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# Mechanisms involved in spironolactone-induced choleresis in the rat Role of multidrug resistance-associated protein 2☆

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**Abstract**

The mechanisms involved in spironolactone (SL, 200 μmol/kg body weight, 3 days i.p.)-induced choleresis were explored in vivo by evaluating bile salt export pump (Bsep)-, multidrug resistance-associated protein 2 (Mrp2)-, and anion exchanger 2 (AE2)-mediated secretory processes in rat liver. Hepatic bile salt metabolism was also analyzed. Total bile flow was significantly increased by SL, primarily due to an increase in bile salt-independent bile flow, whereas bile salt secretion was decreased. SL did not produce any choleresis in $TR^-$ rats. SL decreased the de novo bile salt synthesis rate in concordance with impaired microsomal cholesterol 7α-hydroxylase activity, thus leading to a decrease in endogenous bile salt pool size. In contrast, the maximum secretory rate of tauroursodeoxycholate as well as expression of Bsep protein detected by Western blotting were not affected. Thus, decreased bile salt availability for canalicular transport rather than transport capability itself likely explains reduced biliary secretion of bile salts. Biliary secretion of glutathione, an endogenous substrate of Mrp2, and $HCO_3^-$, the AE2 substrate, were increased by SL, as a main factor explaining enhanced bile salt-independent bile flow. Western blot studies revealed increased expression of Mrp2 in response to SL whereas AE2 content remained unchanged. Enhanced activity and expression of Mrp2 was confirmed by analyzing the excretion rate of dinitrophenyl *S*-glutathione, an exogenous substrate of Mrp2, in isolated hepatocytes and by immunofluorescence microscopy, respectively. We conclude that SL increased bile flow mainly by increasing the biliary secretion of glutathione species and $HCO_3^-$; increased expression of Mrp2 is also involved.


*Keywords:* Spironolactone; Bile flow; Mrp2; Bsep; AE2; Glutathione

## 1. Introduction

In mammals, bile exerts a range of vital physiologic functions from solubilization and digestion of dietary lipids and lipid-soluble nutrients to elimination of lipid-soluble substances, drugs and toxic compounds. Bile formation is initiated at the canalicular domain of hepatocytes and is primarily dependent on active transport of solutes such as bile acids, glutathione and bicarbonate, followed by the passive movement of water. The bile salt export pump (Bsep) mediates the concentrative transport of bile salts across the canalicular membrane [1], and thus generates the bile salt-dependent (BSDF) component of bile flow. Transport of different glutathione species into bile is considered critical to the generation of bile salt-independent bile flow (BSIF) [2]. The multidrug resistance-associated protein 2 (Mrp2, ABCC2) mediates the active transport of oxidized glutathione (GSSG) and glutathione conjugates [3] and also contributes to the active transport of reduced glutathione (GSH) [4]. An electroneutral CI/

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*Abbreviations:* AE2, anionic exchanger 2; BSDF, bile salt-dependent bile flow; Bsep, bile salt export pump; BSIF, bile salt-independent bile flow; CDNB, 1-chloro-2,4-dinitrobenzene; DNP-SG, dinitrophenyl *S*-glutathione; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione *S*-transferase; MPM, mixed plasma membrane; Mrp2, multidrug resistance-associated protein 2; PXR, pregnane X-receptor; SL, spironolactone; Tm, maximum secretory rate; TUDC, sodium tauroursodeoxycholate; UGT, UDP-glucuronosyltransferase

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$HCO_3^-$ exchanger, the anion exchanger 2 (AE2), is responsible for $HCO_3^-$ secretion into bile [5], and also contributes to BSIF formation [6].

Changes in the activity of these transporters, e.g. as a consequence of changes in their rate of synthesis, may critically affect bile formation. Spironolactone (SL), which is widely used as a diuretic in patients with edema or ascites [7], increases bile flow in rats [8,9] though the mechanism is poorly understood. Since this diuretic is an inducer with known effects on several hepatic enzyme systems, including cytochrome P-450 [7], GST [10] and UGT [11] not only in experimental animals but also in humans [7], it is reasonable to speculate that the increase in bile flow may result from increased expression of canalicular transporters. In this regard, it was recently reported that Mrp2 expression is increased in liver membranes from SL-treated rats [12]. Whether this could lead to exacerbated Mrp2-mediated transport of glutathione species into bile, as an explanation for the increased bile flow, is not known. The effect of SL on Bsep expression and activity and on $HCO_3^-$ biliary secretion were not explored. It is of interest to characterize the mechanisms involved in the SL-induced choleresis, which along with its well-known inducer effect could account for an improvement in the biliary clearance of different compounds.

Here we evaluated the effect of SL on parameters determining the bile salt-dependent and -independent fractions of bile flow, particularly those associated with the expression of Mrp2 and Bsep as well as their transport activity. The effect of this steroid on biliary secretion of $HCO_3^-$ was also explored. The data indicate a significant increase in biliary secretion of glutathione species and $HCO_3^-$ as the main cause of SL-induced choleresis.

