respectively, Oxfordbiomed, Michigan, USA) and rabbit antiserum against human pi GST (Immunotech, Marseille, France) as previously described (Catania et al., 2000).

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Because of the different localization of Mrp2 and Mrp3 (apical vs basolateral), and in order to perform a comparative analysis of their expression in response to BDL, we prepared homogenates and total membranes from intestine and kidney, respectively. In addition, because Mrp2 distribution along the intestine is opposite to that of Mrp3 (Mottino et al, 2000; Rost et al., 2002), we examined their expression in representative segments of the whole intestine. Intact intestinal epithelial cells were isolated (Weiser, 1973) from duodenum, proximal jejunum, distal ileum and colon, and homogenized in 50 mM mannitol, 2 mM Tris buffer and protease inhibitors. The material was further sonicated for 20 sec on ice and the total homogenate used in western blot studies. The renal cortex was homogenized in a potassium-HEPES buffer (200 mM mannitol, 80 mM HEPES, and 41 mM KOH, pH 7.5) containing protease inhibitors. Following a low speed centrifugation at 1000 x g for 15 min, the post-nuclear supernatant was subjected to centrifugation at 100,000 x g for 60 min and the pellet resuspended in the same HEPES buffer and used in western blot studies. In preliminary experiments, we found these preparations to be optimal for simultaneous detection of Mrp2 and Mrp3. Protein concentration in these preparations was measured using bovine serum albumin as a standard (Lowry et al., 1951). For Mrp2 detection, the EAG15 polyclonal antibody to rat Mrp2, generously provided by Dr. D. Keppler (Heidelberg, Germany), was used. For Mrp3 detection, a rabbit polyclonal antibody was generated by the Yale Liver Center using a gene fragment of rat Mrp3, generously provided by Dr. H. Suzuki (Tokyo, Japan). A GST-Mrp3 fusion protein (aa837-972) was constructed in pGEX-3X vector BamH1/EcoR1 sites. The fusion protein was purified on glutathione beads and cleaved by Factor Xa. The recovered Mrp3 portion was sent to Chemicon International Inc. (Temecula, CA) for injection into rabbits. The preimmune and anti-Mrp3 sera were evaluated by Western blotting against normal and BDL rat liver fractions and the immune serum only was shown to detect a band of approximately 180 kDa that was highly up-regulated in the BDL livers (data not shown). The antiserum was affinity purified against the rat Mrp3 fusion protein to remove nonspecific reactivities prior to the immunoblotting assay.

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The immunoreactive bands were quantified using the Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD) software.

Assessment of GST activity. Glutathione conjugating activity towards CDNB in cytosol from liver, jejunum, and renal cortex was assayed by a reported procedure (Habig et al., 1974), with some modifications (Catania et al. 2000).

Detection of hepatic, intestinal, and renal content of glutathione. Aliquots of liver, jejunum and renal cortex were homogenized (20% w/v in saline) and 2 volumes of the homogenates were mixed with 1 volume of 10% sulfosalicylic acid, centrifuged at 5000 X g for 5 min, and the supernatants immediately used in assessment of total glutathione (reduced (GSH)+oxidized (GSSG)) and GSSG (Griffith, 1980).

Statistical analysis*.* Data are presented as means ± SD. Statistical analysis was performed using one-way ANOVA, followed by Bonferroni's test, unless otherwise stated. Values of $P < 0.05$ were considered to be statistically significant.

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RESULTS

Liver, small intestine, and kidney mass. Tissue masses did not show differences among Sham rats at 1, 7 and 14 days post-surgery; the data were thus considered as belonging to the same group and referred to as Sham. Table 1 shows that liver weight relative to body weight was not affected in BDL rats at day 1, whereas it was significantly increased by 51% and 57% at days 7 and 14 post-surgery, respectively, when compared to Shams. Likewise, relative kidney mass was increased in BDL7 (+23%) and BDL14 (+25%) groups. In contrast, the relative mass of the fragment of small intestine perfused *in vivo* for transport studies was not affected at any time studied. This portion of the small intestine (~50 cm long) corresponds mainly to jejunum and proximal ileum, where the highest expression and activity of Mrp2 were reported (Gotoh et al., 2000; Mottino et al., 2000).

Expression of Mrp2 and Mrp3. Expression of Mrp2 in plasma membrane from liver, proximal jejunum and renal cortex was estimated by western blotting. A preliminary immunoblot study demonstrated no differences in Mrp2 expression among the different Sham groups (images not shown). For comparison of Sham animals with BDL1 and BDL7 groups in the same immunoblot, and due to limitation in the number of wells per gel, we decided to load membranes from Sham animals studied at day 7 together with those from BDL1 and BDL7 rats. Fig 1 shows a significant decrease in the level of hepatic Mrp2 protein by 44% and 64% in BDL1 and BDL7, respectively. BDL treatment also resulted in a marked reduction of intestinal Mrp2 protein level by 42% in BDL7 compared with Shams, though it remained essentially unchanged in BDL1. In contrast, renal Mrp2 protein was increased significantly by 73% and 123% in BDL1 and BDL7, respectively. Fourteen days after surgery, changes in Mrp2 levels in these three tissues were found to be of similar magnitude as in BDL7 group (images not shown). These data on tissue–dependent regulation of this transporter under conditions of obstructive cholestasis agree well with previous reports from Trauner et al (1997), Lee et al (2001), Tanaka et al. (2002), and Dietrich et al (2004).

