Beneficial Effect of Spironolactone Administration on Ethynylestradiol-Induced Cholestasis in the Rat: Involvement of Up-Regulation of Multidrug Resistance-Associated Protein 2

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ABSTRACT:
The effect of spironolactone (SL) administration on 17α-ethynylestradiol (EE)-induced cholestasis was studied, with emphasis on expression and activity of Mrps. Adult male Wistar rats were divided into the following groups: EE (5 mg/kg daily for 5 days, s.c.), EE + SL (same doses, SL administered the last 3 days of EE treatment), and controls. SL prevented the decrease in bile salt-independent fraction of bile flow induced by EE, in association with normalization of biliary excretion of glutathione. Western blot studies indicate that EE decreased the expression of multidrug resistance-associated protein 2 (Mrp2) by 41% and increased that of Mrp3 by 200%, whereas SL only affected Mrp2 expression (+80%) with respect to controls. The EE + SL group showed increased levels of Mrp2 and Mrp3 to the same extent as that registered for the individual treatments. Real-time polymerase chain reaction studies indicated that up-regulation of Mrp2 and Mrp3 by SL and EE, respectively, was at the transcriptional level. To estimate Mrp2 and Mrp3 activities, apical and basolateral excretion of acetaminophen glucuronide (APAP-glu), a common substrate for both transporters, was measured in the recirculating isolated perfused liver model. Biliary/perfusate excretion ratio was decreased in EE (~88%) and increased in SL (+36%) with respect to controls. Coadministration of rats with SL partially prevented (~53%) impairment induced by EE in this ratio. In conclusion, SL administration to EE-induced cholestatic rats counteracted the decrease in bile flow and biliary excretion of glutathione and APAP-glu, a model Mrp substrate, findings associated with up-regulation of Mrp2 expression. Thus, SL administration to EE-induced cholestatic rats counteracted the decrease in bile flow and biliary excretion of glutathione and APAP-glu, a model Mrp substrate, findings associated with up-regulation of Mrp2 expression.

Bile flow formation is dependent upon concentrative efflux of osmotically active solutes from the hepatocyte to the canaliculus space followed by passive water movement (Nathanson and Boyer, 1991). This step is facilitated by members of the ATP-binding cassette family and involves the concentrative secretion of solutes from the cell into the bile fraction of bile flow, which is comprised of bile acids, taurocholate, and other bile salts. The transmembrane transport of bile salts into the canaliculus (Gerloff et al., 1998), generating the bile salt-dependent component of bile flow (BSDF), and the multidrug resistance-associated protein 2 (Mrp2), which transports lipophilic bile acids, are the main mechanisms contributing to BSDF formation (Meier et al., 1985). Bile formation is affected by several drugs acting at different levels in the hepatocyte. Estrogens are involved in the pathogenesis of both oral contraceptive-induced cholestasis and cholestasis of pregnancy (Vore, 1987; Reyes and Simon, 1993). Ethynylestradiol (EE), a synthetic estrogen, is known to reduce bile flow formation in experimental animals, thus representing a useful model to study estrogen cholestasis (Crocenzi et al., 2001). In the rat, alterations in both BSIF and BSDF by EE have been associated with decreased expression and activity of Bsep and Mrp2 (Trauner et al., 1997; Lee et al., 2000), as well as increased expression and activity of basolateral Mrp3 (Ruiz et al., 2006). This finding would indicate altered disposition of compounds that are common substrates for Mrp2 and Mrp3 with a potential shift in their excretion from bile to bloodstream, as demonstrated for the therapeutic drug acetaminophen (APAP) (Ruiz et al., 2006).