## 2. Materials and methods

### 2.1. Chemicals

SL, leupeptin, phenylmethylsulfonyl fluoride (PMSF), pepstatin A, glutathione (GSH), glutathione reductase, collagenase, 3α-hydroxysteroid dehydrogenase, β-NAD, NADPH, 7β-hydroxycholesterol, sodium isocitrate, isocitrate dehydrogenase, HEPES and 1-chloro-2,4-dinitrobenzene (CDNB) were obtained from Sigma Chemical Company (St. Louis, MO). Sodium tauroursodeoxycholate (TUDC) was a gift from Prodotti Chimici e Alimentari S.p.A. (Italy). Cholesterol oxidase was a gift from Wiener Lab. (Rosario, Argentina). All other chemicals were of analytical grade purity, and used as supplied.

### 2.2. Animals and treatment

Adult male Wistar rats (320–380 g) were used throughout. They were maintained ad libitum on a standard laboratory pellet diet and were allowed free access to water and saline during treatment. Animals were injected with SL dissolved in propylene glycol (60 mM), i.p. at a daily dose of 200 μmol/kg body weight (equivalent to 83.3 mg/kg body weight), for 3 consecutive days before the experiment (SL group). This dose was previously found to maximally increase microsomal UGT [13] and cytosolic GST [10] activities in rat liver. Studies were performed 18 h after the last injection of SL unless otherwise stated. Control rats were injected with propylene glycol (vehicle of SL, 3.3 ml/kg body weight) according to the same schedule described above. Animals were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.) and maintained under this condition throughout the experiment. The femoral vein and the common bile duct were cannulated with polyethylene tubing (PE50 and PE10, respectively). In bile salt depletion studies, appropriate volumes of Krebs–Henseleit-$HCO_3^-$ solution (pH 7.4) containing 2 mg/ml glucose were administered intravenously throughout the experiment to replenish body fluids. Body temperature was maintained between 37.0 and 37.5 °C with a warming lamp to prevent hypothermic alterations of bile flow. All procedures were conducted in accordance with NIH guidelines for the Care and Use of Laboratory Animals.

In a separate set of experiments, bile flow and biliary excretion and hepatic content of glutathione species were determined in Mrp2 congenitally-deficient ($TR^-$) adult male rats (340–390 g) treated with SL or its solvent according to the protocol described above.

### 2.3. Biliary secretory function studies

#### 2.3.1. Basal bile flow and bile salt, $HCO_3^-$ and glutathione excretion rates

Twenty minutes after bile duct cannulation, spontaneously secreted bile was collected for 10 min in preweighed vials and preserved at −20 °C until used in biliary bile salt assessment. Bile volume was measured gravimetrically, assuming a density of 1.0 g/ml, and used to determine the rate of basal bile flow. Total BS concentration in bile samples was measured by the 3α-hydroxysteroid dehydrogenase procedure [14].

Subsequent bile samples were collected for 15 min under 200 μl of liquid Vaseline™ in preweighed tubes, and used to determine biliary $HCO_3^-$ secretion. $HCO_3^-$ concentrations were calculated from pH and $PCO_2$ using the Henderson–Hasselbalch equilibrium equation. pH and $PCO_2$ were measured immediately after bile collection in an automated blood–gas analyzer (Compact 1, AVL Medical Instruments AG, Schaffhausen, Switzerland).

Finally, glutathione species were determined in bile samples collected for another 10 min period or during the equivalent period in $TR^-$ rats in preweighed tubes containing 100 μl of 10% 5-sulfosalicylic acid to minimize oxidation of GSH [15], and immediately used in total glutathione and GSSG assays. To determine intrahepatic

content of glutathione species, livers were perfused for 30 s with cold saline and the median lobe removed and homogenized (20%, w/v, in saline). Two volumes of the homogenate were mixed with 1 volume of 10% 5-sulfosalicylic acid, centrifuged at 5000 × *g* for 5 min, and the supernatant immediately used in total glutathione and GSSG assays. The concentration of total glutathione and GSSG in the supernatants from bile and liver homogenates were determined spectrophotometrically by the enzymatic recycling procedure of Tietze [16], as modified by Griffith [15]. Total glutathione was expressed as equivalents of GSH, and GSSG as equivalents of GSH relative to total glutathione (%).

Biliary excretion rates were calculated as the product of bile flow and solute concentration values.

### 2.4. Bile salt metabolism and transport studies

#### 2.4.1. Bile salt pool size and composition and de novo bile salt synthesis rate

To estimate bile salt pool size and its composition, as well as the de novo hepatic bile salt synthesis rate, we used bile salt-depleted animals as was previously described [17]. The chronic bile fistula model provides a mean for depleting the body of bile salts by eliminating the primary source of these compounds, i.e. the enterohepatic circulation. The bile salt pool size is calculated as the amount of bile salts excreted during the first 10 h of bile drainage, when a complete bile salt pool washout was reached, minus the amount of bile salts resulting from the de novo synthesis and secreted in the same period. The de novo synthesis was estimated as the biliary bile salt excretion rate recorded after complete bile salt pool washout. Total bile salt concentration in bile samples was determined as described above and bile salt composition was assessed by HPLC. The HPLC system consisted of a Waters model M-600 (Waters, Milford, MA) with a C18 column (μBONDAPAK, Waters). Bile salts were eluted isocratically using a mobile phase of methanol: 0.1 M $KH_2PO_4$ (60:40, v/v; pH 4.5) at a flow rate of 1.5 ml/min and detected at 200 nm [18]. Individual bile salts were identified by using appropriate standards.