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The increased expression of hepatic Mrp3, in contrast to the decreased expression of hepatic Mrp2, in liver from BDL rats (Soroka et al., 2001; Donner and Keppler, 2001), suggests a re-direction of Mrp common substrates to blood. To further explore whether expression of Mrp3 relative to Mrp2 is also affected in extrahepatic tissues in BDL rats, we analyzed their level in whole membranes from renal cortex and homogenates from different regions of the intestine 14 days after surgery, when changes in Mrp2 became stable and Mrp3 was maximally induced in liver (Soroka et al., 2001). Fig 2 shows that total cellular content of Mrp2 was decreased by 65% and 60% in duodenum and jejunum, respectively, and increased by 61% in renal cortex, compared with Shams. As previously demonstrated for normal rats (Mottino et al, 2000), expression of Mrp2 was very low in distal ileum or colon irrespective of the treatment (images not shown). Whereas Mrp3 was found to be significantly upregulated in liver from BDL rats 14 days after surgery (Soroka et al., 2001), our current data in Fig 2 shows that Mrp3 expression remained unchanged in renal cortex and different regions of the intestine.

Mrp2 activity and serum level of DNP-SG. DNP-SG and DNP-CG were the major metabolites of CDNB detected in intestinal perfusate or urine. As shown in Fig 3A, intestinal excretion rate of DNP-SG+DNP-CG was not modified in BDL1 and only slightly impaired in BDL7 or BDL14 rats, particularly during the first periods of perfusate collection. The inset in Fig 3A shows that cumulative intestinal excretion of DNP-SG+DNP-CG decreased by 39 and 33% in BDL7 and BDL14 groups, respectively. In contrast, renal excretion of both metabolites was increased in BDL groups, particularly from the 60-min period onwards (see Fig 3B). Their cumulative excretion in urine was significantly increased by 114, 150, and 128% in BDL1, BDL7, and BDL14 rats, respectively (see inset in Fig 3B). In contrast, serum levels of DNP-SG were unchanged in BDL1 and BDL7 groups (see Table 2), as well as in the BDL14 group (0.018 ± 0.002 mM).

***Glutathione content in liver, jejunum and renal cortex*.** Data on glutathione content in these tissues is shown in Table 2. Whereas liver and jejunum exhibited a

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decrease in total glutathione levels in BDL1 and BDL7 animals, they were increased in the renal cortex 7 days post-surgery. All tissues exhibited an increase in the content of GSSG, reflecting an increase in oxidative stress.

Expression and activity of GST. To further explore whether BDL surgery affects the formation of DNP-SG, GST expression and activity were measured in cytosol from liver, jejunum and renal cortex. Glutathione conjugation of CDNB may be mediated by more than one GST class. A preliminary immunoblot study demonstrated no differences in expression of the different GST classes between Sham groups at day 1 and 7 (data not shown). Cytosols from BDL1 and BDL7 groups were thus loaded in a single gel, together with cytosols from Sham animals studied at day 7. Fig 4 shows that expression of GST alpha (αGST) was slightly but significantly decreased by BDL in liver in BDL7 animals, and that GST mu (μGST) remained unaltered. GST pi (πGST) was not detected in liver, irrespective of the treatment. Normally, only traces of this isoform are found in hepatic cytosol, mostly due to contamination with biliary epithelial cells (Parola et al., 1993). Intestinal content of the different GST classes was not affected by BDL either at day 1 or 7. In contrast, renal GSTs belonging to alpha class were increased (+38%) in BDL7 but not in BDL1 group while those from the mu class were increased in BDL7 group by 79%. Pi isoform detected in kidney cytosol was unaffected by BDL.

Table 2 shows that GST activity towards CDNB significantly decreased in liver from BDL1 (-24%) and BDL7 (-37%) groups when compared with Shams, whereas intestinal GST activity remained unchanged. In contrast, renal GST activity was significantly increased by BDL at day 1 (+22%) and 7 (+42%) post surgery. These data correlated well with changes in expression of αGST and μGST, which are major classes involved in CDNB conjugation.