The steroid spironolactone (SL) is a therapeutic drug used as a diuretic in patients with edema or ascites (Ochs et al., 1978) and shows inductive properties on biotransformation enzymes in humans 2 (AE2), is responsible for bicarbonate secretion into bile and also contributes to BSIF formation (Meier et al., 1985). Bile formation is affected by several drugs acting at different levels in the hepatocyte. Estrogens are involved in the pathogenesis of both oral contraceptive-induced cholestasis and cholestasis of pregnancy (Vore, 1987; Reyes and Simon, 1993). Ethynylestradiol (EE), a synthetic estrogen, is known to reduce bile flow formation in experimental animals, thus representing a useful model to study estrogen cholestasis (Crocenzi et al., 2001). In the rat, alterations in both BSIF and BSDF by EE have been associated with decreased expression and activity of Bsep and Mrp2 (Trauner et al., 1997; Lee et al., 2000), as well as increased expression and activity of basolateral Mrp3 (Ruiz et al., 2006). This finding would indicate altered disposition of compounds that are common substrates for Mrp2 and Mrp3 with a potential shift in their excretion from bile to bloodstream, as demonstrated for the therapeutic drug acetaminophen (APAP) (Ruiz et al., 2006).
and experimental animals (Ochs et al., 1978; Catania et al., 2004). SL also induces cholestasis in the rat as a result of a substantial increase in BSIF, whereas BSDF is impaired by this steroid as a result of decreased bile salt pool size (Ruiz et al., 2005). Increased BSIF is in turn associated with increased biliary secretion of glutathione species and up-regulation of Mrp2, and with increased excretion rate of bicarbonate.

Several approaches have been considered to prevent or revert experimental estrogen-induced cholestasis, with emphasis on restoration of bile secretory function (Dumont et al., 2002; Sánchez Pozzi et al., 2003; Crocenzì et al., 2004, 2005; Gerk et al., 2007). However, none of them evaluated the potential benefit of using a classical inducer, known to increase Mrp2 expression and, concomitantly, its activity. Preserved normal Mrp2 function at the canalicular level is relevant not only to BSIF formation, but also to biliary elimination of endogenous compounds such as bilirubin glucuronides and a wide variety of xenobiotics (Catania et al., 2004). Thus, the purpose of the current study was to evaluate the ability of SL to normalize bile flow formation and Mrp2 function in EE intrahepatic cholestasis. The data indicate that SL was able to prevent the impairment in bile flow and glutathione biliary secretion, in association with an increased expression of Mrp2. In addition, SL partially restored the capability for biliary secretion of APAP glucurionate (APAP-glu), found to be substantially deteriorated in EE rats.

**Materials and Methods**

**Chemicals.** SL, EE, leupeptin, phenylmethylsulfonyl fluoride, pepstatin A, glutathione (GSH), glutathione reductase, 3α-hydroxysteroid dehydrogenase, β-NAD, NADPH, HEPES, palmityl lysophosphatidylcholine, d-saccaric acid 1,4-lactone, UDP-glucuronic acid, APAP, and APAP-glu were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical-grade purity and used as supplied.

**Animals and Treatment.** Adult male Wistar rats (300–360 g) were used throughout. They were maintained ad libitum on a standard laboratory pelleted diet and were allowed free access to water and saline during treatment. Animals were randomly divided into four experimental groups. 1) In the EE group, EE was dissolved in propylene glycol (33.7 mM) and administered at a daily dose of 5 mg/kg b.wt. s.c., for 5 consecutive days (Crocenzì et al., 2001; Ruiz et al., 2006). 2) In the SL group, rats were injected i.p. with SL dissolved in propylene glycol (60 mM), at a daily dose of 200 μmol/kg body weight (equivalent to 83.3 mg/kg body weight), for 3 consecutive days (Ruiz et al., 2005). 3) In the EE + SL group, rats were injected with EE, as in the EE group, for 5 days, and simultaneously with SL, as in the SL group, for the last 3 days of the EE protocol. 4) In the control group, rats were injected with vehicle (propylene glycol) either s.c. for 5 days (0.5 ml/kg b.wt.) or i.p. for 3 days (3.3 ml/kg b.wt.) or both simultaneously. 

**Western Blotting Studies.** Western blot analyses of Mrp2, Bsep, and AE2 were performed in MPM as described (Ruiz et al., 2005). Detection of Mrp3 was performed also in MPM using a rabbit polyclonal antibody to rat Mrp3 (Ogawa et al., 2000), as previously described (Ghanem et al., 2005). Detection of UDP-glucuronosyltransferases (UGTs) was performed in microsomal preparations using polyclonal anti-peptide antibodies that specifically recognize the 1A6 and 1A7 isoforms belonging to UGT family 1 (Ikushiro et al., 1995), as previously reported (Luquita et al., 2001). Immunoreactive bands in the different studies were quantified using the Gel-Pro Analyzer software (Media Cybernetics, Bethesda, MD).