#### 2.4.2. Cholesterol 7α-hydroxylase activity

The effect of SL on the initial and rate-limiting step in the biosynthesis of bile salts was evaluated by measuring the microsomal cholesterol 7α-hydroxylase activity as was described [19].

#### 2.4.3. Maximum secretory rate (Tm) of bile salts

To estimate the maximal capability of the liver to transport bile salts into bile, TUDC was infused to another set of rats. TUDC was selected for this purpose due to its extremely low toxicity, in contrast to other naturally occurring bile salts, whose apparent maximum transport is largely determined by the cytotoxic nature of the bile salt rather than by the saturation of its canalicular carrier. The Tm of TUDC was assessed by infusing i.v. this bile salt as was previously described [17], and calculated as the mean of the three highest consecutive 10 min secretory rates recorded over the whole infusion period [20]. These animals were also used to estimate the BSIF by the conventional extrapolation to zero bile salt output of the regression line between the bile flow and the bile salt output, as stimulated by TUDC infusion [21].

### 2.5. Mrp2, Bsep and AE2 expression studies

#### 2.5.1. Specimen collection and plasma membrane preparation

Another set of animals were sacrificed by exsanguination under sodium pentobarbital anesthesia and the livers were rinsed in situ with cold saline. The major lobe was removed and a portion was snap frozen in liquid nitrogen and preserved at −70 °C until use for plasma membrane preparation and total RNA isolation. Another portion was frozen in 2-methylbutane (Acros Organics, Pittsburgh, PA) for immunofluorescence analysis. In additional experiments, rats were sacrificed 3 h after the last injection of SL and the major lobe removed and used only for total RNA extraction. For membrane preparation, liver samples were homogenized in 0.3 mol/l sucrose containing 0.1 mmol/l PMSF, 25 μg/ml leupeptin and 5 μg/ml pepstatin A. Membrane fractions enriched in plasma membrane (mixed plasma membrane, MPM), were prepared from the homogenates by differential centrifugation as described [22]. Protein concentration was measured using bovine serum albumin as standard [23].

#### 2.5.2. Western blot analysis of Bsep, Mrp2 and AE2

Western blotting was performed in MPM using an amount of protein in the gels that was found to give a densitometric signal in the linear range of the response curve for the antibodies used (data not shown). Preparations were loaded onto 8% SDS–polyacrylamide gel and subjected to electrophoresis. After electrotransfer onto nitrocellulose membranes (Protran, Schleicher and Schuell, Keene, NH), the blots were blocked overnight at 4 °C with PBS containing 0.3% Tween 20 and 5% nonfat dry milk, and then incubated overnight with goat polyclonal antibody to mouse Bsep (P-18, Santa Cruz Biotechnologies, Santa Cruz, CA; 1:500) or with rabbit polyclonal antibody to rat AE2 (Alpha Diagnostic International Inc, San Antonio, TX; 1:250) or for 2 h with monoclonal antibody to human Mrp2 (M2III-6, Alexis Biochemicals, Carlsbad, CA; 1:2000). Immune complexes were detected by incubation with horseradish peroxidase-linked secondary antibodies (1:5000) for 1 h. Immunoreactive bands were detected using a chemiluminescence kit (ECL + Plus, Amersham Pharmacia Biotech, Inc) and quantified using the Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD) software.

#### 2.5.3. Immunofluorescence detection of Mrp2

Liver slices (5 μm) were prepared with a Zeiss Microm HM500 microtome cryostat, air dried for 2 h, and fixed for 10 min with cold acetone (−20 °C). For labeling, tissue sections were incubated overnight with the Mrp2 antibody (1:100). Sections were then washed five times with PBS, and incubated with Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA) (1:200) for 2 h. After washing three times with PBS and once with distilled water, slices were air dried and mounted.

The images were captured on a Zeiss Axiovert 25 CFL inverted microscope. To ensure comparable staining and image capture performance for SL and control groups, liver slices were prepared the same day, mounted on the same glass slide, and subjected to the staining procedure and microscopy analysis simultaneously.

#### 2.5.4. Northern blot analysis of Mrp2 mRNA

Total RNA was isolated from liver samples using a commercial kit (SV total RNA isolation system, PROMEGA, Madison, Wl) following the instructions provided by the manufacturer. Northern blot studies were performed as previously described in detail for UDP-glucuronosyltransferase [24]. Mrp2 cDNA represents ∼1.0 kb from 2222 to 3248 of rat Mrp2 gene (accession number: L49379). The Mrp2 probe was labeled with digoxigenin-dUTP by a random-primed methodology and detected with an alkaline phosphatase-conjugated antibody followed by chemiluminescent reaction (kit 1585614, Boehringer Mannheim). The bands were quantified using the Gel-Pro Analyzer software. The densitometry was done in the linear range of the film.