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DISCUSSION

Previous studies in humans indicate that expression of MRP2 is downregulated in the intestine in obstructive cholestasis, suggesting a minor role of this tissue in compensating for altered liver Mrp2-mediated secretory function (Dietrich et al, 2004). A similar finding was confirmed in rats with obstructive experimental cholestasis (Dietrich et al, 2004). A role for increased levels of interleukin-1β (IL-1β) was implicated in downregulation of Mrp2 in both liver and intestine from rats undergoing BDL (Dietrich et al, 2004, Denson et al, 2002). It was demonstrated that decreased binding of RARα:RXRα nuclear receptor dimer to the promoter region of Mrp2 in BDL rats, due to increased levels of IL-1β, accounts for Mrp2 downregulation in liver (Denson et al, 2002). In contrast, renal cortex exhibits upregulation of expression and function of Mrp2 in BDL rats (Lee at al, 2001, Tanaka et al, 2002), which likely results from preserved levels of RARα:RXRα dimmer in this tissue Though tissue related changes in Mrp2 expression and function have been characterized mechanistically, no studies have evaluated the impact of these specific regulations in the overall metabolism and disposition of a model Mrp substrate in the intact animal. Due to its hydrophobicity, CDNB can easily enter cells for further conjugation with glutathione and conversion to the prototypical Mrp2 substrate, DNP-SG. Coordinated metabolism and transport of these two compounds represents sequential phase II reaction-transport processes that affect a wide variety of natural occurring xenobiotics and therapeutic drugs (Catania et al., 2004).

Using an *in situ* perfused intestinal model with simultaneous collection of urine samples, we found that the kidneys were the major contributors to overall CDNB metabolism and DNP-SG elimination in BDL rats, and that the highest contribution was observed from 7 days post-surgery onwards. Induction of expression of αGST and μGST classes involved in CDNB conjugation (Hayes and Pulford, 1995) and Mrp2, strongly suggest simultaneous upregulation of genes involved in metabolism and elimination of CDNB. Whether a common molecular mechanism/mediator is involved in their modulation remains unknown. This evidence, together with increased cytosolic GST activity towards CDNB and availability of glutathione, the cosubstrate

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of GST reaction, identifies renal tubular cells as an important supplier of urinary DNP-SG under BDL conditions. The fact that hepatic GST expression and activity and glutathione content are decreased in BDL7 and that serum levels of DNP-SG were not increased in spite of induction of liver basolateral Mrps further support this assumption.

Because of its highly hydrophobic nature, it is assumed that CDNB freely enters cells by diffusion, and thus GST activity and the availability of the cosubstrate GSH must determine CDNB conjugation. Decreased hepatic output and plasma concentration of GSH are common features of liver injury in animals (Purucker et al., 1995) and humans (Burgunder and Lauterburg, 1987). GSH availability in extrahepatic tissues highly depends on transfer from liver (Deneke and Fanburg, 1989). Whereas liver and intestinal content of GSH were reduced in BDL rats (see Table 2), renal content was significantly increased at day 7 post-surgery, consistent with the induction of GSTs and maximum increase in urinary excretion of DNP-SG observed *in vivo.* Our data on increased renal content of GSH are in agreement with a previous study from Purucker et al. (2002), who postulated that reduced renal efflux of GSH by competition with organic anions at membrane transporter level leads to its decreased turnover and increased cellular accumulation. We postulate that this intracellular pool of GSH is critical for increased conversion of CDNB to DNP-SG for subsequent excretion into urine in rats with obstructive cholestasis.

Although glomerular filtration of amphiphilic compounds such as sulfate-conjugated bile acids and bilirubin glucuronides may account for their urinary excretion, increased renal tubular excretion of these compounds have been demonstrated in conditions associated with cholestasis (Gutmann et al., 2000; Tanaka et al., 2002), implicating Mrp2 and other specific conjugate transporters. Our data on increased urinary extraction of DNP-SG and DNP-SC agree well with these latter findings and highlight the importance of Mrp2 induction in the elimination of hydrophobic xenobiotics. The enhanced renal secretory function was maintained at day 14 post-surgery, suggesting a role for GST and Mrp2 induction under conditions of persistent cholestasis.

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Additionally, we found that expression of Mrp3 was preserved in renal cortex in BDL14 group. Increased expression of Mrp2 relative to Mrp3 in the kidney indicates a preferential apical vs basolateral disposition of common Mrp substrates.

Intestinal secretion of DNP-SG was unchanged in BDL1 group and only slightly affected in BDL7 or BDL14 groups in spite of reduced Mrp2 expression in brush border membranes (Fig 1) and total homogenates (Fig 2). By comparing maximum transport capacity of DNP-SG in isolated intestinal segments (Mottino et al, 2001) with *in vivo* transport capability, we hypothesized than Mrp2 is not saturated after i.v. administration of 30 µmol/Kg of CDNB in normal rats (Villanueva et al., 2005). Taken together with the present findings of preserved GST expression and activity in jejunum and the high content of GSH relative to other extrahepatic tissues such as kidney of BDL rats (see Table 2), this may explain the slight discrepancy in intestinal secretion of DNP-SG at days 7 or 14 post-surgery and the *in vitro* assays of Mrp2 expression. Although the contribution of the jejunum to the overall clearance of systemically administered CDNB was only minimally affected in our study, impairment in Mrp2 expression in enterocytes in obstructive cholestasis substantially affects Mrp2 function as a membrane barrier against absorption of xenobiotics available luminally according to Dietrich et al (2004). The differences observed in Mrp2 function in BDL animals when substrates are administered systemically vs luminally may result from differences in the type of substrates administered and/or in the use of non-saturating vs saturating conditions. We have observed that significant downregulation of intestinal Mrp2 also occurred 14 days post-surgery, and additionally, that Mrp3 expression was preserved in this latter group. Since Mrp3 is located at the basolateral level and shares some substrate specificity with Mrp2, we predict a substantial increase in bioavailability for orally incorporated drugs and xenobiotics, common substrates for Mrps, in obstructive cholestasis.