**Quantitative Real-Time PCR.** Total RNA was isolated from liver samples using TRIzol (Invitrogen, Carlsbad, CA), following the manufacturer’s protocol. cDNA was produced by using the SuperScript Preamplification System for first-strand cDNA synthesis according to the manufacturer’s instructions (Invitrogen). Real-time quantitative PCR was performed on cDNA samples using the MiniOpticon System (Bio-Rad Laboratories, Hercules, CA). Sequences of primer pairs and conditions for Mrp3 and Bsep were as described previously (Cao et al., 2002; Gao et al., 2004), and for Mrp2 and 18S, they were designed to optimally detect the respective mRNAs. All sequences and conditions are summarized in Table 1. The amplified product size by each pair of primers was 450, 573, 164, and 150 bp, for Mrp2, Mrp3, Bsep, and 18S, respectively. At the end of these experiments, PCR products were removed from tubes and analyzed by gel electrophoresis to confirm the product of interest. Quantification of the target cDNAs in all samples was normalized to 18S ribosomal RNA (Ct_target − Ct_18S = ΔCt) and the difference in expression for each target cDNA in the treated groups was expressed to the amount in the control group (ΔCt_treated − ΔCt_control = ΔΔCt). Fold changes in target gene expression were determined by taking 2 to the power of this number (2ΔΔCt).

**Mrp Transport Activity in Recirculating IPL.** To evaluate to what extent the different treatments affect normal biliary secretion of a common substrate for both Mrp2 and Mrp3, the apical and basolateral transport rates for APAP-
glu were measured. After a low, nontoxic dose of APAP is injected in vivo to rats or incorporated to the rat IPL system, it is efficiently converted to its glucuronide (Kessler et al., 2002). Subsequent APAP-glu excretion into bile and perfusate gives an estimation of its transport by Mrp2 (Xiong et al., 2000) and Mrp3 (Manautou et al., 2004), respectively. The livers were isolated and perfused for 60 min with oxygenated Krebs-Ringer bicarbonate buffer containing APAP (2 μmol/ml) as described previously (Ruiz et al., 2006). Liver viability was maintained controlled throughout by monitoring LDH leakage into the perfusate (with values <10 IU/l between the start and the end of the experiment). The content of APAP-glu in bile, perfusate, and liver homogenate was determined by HPLC (Ghanem et al., 2005).

Figure 3A shows that the content of APAP-glu in perfusate determined at 60 min, equivalent to the amount of the metabolite accumulated during a single perfusion period. The results are shown in the figure. The difference in APAP-glu content between control and experimental groups was statistically significant (p < 0.05).

Table 1 summarizes the data. The table includes the mean and standard deviation of APAP-glu content in perfusate, bile, and liver homogenate for different experimental groups.

### Results

#### Biliary Secretory Function

Individual treatment with EE or SL as well as the coadministration of both compounds caused an increase in the liver to body weight ratio with respect to controls (Table 2), mainly due to an increase in liver weight (data not shown). As expected from previous studies (Koopen et al., 1998; Croczeni et al., 2001), basal bile flow and bile acid, bicarbonate, and glutathione excretion rates were reduced by EE. SL increased bile flow and bicarbonate and glutathione excretion rates, whereas bile salt output was decreased. Administration of SL to EE-treated rats for the last 3 days of the EE protocol normalized bile flow through selective induction of glutathione and bicarbonate biliary output. Normalization in bile flow formation occurred despite the fact that bile salt output remained impaired. The hepatic content of glutathione was not different among groups (Table 2), despite the significant changes detected in its biliary excretion in rats treated with EE or SL alone. This finding could be tentatively explained by adaptive changes in the same direction in its hepatic synthesis or, alternatively, by changes in the opposite direction in its basolateral efflux. Alkaline phosphatase was increased by EE as expected from a cholestatic model and returned to control values in the EE+SL group. Serum bile salt levels were significantly increased in EE-treated rats, probably as a consequence of decreased biliary secretion (Croczeni et al., 2001) and hepatic uptake (Bossard et al., 1993). Increased basolateral efflux of bile salts via Mrp3 (Ruiz et al., 2006) may have also contributed to explain their increased serum levels. Experimental groups receiving SL showed no changes in serum bile salt levels with respect to controls.