### 2.6. Mrp2 transport activity in isolated hepatocytes

In a different set of animals the whole liver was used for isolation of hepatocytes by collagenase perfusion and mechanical dissociation [25]. Hepatocytes, suspended in Krebs–Henseleit Ringer–Hepes buffer, pH 7.4, were used for determination of Mrp2 transport activity. Protein concentration in the suspensions was determined using bovine serum albumin as standard [23]. Cell viability was determined by trypan blue exclusion and was always greater than 87%. To evaluate Mrp2 activity, the transport rate of dinitrophenyl *S*-glutathione (DNP-SG), was measured in isolated hepatocytes as described [26]. Briefly, hepatocytes were loaded with DNP-SG by incubation with its precursor CDNB (100 μM) for 20 min at 10 °C. Cells were washed twice with the above mentioned buffer without CDNB at 10 °C and cooled at 0 °C. Aliquots were taken, loaded in the test tubes (0.4 ml polyethylene conical tubes) containing a lysis solution (3 M CINa, 0.1%Triton X-100) and a silicone layer (Wacker-Chemie GmbH, Munich, Germany) and incubated at 37 °C for 0, 30, 60 and 90 s. Then they were centrifuged at 12000 rpm (Beckman Microfuge) for 20 s causing the cells to filter through the silicone layer down to the lysis medium. DNP-SG was determined in supernatants (previous deprotenization with 70%, v/v, $HClO_4$) and cells (previous lysis after at least 24 h of incubation at room temperature) by HPLC. Separation of the conjugate was performed by a isocratic elution with a mobile phase of acetonitrile: 0.01% $H_3PO_4$ (1:3, v/v) at a flow rate of 1.0 ml/min [27]. DNP-SG was detected at 365 nm and quantified by the external standard method [28]. Transport rate was estimated by the slope of the regression of pmol of DNP-SG excreted per mg of hepatocyte protein versus time.

### 2.7. Statistical analysis

Data are presented as mean ± S.D. Statistical analysis was performed using the Student's *t*-test. Values of $p < 0.05$ were considered to be statistically significant.

## 3. Results

### 3.1. Effect of SL on biliary secretory function

Table 1 shows that bile flow was significantly increased in SL-treated rats (62%) as was previously documented [8,9]. Treatment with SL also increased the liver weight/body weight ratio (see Table 1) as described [10]. Whereas, the secretory rate of total glutathione and $HCO_3^-$ were increased in response to SL (138 and 59%, respectively), basal bile salt secretion was decreased by 31%. These data

Table 1
Bile flow and biliary secretion of bile salts, glutathione and $HCO_3^-$

| | Control | SL |
|---|---|---|
| Bile flow (μl/min/g liver) | 1.94 ± 0.09 ($n$ = 13) | 3.14 ± 0.21* ($n$ = 11) |
| Liver weight/body weight × 100 | 3.2 ± 0.3 ($n$ = 13) | 3.7 ± 0.3* ($n$ = 11) |
| Basal total bile salt secretion (nmol/min/g liver) | 71 ± 28 ($n$ = 11) | 48 ± 14* ($n$ = 10) |
| Biliary total glutathione excretion rate (nmol/min/g liver) | 5.4 ± 2.3 ($n$ = 8) | 12.8 ± 1.5* ($n$ = 6) |
| Biliary GSSG concentration (%) | 37 ± 12 ($n$ = 4) | 35 ± 8 ($n$ = 4) |
| Hepatic total glutathione content (nmol/g liver) | 4940 ± 664 ($n$ = 3) | 4998 ± 327 ($n$ = 3) |
| Hepatic GSSG content (%) | 3.1 ± 1.7 ($n$ = 3) | 3.2 ± 1.0 ($n$ = 3) |
| Biliary $HCO_3^-$ excretion rate (nmol/min/g liver) | 47 ± 4 ($n$ = 5) | 75 ± 4* ($n$ = 4) |

Total glutathione was expressed as equivalents of GSH, and GSSG as equivalents of GSH relative to total glutathione (%). Data are presented as means ± S.D.
* Significantly different from controls, $p < 0.05$.

Table 2
Bile flow and biliary secretion and liver content of glutathione in $TR^-$ rats

| | $TR^-$ control | $TR^-$ SL |
|---|---|---|
| Bile flow (μl/min/g liver) | 0.98 ± 0.15 | 1.19 ± 0.24 |
| Liver weight/body weight × 100 | 3.0 ± 0.5 | 3.5 ± 0.6 |
| Biliary total glutathione excretion rate (nmol/min/g liver) | 0.16 ± 0.02 | 0.22 ± 0.05 |
| Biliary GSSG concentration (%) | 30 ± 4 | 35 ± 2 |
| Hepatic total glutathione content (nmol/g liver) | 15542 ± 1781 | 16106 ± 1887 |
| Hepatic GSSG content (%) | 2.6 ± 0.8 | 3.5 ± 0.6 |

Total glutathione was expressed as equivalents of GSH, and GSSG as equivalents of GSH relative to total glutathione (%). Data are presented as means ± S.D. ($n = 3$).

suggest that the increased secretion of the main components of BSIF, rather than changes in secretion of bile salt, is responsible for the choleretic effect of SL. The increased secretory rate of total glutathione in response to SL without any change in liver content would indicate increased ability for transport at the canalicular level, as was suggested for other inducers [29]. The relative content of GSSG in bile and liver was similar in SL-treated and control rats. SL did not produce any choleresis in $TR^-$ rats (Table 2). Similarly, $TR^-$ rats treated with SL did not exhibit any difference either in biliary excretion of reduced or oxidized glutathione or in their liver content when compared to the respective controls.