Renal excretion of DNP-SG and DNP-CG represented approximately 59% of biliary excretion in Sham rats, when expressed as % dose per mass unit (Fig 5). Under BDL conditions, urinary excretion was even higher than biliary excretion in Shams (+13%,

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+63%, and +22% for BDL1, BDL7 and BDL14, respectively). However, because of the substantial difference between liver and kidney masses, overall contribution of the kidneys could only partially compensate for impaired biliary secretory function. Nevertheless, this renal adaptation may facilitate the clearance of hydrophobic xenobiotics during impairment of hepatic phase II metabolism and apical excretion until normal liver function is restored. In contrast, the participation of the intestine was minimal irrespective of the treatment, contrasting with its much greater role in limiting the absorption of xenobiotics and drugs administered luminally.

Whether our results can be extrapolated to Mrp substrates suffering phase II metabolism, other than glutathione conjugation, is uncertain. The endogenous Mrp2 substrate bilirubin needs to be conjugated, e.g. with glucuronic acid, previous to its transport by Mrp2 (Kamisako et al., 2000;Keppler et al., 2000). It is well established that bilirubin mono- and di-glucuronide can reach the urine either by glomerular filtration or tubular secretion. Urinary excretion of bilirubin conjugates is significantly increased in BDL rats (Tanaka et al., 2002), consistent with increased expression of Mrp2 in renal cortex. Because plasma concentration of conjugated bilirubin is also substantially increased in obstructive cholestasis in association with Mrp3 induction, and bilirubin glucuronides are also substrates for Mrp3 (Keppler et al., 2000;Belinski et al., 2005), it is possible that liver basolateral secretion represents an important source of conjugated pigment for its subsequent urinary elimination. Consistent with this possibility, we found that expression of UDP-glucuronosyltransferase 1A1 (UGT1A1), the main isoform involved in bilirubin conjugation, is preserved in liver from BDL rats (unpublished results), in contrast to decreased GST-mediated conjugation of CDNB as currently observed. We also observed that, unlike GST, renal UGT1A1 remained unchanged (unpublished results). Taken together, the evidence would indicate that the role of the liver in providing Mrp substrates for subsequent urinary elimination may vary depending on the metabolic pathway involved in its generation, e.g. GST vs UGT. Contrasting with renal findings, the role of intestinal excretion of bilirubin in BDL conditions seems to be minor since no substantial

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amount of pigment was present in the intestinal perfusate from BDL animals either at day 1 or 7 post-surgery (unpublished results).

In conclusion, the current data demonstrate the relevance of the association between renal GST and Mrp2 for elimination of hydrophobic xenobiotics, particularly under conditions of impaired biliary secretory function, as occurs in obstructive cholestasis.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. ***Western blot analysis of Mrp2 in plasma membrane.***

Ten µg of protein from liver mixed plasma membranes and brush border membranes from renal cortex, and 30 µg of protein from intestinal brush border membranes were loaded in the gels for Mrp2 analysis. Western blot detection of Mrp2 was performed by using a monoclonal antibody to human Mrp2 (M_2 III-6, Alexis Biochemicals, Carlsbad, CA). Data on densitometry are means ± SD of 3 animals per group. Sham group consists of animals studied at day 7.

∗ significantly different from Sham ($P < 0.05$).

Fig. 2. Expression of Mrp3 relative to Mrp2 in kidney and intestine.

Fifty µg of protein from intestinal cell homogenates and renal cortex total membranes were loaded in the gels for simultaneous detection of Mrp2 and Mrp3. For Mrp2 detection, the EAG15 polyclonal antibody to rat Mrp2, generously provided by Dr. D. Keppler (Heidelberg, Germany), was used. Data on densitometry are means ± SD of 3 animals per group. Statistical analysis was performed by Student *t* test. Sham group consists of animals studied at day 14.

∗ significantly different from Sham ($P < 0.05$).

Fig. 3. ***Intestinal and urinary excretion of DNP-SG.***

Excretion of the prototypical substrate of Mrp2, DNP-SG, and its derivative DNP-CG in intestinal perfusate (panel A) and urine (panel B) was expressed per 15 or 30 min, respectively. Insets depict cumulative excretion of DNP-SG and DNP-CG by 90 min. Data are means ± SD of 9 (Sham) or 3 (BDL) animals per group. Sham group consists of animals studied at day 1, 7, and 14 (3 each).

∗ significantly different from BDL7 and BDL14 ($P < 0.05$).