#### Expression of Mrp2, Mrp3, Bsep, and AE2

Western blot studies (Fig. 1) indicate that Mrp2 protein expression was significantly reduced (~41%) after EE treatment and increased (+60%) after SL administration, as was previously reported (Trauner et al., 1997; Lee et al., 2000; Ruiz et al., 2005, 2006). Coadministration to animals of both steroids up-regulated Mrp2 levels by 50% over controls, consistent with the effect registered for SL alone. Figure 1 also shows that whereas EE produced a marked increase in Mrp3 protein expression (+200%), as we reported previously (Ruiz et al., 2006), SL did not show any effect. The conjoint treatment increased Mrp3 protein level by 260%, which was similar to the extent registered for EE alone. Thus, in the EE+SL group was observed a preferential induction of Mrp3 versus Mrp2 compared with controls. Bsep expression tended to decrease in response to EE, although it did not reach statistical significance. SL administration did not affect Bsep levels. Figure 1 shows that AE2 also remained unaffected in all groups.

It is expected that increased synthesis of mRNA precedes induction of protein synthesis under conditions of transcriptional regulation. To establish whether up-regulation of Mrp2 and Mrp3 as shown in Fig. 1 results from increased expression of their respective mRNAs, we assessed their levels by real-time PCR up to 12 h after the last injection of SL and/or EE. Because induction of mRNA levels may be transient, we selected 3, 6, and 12 h postinjection as time points. Data in Fig. 2 are presented as percentages and were referred to the respective control, considered as 100%. In the EE group, Mrp2 mRNA expression remained invariant, whereas Mrp3 mRNA increased at 6 and 12 h to a similar extent. In contrast, SL significantly increased Mrp2 mRNA expression at 12 h without affecting Mrp3 mRNA levels. Conjoint treatment resulted in increased levels for both mRNAs at both 6 and 12 h, as expected from their individual effects, although some differences in the magnitude of induction between individual and simultaneous treatment were noted. Although we only found a trend to decrease in Bsep protein in response to EE, other authors showed a moderate but significantly decrease with the same protocol (Trauner et al., 1997; Lee et al., 2000). We further decided to evaluate the potential changes in the corresponding mRNA in our groups. We found no variations in this measure at any time or group studied (data not shown).

#### Mrp2 and Mrp3 Transport Activity in IPL

In view of the selective induction of Mrp2 and Mrp3 produced by SL and EE, respectively, it was of interest to evaluate their impact on transport function. The influence of the relative contribution of Mrp2 versus Mrp3 to preferential biliary versus basolateral disposition of the common substrate APAP-glu was evaluated using the IPL model. Figure 3A shows that the content of APAP-glu in perfusate determined at 60 min, equivalent to the amount of the metabolite accumulated during this time, was increased by 60% in the EE group, by 77% in the SL group, and by 130% in the EE+SL group over controls. Cumulative biliary excretion of APAP-glu by this same period significantly decreased in response to EE (~80%), increased in response to SL (+110%), and was not affected in the EE+SL group, compared