To determine the mechanisms that led to a decrease in basal biliary secretion of bile salts in the SL group, we further studied bile salt synthesis, bile salt excretion and bile salt pool size as well as transport capacity at the canalicular level. Table 3 shows that the size of the endogenous bile salt pool was decreased by 30% by SL. Further data in Table 3 indicate that a decrease in de novo synthesis (33%) in the SL group was involved. Table 3 also shows that the relative composition of major bile salts assessed by HPLC was not affected by SL, suggesting that inhibition of the first step in their biosynthesis is likely involved. The finding that SL diminished cholesterol 7α-hydroxylase activity by 64% (Table 3) confirmed this presumption.

Studies on transport capacity of bile salts by the hepatocyte were performed by assessment of the Tm for TUDC in vivo. This parameter is well accepted to estimate the number of functional transporter units at the canalicular level. The Tm of TUDC was not affected by SL (969 ± 298 and 961 ± 169 nmol/min/g liver for control and SL group, respectively, $n = 3$), indicating a similar number of transport units for both groups. When the relationship between bile flow and bile salt output obtained during TUDC infusion was examined, it was evident (Fig. 1) that BSIF (estimated through the *y*-intercept of the regression line) was higher (50%) in SL-treated rats, whereas the choleretic efficiency of the secreted bile salts (estimated through the slope of the regression line) was unchanged.

### 3.2. Transport of DNP-SG in isolated hepatocytes

We also analyzed the secretion rate of DNP-SG, a Mrp2 exogenous model substrate, by isolated hepatocytes. As seen in Fig. 2, DNP-SG transport into the medium was significantly increased by SL (321 ± 16 and 478 ± 44 pmol/min/mg protein for control and SL groups, respectively, $p < 0.05$, $n = 3$). In contrast, intracellular levels of DNP-SG did not change in response to SL (32 ± 7 versus 36 ± 4 nmol/mg protein for control and SL hepatocytes, respectively, $n = 3$).

Table 3
Bile salt metabolism and transport

| | Control | SL |
|---|---|---|
| Bile salt pool size (μmol/kg body weight) | 389 ± 134 ($n = 5$) | 273 ± 72* ($n = 8$) |
| Relative content of individual bile salts in the bile salt pool (%) ($n = 4$) | | |
| α + βMC | 48.5 ± 3.3 | 41.3 ± 3.4 |
| UDC | 2.9 ± 1.6 | 2.3 ± 0.5 |
| HDC | 5.0 ± 1.6 | 8.5 ± 4.8 |
| C | 36.9 ± 3.0 | 39.5 ± 5.6 |
| CDC | 3.2 ± 1.3 | 3.6 ± 3.2 |
| CD | 2.2 ± 0.9 | 3.4 ± 1.7 |
| de novo bile salt synthesis rate (nmol/min/g liver) | 6.0 ± 1.6 ($n = 4$) | 4.0 ± 1.1* ($n = 8$) |
| Cholesterol 7α-hydroxylase activity (pmol of 7α-hydroxycholesterol/min/mg protein) | 33 ± 5 ($n = 4$) | 12 ± 1* ($n = 4$) |

α + βMC: glyco- and tauro-muricholate, UDC: glyco- and tauro-ursodeoxycholate, HDC: glyco- and tauro-hyodeoxycholate, C: glyco- and tauro-cholate, CDC: glyco-and tauro-chenodeoxycholate and DC: glyco- and tauro-deoxycholate. Results are means ± S.D.

* Significantly different from controls, $p < 0.05$.

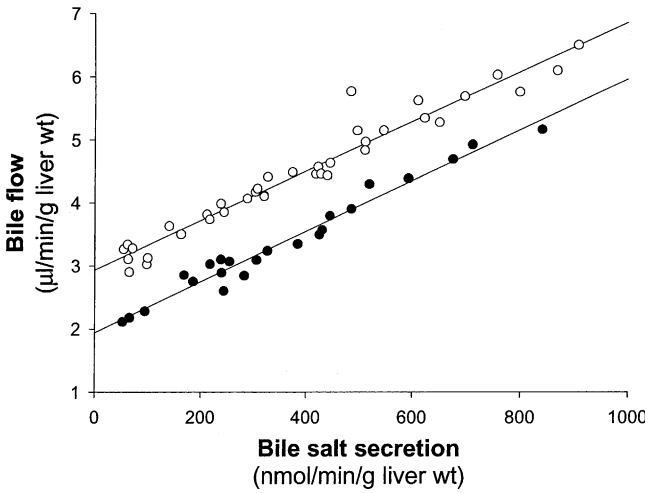


Fig. 1. Sequential changes in bile flow and total bile salt secretion in TUDC-infused animals. Control (filled circles) and SL-treated rats (empty circles) were infused i.v. with TUDC at stepwise-increasing rates (2.0; 2.3; 5.0; 6.5; 12.0 and 16.0 μmol/min/100 g body weight, $n = 3$). Regression analysis yielded the following equations: control: $y = 0.0040x + 1.94$ ($r = 0.97$) and SL: $y = 0.0039x + 2.94$ ($r = 0.95$). BSIF was estimated through the $y$-intercept of the regression line.