∗∗ significantly different from BDL1, BDL7, and BDL14 ($P< 0.05$).

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Fig. 4*. Western blot analysis of cytosolic GST.*

Four µg of hepatic cytosolic protein and 10 µg of intestinal and renal cortex cytosolic protein were loaded in the gels for GST classes analysis. Data are means ± SD of 3 animals per group. Sham group consists of animals studied at day 7.

* significantly different from Sham ($P < 0.05$).

Fig. 5. *Cumulative excretion of CDNB metabolites.*

Cumulative biliary, urinary and intestinal excretion of DNP-SG and DNP-CG by 90 min were expressed as percentage of the CDNB administered dose, and referred either as per gram (panel A) or whole (panel B) tissue. Data are means ± SD of 9 (Sham) or 3 (BDL) animals per group. Sham group consists of animals studied at day 1, 7, and 14 (3 each).

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Table 1: Liver, intestine and kidney masses.

	Sham (N=9)	BDL1 (N=3)	BDL7 (N=3)	BDL14 (N=3)
Liver mass (g/100 g bw)	3.62 ± 0.07	3.19 ± 0.28	5.47 ± 0.82∗ #	5.69 ± 0.23∗ #
Intestine mass (g/100 g bw)	1.77 ± 0.07	1.71 ± 0.45	1.86 ± 0.24	1.93 ± 0.20
Kidney mass (g/100 g bw)	0.60 ± 0.05	0.61 ± 0.03	0.74 ± 0.04∗ #	0.75 ± 0.05∗ #

Intestine mass corresponds to the segment perfused in the *in vivo* experiments and represents about half the mass of the whole small intestine. This segment corresponds approximately to whole jejunum and proximal ileum. Kidney mass includes total weight of bilateral organs. Data are means ± SD. Sham group consists of animals studied at day 1, 7, and 14 (3 each).

∗ significantly different from Sham group ($P< 0.05$).

significantly different from BDL1 group ($P< 0.05$).

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Table 2: Serum level of DNP-SG, and GST activity and Glutathione content in liver, jejunum and renal cortex.

	Sham (N=6)	BDL1 (N=3)	BDL7 (N=3)
Serum concentration of DNP-SG (mM)	0.024 ± 0.004	0.023 ± 0.002	0.019 ± 0.004
Liver GST activity (nmol/min/mg protein)	1610 ± 81	1228 ± 100*	1018 ± 236*
Intestinal GST activity (nmol/min/mg protein)	443 ± 51	516 ± 37	512 ± 9
Renal GST activity (nmol/min/mg protein)	352 ± 10	430 ± 34*	500 ± 12* #
Hepatic total glutathione content (nmol/g liver)	4544 ± 579	3193 ± 171*	2764 ± 744*
Hepatic GSSG content (nmol/g liver)	109 ± 30	238 ± 12*	276 ± 59*
Intestinal total glutathione content (nmol/g liver)	1958 ± 59	1115 ± 122*	1274 ± 113*
Intestinal GSSG content (nmol/g liver)	27 ± 4	36 ± 4*	30 ± 2

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Renal total glutathione content (nmol/g liver)	462 ± 35	614 ± 62	1174 ± 168∗ #
Renal GSSG content (nmol/g liver)	18 ± 1	38 ± 4∗	71 ± 10∗ #

Level of DNP-SG in serum was assessed by HPLC, 5 min after administration of CDNB. GST activity towards CDNB was assessed in cytosolic fractions. Tissue content of glutathione was assessed in homogenates from hepatic and extrahepatic tissues. Data are means ± SD. Sham group consists of animals studied at day 1 and 7 (3 each).

∗ significantly different from Sham group ($P< 0.05$).

significantly different from BDL1 group ($P< 0.05$).