### Table 2

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<th>Control</th>
<th>EE</th>
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<th>EE+SL</th>
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<tr>
<td>Liver weight/body weight (%)</td>
<td>3.25 ± 0.14</td>
<td>3.83 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.7 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.4 ± 0.2&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<td>Bile flow (μl/min/liver)</td>
<td>2.00 ± 0.17</td>
<td>0.98 ± 0.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.43 ± 0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.84 ± 0.17&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Bile salt output (nmol/min/g liver)</td>
<td>58.2 ± 9.7</td>
<td>36.7 ± 15.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.7 ± 7.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.2 ± 7.5&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Biliary excretion of bicarbonate (nmol/min/g liver)</td>
<td>51.2 ± 4.0</td>
<td>23.8 ± 7.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90.1 ± 10.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.2 ± 4.1&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<td>Biliary excretion of total glutathione (nmol/min/g liver)</td>
<td>2.53 ± 0.46</td>
<td>0.38 ± 0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.79 ± 0.71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.11 ± 0.16&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Hepatic content of total glutathione (nmol/g liver)</td>
<td>5984 ± 562</td>
<td>5391 ± 773</td>
<td>5623 ± 1466</td>
<td>4344 ± 977</td>
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<td>Alkaline phosphatase (U/l)</td>
<td>260 ± 47</td>
<td>333 ± 24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>143 ± 12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>199 ± 28&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Serum bile acids (μM)</td>
<td>213 ± 79</td>
<td>1171 ± 489&lt;sup&gt;a&lt;/sup&gt;</td>
<td>145 ± 96&lt;sup&gt;c&lt;/sup&gt;</td>
<td>272 ± 77&lt;sup&gt;c&lt;/sup&gt;</td>
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<sup>a</sup> Significantly different from controls (p < 0.05).
<sup>b</sup> Significantly different from EE (p < 0.05).
<sup>c</sup> Significantly different from SL (p < 0.05).
with controls (Fig. 3B). This latter result indicates that SL was able to restore EE-induced impairment in biliary secretory function associated with Mrp2. Figure 3C shows that hepatic content of APAP-glu, determined at the end of the experiment, was affected by SL only when administered alone, with an increase of 107% over controls. The total amount of APAP-glu produced by the liver throughout the experiment was estimated as a summation of cumulative biliary and perfusate excretions and liver content, and is shown in Fig. 3D. This measure was significantly increased in response to SL, when administered either alone (+90%) or together with EE (+78%). Clearly, SL stimulated the synthesis of APAP-glu, thus increasing availability of the common Mrp substrate for subsequent secretion via Mrp2 or Mrp3. In this model, transport activity is conditioned by the substrate availability in addition to transporter expression. Thus, it is not clear whether SL administration leads to an increase in intrinsic activity of Mrp2. To eliminate the influence of increased production of APAP-glu in SL and EE/SL groups, we referred the biliary and perfusate excretion, as well as the hepatic content, to the total amount of APAP-glu formed (see Table 3). Relative biliary excretion of this metabolite was impaired by EE and by EE/SL treatments by 83 and 44%, respectively, and slightly but significantly increased in SL group (19%). Relative basolateral excretion was increased only by EE (45%) and EE/SL (+25%) groups. Table 3 also shows that the relative hepatic content exhibited a decrease in EE (−42%) and EE/SL (−51%) groups, whereas this measure was not affected by SL alone. The biliary/perfusate elimination ratio was substantially decreased by EE (−88%) and only partially decreased by EE/SL (−53%), compared with controls. Administration of SL alone slightly but significantly increased (+36%) this same ratio.

**UGT Expression and Activity.** We have previously characterized SL as an inducer of selective phase II biotransformation enzymes, including UGT (Catania et al., 2004). To establish whether modifications in UGT activity mediate the induction of APAP-glu synthesis as detected in IPL, we further assessed its activity toward APAP. Figure 4A shows that UGT activity was increased by 100% in rats receiving SL, either when administered alone or conjointly with EE, consistent with the findings in IPL. EE itself did not affect this measure. The higher activity in SL and EE/SL groups is associated, at least in part, with induction of expression of isof orm UGT1A7 (78% and 129%, respectively), whereas UGT1A6, another isof orm
involved in conjugation of APAP (Kessler et al., 2002), was not significantly different from SL, as in the EE group. This could explain why the combined treatment did not lead to restoration in biliary excretion of APAP-glu to the same extent shown for SL alone. We also observed that SL administered alone or in combination with EE increased expression and activity of UGT involved in APAP conjugation, thus increasing the availability of APAP-glu generated endogenously in IPL for subsequent disposition. The partial restoration of biliary secretory function for APAP-glu is better illustrated in Table 3, where excretion rates and hepatic content were referred to total amount of this derivative.