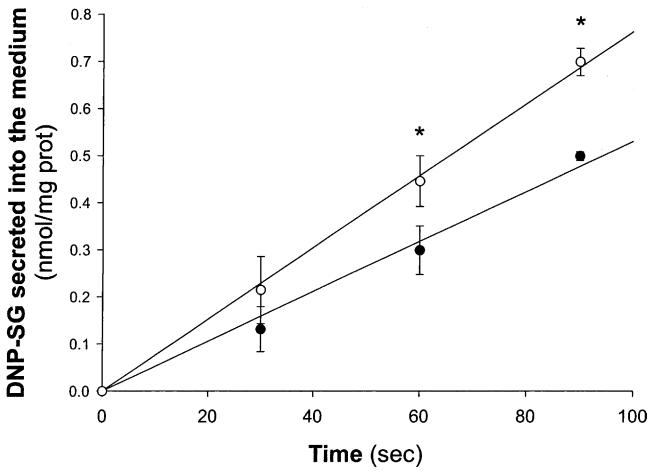


Fig. 2. DNP-SG excretion rate vs. time in isolated hepatocytes. DNP-SG transport was evaluated in triplicates in isolated hepatocytes from control (filled circles) and SL-treated rats (empty circles). The measurement was made by quadruplicate. Data represent means ± S.D. of three rats per group. *Significantly different from controls, $p < 0.05$.

### 3.3. Effect of SL on expression of Bsep, Mrp2 and AE2

Western blot studies were carried out in MPM. As shown in Fig. 3, densitometry reveals that SL did not modify the levels of Bsep, agreeing well with unchanged Tm values. In contrast, expression of Mrp2 protein was significantly increased by SL (about 70% over controls), consistent with increased ability of the SL livers to secrete glutathione and hepatocytes to export DNP-SG into the medium. There was no change in expression of AE2 protein between groups despite increased biliary $HCO_3^-$ secretion in SL-treated rats.

To confirm the presence of increased levels of Mrp2 in liver from SL rats in situ, we performed an immunofluor-

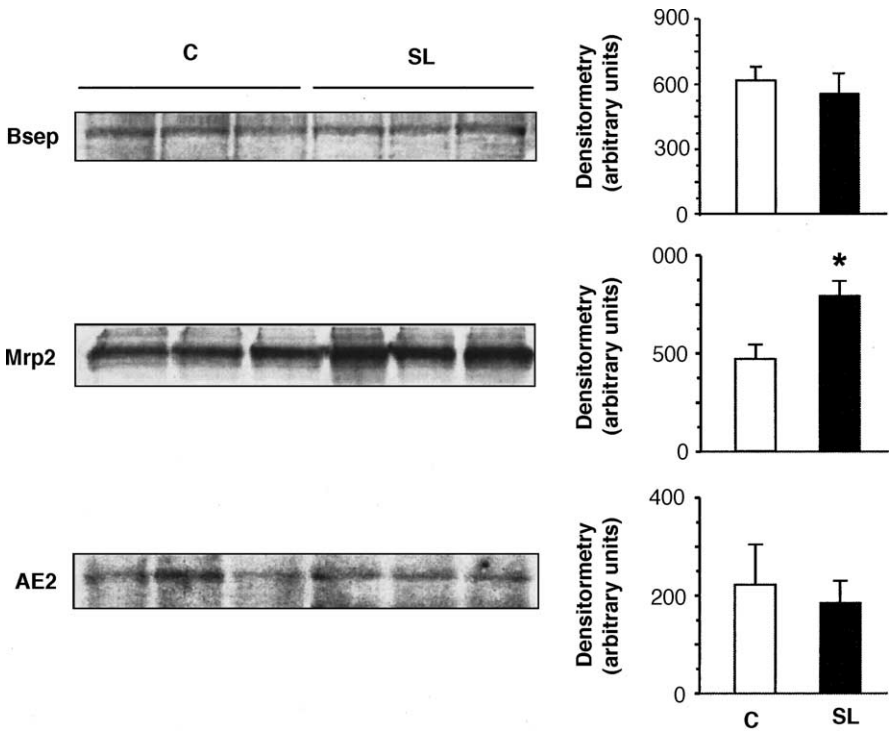


Fig. 3. Detection of Bsep, Mrp2 and AE2 by Western blotting. Canalicular transporters were detected in mixed plasma membranes. Equal amounts of total protein (15 μg for Bsep and Mrp2 detection and 30 μg for AE2 detection) were loaded in all lanes. Data on densitometric analysis represent means ± S.D. of three rats per group. *Significantly different from controls, $p < 0.05$.