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Figure 1

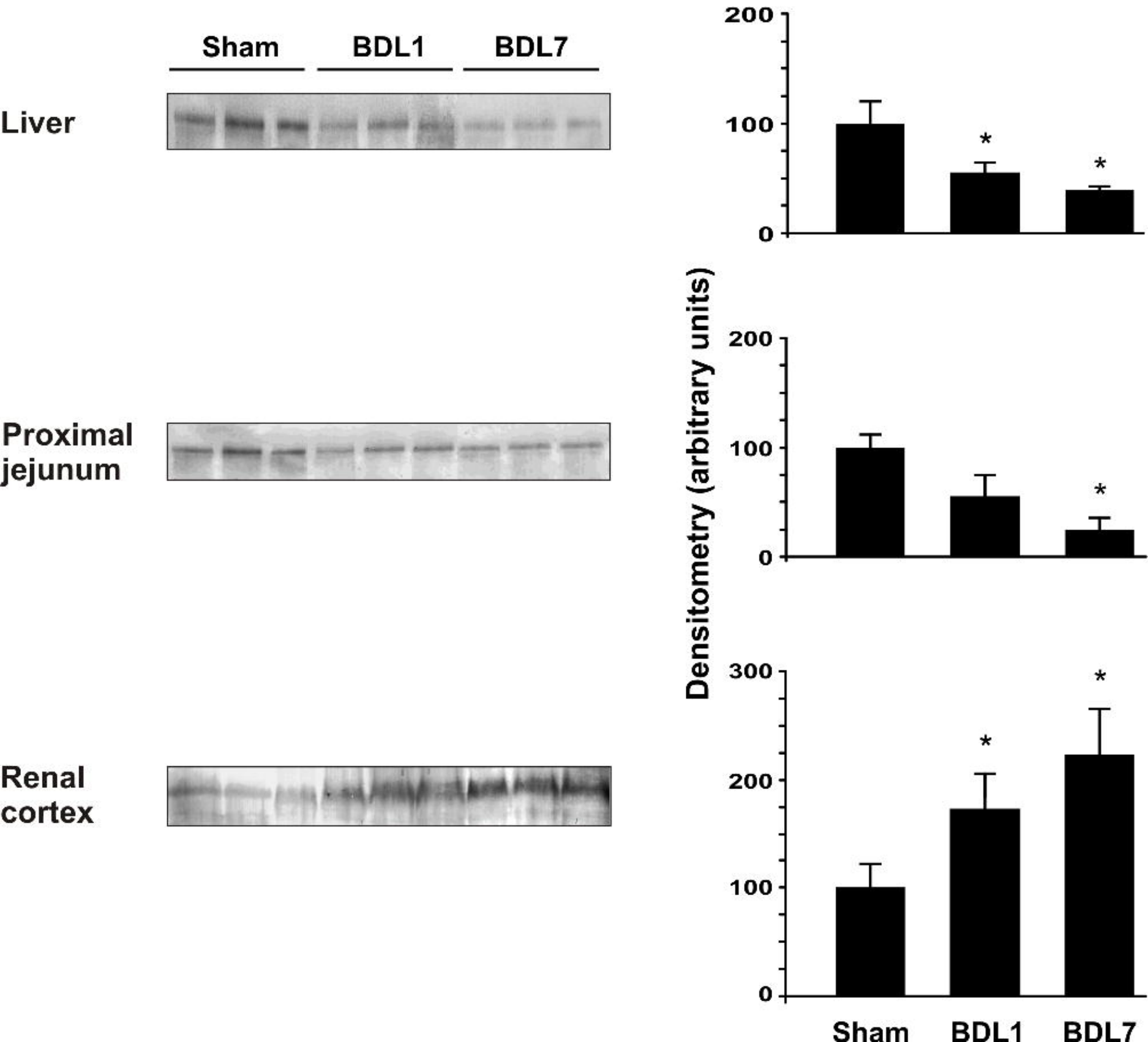

Sham
BDL1
BDL7
Liver
Proximal jejunum
Renal cortex
Densitometry (arbitrary units)
200
100
0
200
100
0
300
200
100
0
*
*
*
*
*
Sham
BDL1
BDL7

Figure 2

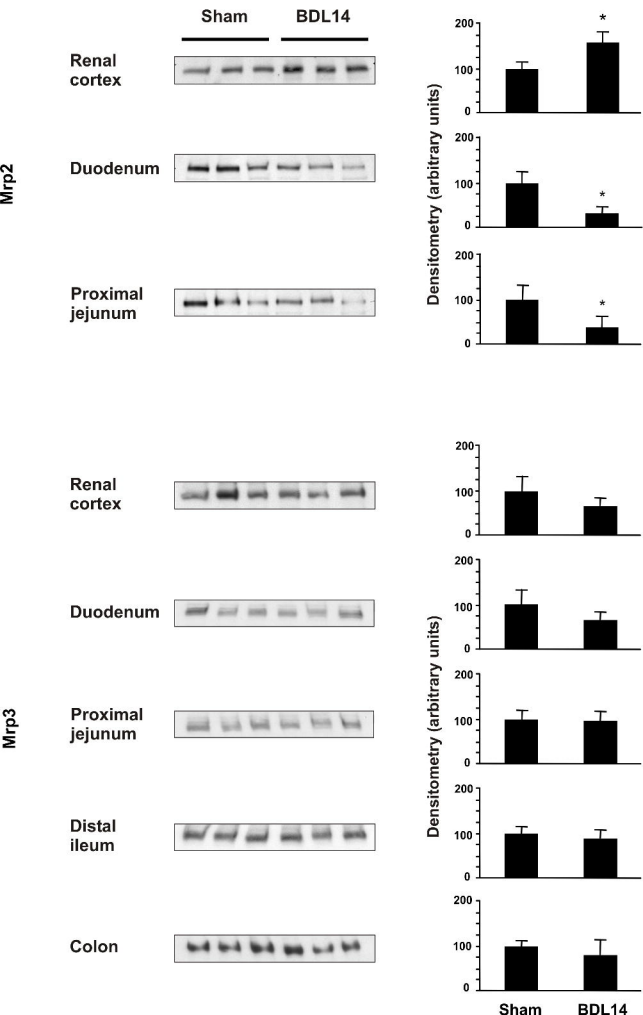

Sham
BDL14
Mrp2
Renal cortex
Duodenum
Proximal jejunum
Mrp3
Renal cortex
Duodenum
Proximal jejunum
Distal ileum
Colon
Densitometry (arbitrary units)
200
100
0
*
Sham
BDL14