**Discussion**

EE administration to rats represents a well characterized model of drug-induced intrahepatic cholestasis, which has been largely tested to develop therapeutic approaches of potential applicability in humans. Decreased expression and function of canalicular Bsep and Mrp2 are a major factor of deteriorated bile flow formation in this model (Trauner et al., 1997; Lee et al., 2000; Crocenzi et al., 2001). Ursodeoxycholate is a bile salt commonly used in the treatment of cholestatic diseases (Paumgartner and Beuers, 2002), including cholestasis of pregnancy (Palma et al., 1997). Dumont et al. (2002) reported that ursodeoxycholic acid restored bile flow in EE-treated rats by increasing bile acid secretion, possibly as a result of normalization in Bsep expression. They also observed that this treatment did not improve glutathione output or bile acid-independent flow. More recently, Crocenzi et al. (2005) demonstrated that ursodeoxycholate did not prevent down-regulation of Mrp2 induced by EE but positively modulates its activity, as detected through assessment of biliary excretion of dinitrophenyl S-glutathione, a known Mrp2 substrate. This could result from a direct interaction of this therapeutic bile salt with Mrp2 (Gerk et al., 2007), thus leading to partial restoration of its transport function. The flavonoid silymarin was also effective in partially preventing EE-induced cholestasis in rats mainly by normalizing the diminished bile salt pool size and bicarbonate output (Crocenzi et al., 2001). Neither of these therapeutic agents was able to prevent the decrease in Mrp2 protein level and GSH biliary secretory rate. In the current study we explore for the first time the benefit of using a classical enzyme inducer to prevent deterioration of Mrp2 expression and function produced by EE. Indeed, SL is a well known phase I and II enzyme inducer (Ochs et al., 1978; Catania et al., 2004) with additional choleretic properties due to increased expression of Mrp2 and BSIF formation (Ruiz et al., 2005). This feature makes SL a good candidate to test its ability to prevent EE-induced cholestasis in the rat.

Our current in vivo studies on biliary secretory function confirmed previously reported cholestatic/choleretic activity of EE/SL and further demonstrate that coadministration of the inducer with EE, for the last 3 days of the EE protocol, completely prevented impairment in bile flow formation. This occurred despite the fact that bile salt secretion remained deteriorated, probably as a consequence of decreased hepatic synthesis of bile salts, which was demonstrated individually for both EE and SL (Koopen et al., 1999; Ruiz et al., 2005). This is the most suitable explanation since Bsep expression and the levels of serum bile salts were normal in the EE+SL group. Rather, restoration of bile flow formation was based on preserved biliary secretory rate for both GSH (associated to up-regulation of Mrp2) and bicarbonate. Despite the preserved levels of AE2, an activation of the exchanger by SL cannot be ruled out because of the dramatic increase in bicarbonate biliary excretion rate. Alternatively, the source of biliary bicarbonate could result from exacerbated ductular secretion. Confirmation that cholestasis was counteracted by SL comes from analysis of the data on the serum markers alkaline phosphatase and total bile acids, which were elevated in EE and completely normalized in the EE+SL group, compared with controls.

Consistent with restored biliary secretion of GSH described in in vivo experiments, administration of SL together with EE countered EE-induced impairment in biliary secretion of APAP-glu in IPL. This major APAP metabolite is a common substrate for both Mrp2 and Mrp3, although it presents higher affinity for Mrp3 (Manautou et al., 2004). Thus, partial restoration of its biliary secretion in the EE+SL group is of relevance, considering that these same rats exhibited significant up-regulation of Mrp3 and that this transporter efficiently delivers APAP-glu to the perfusate (Ruiz et al., 2006). Indeed, up-regulation of Mrp3 was present in EE+SL and reached the same level as in the EE group. This could explain why the combined treatment did not lead to restoration in biliary excretion of APAP-glu to the same extent shown for SL alone. We also observed that SL administered alone or in combination with EE increased expression and activity of UGT involved in APAP conjugation, thus increasing the availability of APAP-glu generated endogenously in IPL for subsequent disposition. The partial restoration of biliary secretory function for APAP-glu is better illustrated in Table 3, where excretion rates and hepatic content were referred to total amount of this derivative.
Irrespective of APAP-glu production, it is clearly shown that its preferential biliary versus basolateral disposition in normal rats was inverted in rats receiving EE, and that these same rats exhibited a more efficient capability for elimination of this metabolite from the cell, probably reflecting a higher affinity toward APAP-glu reported for Mrp3 versus Mrp2 (Manautou et al., 2004).