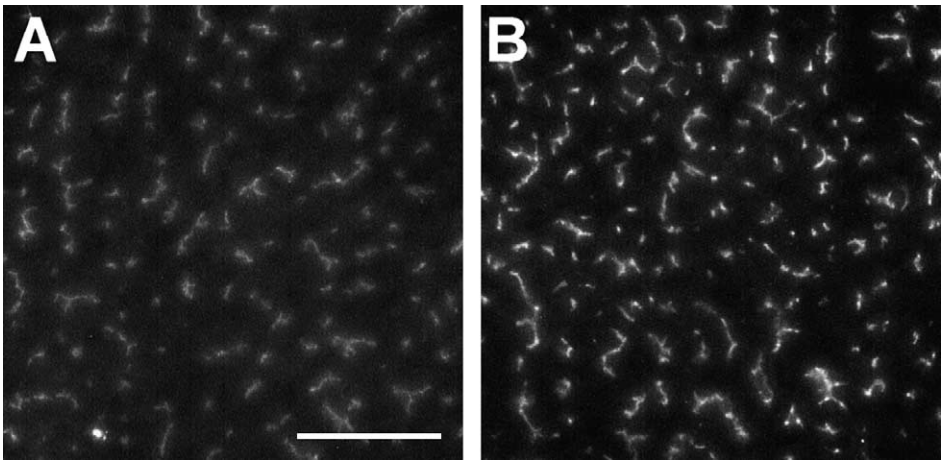


Fig. 4. Detection of Mrp2 by immunofluorescence. The images show immunodetection of Mrp2 at the canalicular level in control (A) and SL-treated rats (B). Detection of Mrp2 is more evident in SL group. Pictures are representative images from at least three independent experiments per group. White bar represents 40 μm.

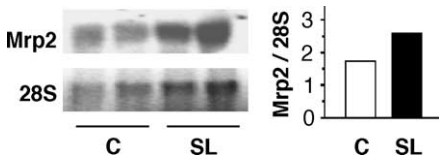


Fig. 5. Detection of Mrp2 mRNA by Northern blotting. Total RNA was extracted from liver of rats sacrificed 3 h after the last injection of SL. To correct for differences in total RNA loading and transfer among the lanes, the content of 28S rRNA was also estimated. The densitometric analysis is shown as the ratio of Mrp2 to 28S rRNA (mean values, $n = 2$). A similar increase in this ratio was detected in two additional experiments per group (images not shown).

escence microscopy study. Although no quantitative analysis of Mrp2 expression was performed with this methodology, the images in Fig. 4 clearly demonstrate that immunoreactivity, mainly localized at the canalicular level, was higher in SL group.

We further carried out Northern blot studies to establish whether induction of Mrp2 was at the transcriptional level. Fig. 5 shows that the level of mRNA encoding Mrp2 was increased by 50% 3 h after the last dose of SL. The same study performed with liver samples taken from rats sacrificed 18 h after the last injection of SL did not show variations between groups (image not shown). Thus, our data confirm previous reports demonstrating that mRNA Mrp2 levels vary with time after treatment with an inducer, variations being detectable for only a few hours after every injection [29]. Our analysis of mRNA levels 3 h after the last SL dose indicates that transcriptional regulation is at least partially involved in SL-induced Mrp2 expression.

## 4. Discussion

Administration of SL to experimental animals increases bile flow under basal conditions [8,9], though the mechanism is poorly known. We characterized the mechanisms involved in the choleresis induced by SL by evaluating its influence on the parameters determining the bile salt-dependent and -independent fractions of bile flow, particularly those concerning the expression and transport activity of Bsep and Mrp2. The data show that SL produced a reduction in BSDF as a consequence of a decrease in the size of the endogenous bile salt pool.

Regulation of the bile salt pool size depends on a balance among basolateral uptake, bile salt synthesis and secretion, and intestinal absorption. As SL did not modify either the Tm for TUDC or the expression of Bsep, the decrease in the BSDF is unlikely due to an alteration in canalicular secretion of bile salts. The current data indicate that a decrease in de novo synthesis of bile salts is involved. A key point in the regulation of bile salt synthesis is the microsomal enzyme cholesterol 7α-hydroxylase, the initial rate-limiting enzyme controlling the overall (cholate plus chenodeoxycholate) bile salt synthesis [30,31]. Our results showing a preserved relative composition of individual bile salts of the pool together with decreased cholesterol 7α-hydroxylase activity in response to SL administration indicated an inhibitory action on that particular step. Bile salt synthesis is inhibited by pregnane-X-receptor (PXR) ligands such as litocholic acid and pregnenolone 16α-carbonitrile at the level of cholesterol 7α-hydroxylase expression [31,32]. Furthermore, SL is able to activate the expression of *CYP3A23* gene in the LLC-PK1 cell line via PXR [33]. It is possible that PXR mediates the overall reduction in bile salt synthesis induced by SL in the current experimental conditions. In support to this assumption, we observed that SL produced a 3-fold increase in hepatic PXR mRNA levels detected by RealTime PCR (unpublished results). The possibility that SL also alters bile salt recirculation at the level of intestinal absorption or liver uptake can not be ruled out.