Figure 3

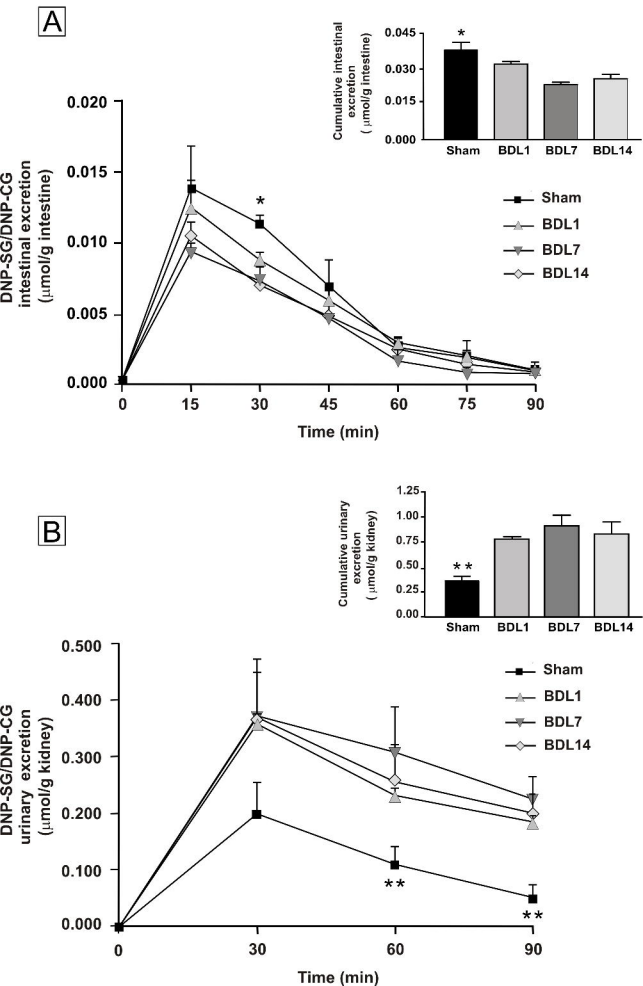

A
DNP-SG/DNP-CG intestinal excretion (μmol/g intestine)
0.020
0.015
0.010
0.005
0.000
0
15
30
45
60
75
90
Time (min)
*
Sham
BDL1
BDL7
BDL14
Cumulative intestinal excretion (μmol/g intestine)
0.045
0.030
0.015
0.000
Sham
BDL1
BDL7
BDL14
B
DNP-SG/DNP-CG urinary excretion (μmol/g kidney)
0.500
0.400
0.300
0.200
0.100
0.000
0
30
60
90
Time (min)
**
Sham
BDL1
BDL7
BDL14
Cumulative urinary excretion (μmol/g kidney)
1.25
1.00
0.75
0.50
0.25
0.00
Sham
BDL1
BDL7
BDL14