Expression of hepatobiliary transporters is regulated by nuclear receptors, as has been demonstrated for phase I and II biotransformation enzymes. Kast et al. (2002) have reported that MRP2/Mrp2 genes are modulated by pregnane X receptor (PXR), farnesoid X receptor, and constitutive androstane receptor in human and rodents. These three distinct nuclear receptor signaling pathways converge on a common response element in the 5′-flanking region of the gene. PXR activation is dependent upon the formation of the heterodimer with the retinoid X receptor. SL is a well known ligand of PXR, as was also demonstrated for the steroids pregnenolone 16α-carbonitrile, 5β-pregnane-3,20-dione, dexamethasone, and so on (Schuetz et al., 1998; Kliewer et al., 2002). Thus, our data on increased expression of Mrp2 and Bsep in the rat model as a major factor contributing to cholestasis (Trauner et al., 1997; Lee et al., 2000). More recently, it

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<th>Control</th>
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<th>SL</th>
<th>EE+SL</th>
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<tr>
<td>Perfusate (%)</td>
<td>64 ± 4</td>
<td>93 ± 1a</td>
<td>56 ± 5b</td>
</tr>
<tr>
<td>Biliary (%)</td>
<td>36 ± 4</td>
<td>6 ± 1a</td>
<td>43 ± 5a,b</td>
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<tr>
<td>Hepatic content (%)</td>
<td>0.057 ± 0.013</td>
<td>0.033 ± 0.008a</td>
<td>0.066 ± 0.015a</td>
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<td>Biliary/perfusate ratio</td>
<td>0.57 ± 0.10</td>
<td>0.07 ± 0.02a</td>
<td>0.78 ± 0.16b</td>
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a Significantly different from controls (p < 0.05).
b Significantly different from EE (p < 0.05).
c Significantly different from SL (p < 0.05).

Fig. 4. UGT activity and immunodetection of UGT isoforms. UGT activity was assessed using APAP as a substrate (A). Only major UGT isoforms belonging to family 1 and involved in APAP glucuronidation were assessed in microsomal membranes (B). Equal amounts of total protein (15 μg) were loaded in the gels. This amount of protein gave a densitometric signal in the linear range of the response curve for the different antibodies. Uniformity of loading and transfer from gel to nitrocellulose membrane was controlled with Ponceau S. Data on densitometric analysis represent means ± S.D. of three rats per group. a Significantly different from controls, p < 0.05; b significantly different from EE, p < 0.05.
was demonstrated in mice that the effect on Bsep was mediated by binding of EE to the estrogen receptor α (Yamamoto et al., 2006). Further studies are necessary to establish whether this same receptor mediates transcriptional up-regulation of Mrp3, as currently demonstrated.

Patients with liver disease, mostly cirrhosis, often receive SL as a diuretic. However, prolonged administration may lead to pharmacologic effects other than that of aldosterone antagonist. Miquet et al. (1980) demonstrated that the administration of SL to humans with alcoholic cirrhosis accelerates the clearance of coadministered drugs, basically as a result of its inducing properties on liver drug metabolism. We postulate that Mrp2 up-regulation by SL may represent an additional mechanism explaining drug-drug interaction when the steroid is administered as a therapeutic agent. Feher et al. (1976) reported that SL administration to patients with cirrhosis or different forms of bile duct obstruction decreased plasma bile acid levels by more than 50%, which could be tentatively explained by an inhibitory action on bile salt synthesis (Rui et al., 2005). This action of SL, leading to the attenuation of deleterious effects of bile salts accumulated in cholestatic liver, together with the significant restoration of Mrp2 expression and activity, as currently demonstrated, may suggest the potential clinical application of SL in intrahepatic cholestasis. Exploring the action of SL in human models, such as primary hepatocytes culture or liver cell lines, would represent a preliminary approach to validate this possibility.

In conclusion, SL counteracted the alterations in bile flow formation induced by EE through transcriptional up-regulation of Mrp2 and the associated increase in GSH biliary excretion. Restoration of Mrp2 expression coexisted with up-regulation of Mrp3, as induced by EE itself, and led to partial prevention in impairment of biliary secretion of a common Mrp substrate induced by this cholestatic estrogen.

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References


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