It has been demonstrated that glutathione and glutathione conjugates provide an important osmotic force

that significantly contributes to BSIF formation [34]. Our experiments demonstrate that SL similarly increased biliary excretion of both GSH and GSSG. This, together with the increase in the biliary secretion of $HCO_3^-$, an additional contributor to BSIF, could explain the increment in this fraction of bile flow. Augmented excretion of GSH species can be attributed to increased Mrp2 transport activity, as confirmed by the accelerated secretion of DNP-SG by isolated hepatocytes and by the absence of a choleretic effect of SL in $TR^-$ rats. In contrast, SL did not induce the AE2 protein expression. Consequently, the increased $HCO_3^-$ secretory rate observed in the SL group may result from a different, at present unknown, effect of the inducer. This could include activation of the exchanger, that depends on the variations in the intracellular pH [35] and/or an additional source other than canalicular secretion (i.e. cholangiocytes) [36]. The possibility that osmotically active SL metabolites could contribute, at least partially, to the choleresis observed in treated rats can not be excluded.

Because BSDF was impaired by SL, we speculate that the enhancement in biliary secretion of glutathione species and $HCO_3^-$ in the SL group was able to override the negative impact of reduced bile salt secretion. Experimental cholestasis in rats has been associated with impaired Mrp2-mediated transport as well as down-regulation of the protein [37]. It would be of interest to determine whether SL is able to reverse the altered liver secretory function in rats under conditions of impaired Mrp2 expression and activity. In addition, we demonstrated that SL decreased the bile salt pool size, which could be instrumental in neutralizing the deleterious effect of bile salts accumulated as a result of cholestasis. In support to this possibility, Feher et al. [38] reported that SL administration to cirrhotic or bile-duct obstructed patients decreased plasma bile acid levels by more than 50%. These authors postulated that the reported effect could result from an accelerated microsomal metabolism of bile salts and pointed out the significance of giving SL to cirrhotic patients exhibiting raised serum bile salt levels who are not receiving the drug. In view of our results, an inhibitory effect on bile salt synthesis is likely an alternative mechanism to explain the SL-induced decrease in serum bile salt levels reported by these authors.

Increasing evidence indicates that transport of metabolites from endogenous compounds and drugs at the canalicular level, rather than metabolism itself, is the rate-limiting step in their overall liver transport, particularly under saturating conditions. Thus, under inducing conditions, upregulation of transport systems at the apical pole of the hepatocyte, along with increased metabolism, may be instrumental in increasing the hepatic clearance of common substrates. We previously reported that SL increases the in vivo Tm of bilirubin under unconjugated bilirubin overloading conditions in rats [39], in concert with increased expression of the main isoforms of UGT involved in pigment glucuronidation, UGT1A1 and UGT1A5 [11]. Conjugation of bilirubin with UDP-sugars is likely a prerequisite for Mrp2-mediated secretion, since conjugated bilirubin is a known Mrp2 substrate [40]. We also demonstrated an increase in expression and activity of GST in liver from SL-treated rats [10]. These previous data, together with the current results on up-regulation of Mrp2 by SL, support the assumption that induction of phase II metabolizing enzymes together with Mrp2 would improve liver transport of compounds that are substrates for both systems. Simultaneous upregulation of phase II enzymes and Mrp2 can result from a coordinated mechanism such as binding of SL to a nuclear receptor commonly regulating both systems. A possible candidate is PXR since other known ligands of this nuclear receptor, namely, dexamethasone and pregnenolone 16α-carbonitrile were previously found to similarly upregulate Mrp2 and specific forms of cytochrome P-450, GST and UGT enzyme systems in liver [41]. Our recent experiments demonstrating increased levels of PXR mRNA in liver from SL-treated rats support this possibility.

In line with the effects of SL here reported, other authors demonstrated that the administration of this drug to patients as a diuretic agent accelerates the clearence of coadministered drugs, basically as a result of its inducing properties on liver drug metabolism [42,43]. We postulate that upregulation of expression of major liver drug transporters by SL may represent an additional mechanism explaining drug–drug interaction when this steroid is administered as a therapeutic agent. The dose of SL administered to patients (e.g. 100 mg/day) is usually far below the dose administered to rats in the current study. However, the therapeutic protocol requires chronic administration and could represent a much more sustained action leading to alteration of the parameters here evaluated.

In conclusion, the present study demonstrates that SL increases the total bile flow by increasing the bile salt-independent fraction in the rat. Increased Mrp2-mediated biliary secretion of glutathione species, together with increased secretion of $HCO_3^-$ are likely involved. In contrast, the bile salt dependent fraction of bile flow was diminished by SL as a consequence of a decrease in de novo synthesis of bile salts. Because of the previous findings on increased activity of phase II enzymes in SL-treated animals, we postulate a coordinated induction of metabolism and transport of phase II substrates by this compound.

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