Figure 4

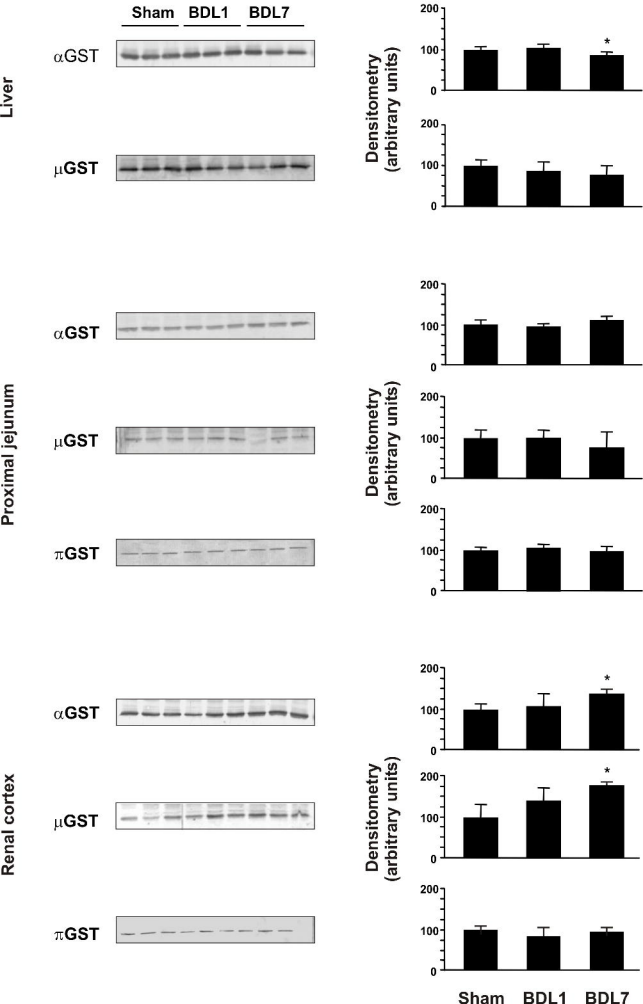
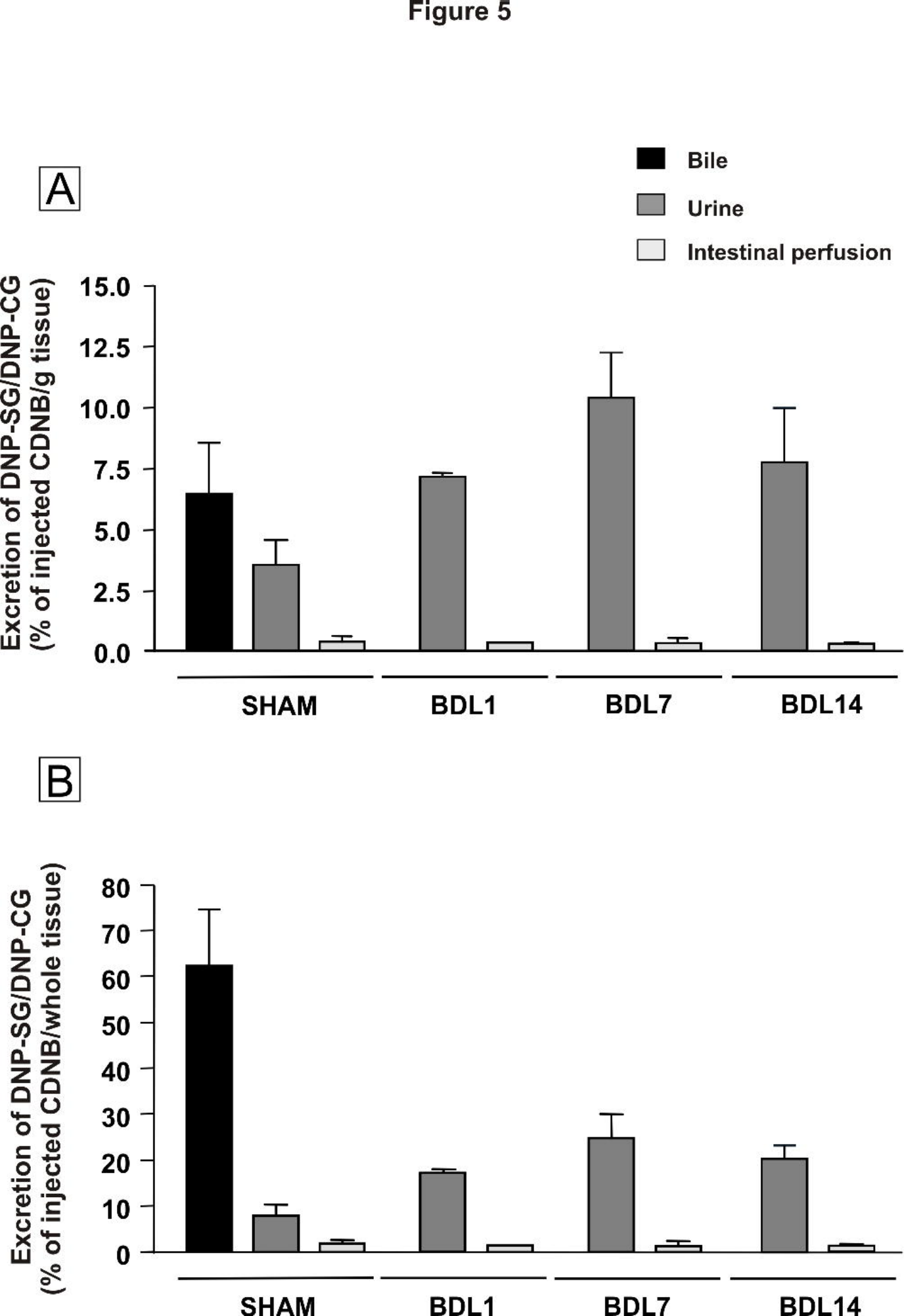

Sham
BDL1
BDL7
Liver
αGST
μGST
Proximal jejunum
αGST
μGST
πGST
Renal cortex
αGST
μGST
πGST
Densitometry (arbitrary units)
200
100
0
*
Sham
BDL1
BDL7

Figure 5


A
Bile
Urine
Intestinal perfusion
Excretion of DNP-SG/DNP-CG
(% of injected CDNB/g tissue)
15.0
12.5
10.0
7.5
5.0
2.5
0.0
SHAM
BDL1
BDL7
BDL14
B
Excretion of DNP-SG/DNP-CG
(% of injected CDNB/whole tissue)
80
70
60
50
40
30
20
10
0
SHAM
BDL1
BDL7
BDL14