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BENEFICIAL EFFECT OF SPIRONOLACTONE ADMINISTRATION ON ETHYNYLESTRADIOL-INDUCED CHOLESTASIS IN THE RAT. INVOLVEMENT OF UP-REGULATION OF Mrp2.

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Abstract

The effect of spironolactone (SL) administration on 17α-ethynylestradiol (EE)-induced cholestasis was studied, with emphasis in expression and activity of Mrps. Adult male Wistar rats were divided in the following groups: EE (5 mg/kg daily for 5 days, s.c.), SL (200 μmol/kg daily for 3 days, i.p.), 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 Mrp2 by 41% and increased that of Mrp3 by 200%, whereas SL only affected Mrp2 expression (+60%) with respect to controls. EE+SL group showed increased levels of Mrp2 and Mrp3 to the same extent as registered for the individual treatments. Real time PCR studies indicated that up-regulation of Mrp2 and Mrp3 by SL and EE, respectively, were at 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. Co-administration of rats with SL partially prevented (-53%) from 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.

Introduction

Bile flow formation is dependent upon concentrative efflux of osmotically active solutes from the hepatocyte to the canalicular space followed by passive water movement (Nathanson and Boyer, 1991). This step is facilitated by members of the ATP-Binding Cassette (ABC) family of membrane transporters that mainly include the bile salt export pump (Bsep), that mediates the concentrative transport of monovalent 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) that transports lipophilic substances conjugated with glutathione, glucuronate or sulfate, and glutathione (oxidized and reduced) (Rebbeor et al., 2002) contributing to the formation of bile salt-independent flow (BSIF). Besides, an electroneutral chloride/bicarbonate exchanger, the anion exchanger 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 to decreased expression and activity of Bsep and Mrp2 (Trauner et al., 1997; Lee et al., 2000) as well as to 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 ascitis (Ochs et al., 1978) and shows inductive properties on biotransformation enzymes in humans and experimental animals (Ochs et al., 1978; Catania et al., 2004). SL also induces choleresis 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 in restoration of bile secretory function (Dumont et al., 2002; Sánchez Pozzi et al., 2003; Crocenzi et al., 2004; Crocenzi et al., 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 Mrp2 normal 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 normalized 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. Additionally, SL partially restored the capability for

biliary secretion of APAP glucuronide (APAP-glu), found to be substantially deteriorated in EE rats.

Materials and Methods

Chemicals. SL, EE, leupeptin, phenylmethylsulfonyl fluoride (PMSF), pepstatin A, glutathione (GSH), gluthatione reductase, 3α-hidroxysteroid dehydrogenase, β-NAD, NADPH, HEPES, palmitoyl lysophosphatidylcholine, D-saccharic acid 1,4 lactone, UDP-glucuronic acid, APAP and APAP-glu were obtained from Sigma Chemical Company (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 standard laboratory pellet diet and were allowed free access to water and saline during treatment. Animals were randomly divided in four experimental groups:

1- EE group: EE was dissolved in propylene glycol (33.7 mM), and administered at a daily dose of 5 mg/kg body wt s.c., for five consecutive days (Crocenzi et al., 2001; Ruiz et al., 2006).

2- 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- EE+SL group: rats were injected with EE, as in EE group, for 5 days, and simultaneously with SL, as in SL group, for the last 3 days of EE protocol.

4- Control groups: rats were injected with vehicle (propylene glycol) either s.c. for 5 days (0.5 ml/kg body wt) or i.p. for 3 days (3.3 ml/kg body wt) or both

simultaneously. Preliminary studies demonstrated no differences among these groups in functional studies performed either *in vivo* or in isolated perfused liver (IPL) model, as well as in western blot studies, so that only data from rats receiving propylene glycol both i.p and s.c. are presented in Results section.

All procedures involving animals were conducted in accordance with NIH guidelines for the Care and Use of Laboratory Animals. Studies were performed 18 h after the last injection of vehicle or steroids, unless otherwise stated. Basal bile was collected for determination of bile flow and biliary excretion of bile acids, glutathione and bicarbonate (Ruiz et al., 2005). At the end of the experiments, the animals were sacrificed by exsanguination and serum aliquots were used in determination of the cholestasis markers alkaline phosphatase and total bile acids, using commercial kits (Wiener Lab, Rosario, Argentina, and Randox Lab, Crumlin, UK; respectively). Livers were immediately rinsed *in situ* with cold saline, removed, and the liver weight/body weight ratio calculated. Portions of hepatic tissue were used for assessment of total glutathione content (Ruiz et al., 2005) and preparation of microsomal and mixed plasma (MPM) membranes (Ruiz et al., 2006).

Evaluation of Mrp2, Mrp3 and Bsep mRNA levels in response to EE and/or SL treatments was performed at different times post-administration of the corresponding protocols. Thus, different sets of animals were sacrificed at 3, 6 or 12 h after the last injection of these steroids. Parallel groups of animals were injected with solvent i.p. and/or s.c. and sacrificed at the times indicated. The livers were removed, snap frozen in liquid nitrogen and preserved at -70 °C until use for total RNA isolation.

Western blotting studies. Western blot analysis 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 recognizes 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 (Media Cybernetics, Silver Spring, MD) software.

Quantitative Real Time PCR. Total RNA was isolated from liver samples using Trizol (Invitrogen, 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, CA). Real-time quantitative PCR was performed on cDNA samples using the MiniOpticom System (BioRad Laboratories, Hercules, CA). Sequences of primer pairs and conditions for Mrp3 and Bsep were as described (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 product of interest. Quantification of the target cDNAs in all samples was normalized to 18S ribosomal

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RNA ($Ct_{target} - Ct_{18S} = \Delta Ct$) and the difference in expression for each target cDNA in the treated groups was expressed to the amount in the control group ($\Delta Ct_{treated} - \Delta Ct_{control} = \Delta\Delta Ct$). Fold changes in target gene expression were determined by taking 2 to the power of this number ($2^{-\Delta\Delta Ct}$).

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, non toxic 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). APAP-glu subsequent 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 (Ruiz et al., 2006). Liver viability was 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).

UGT activity. The conjugating activity of microsomal UGT towards APAP was evaluated as described (Ruiz et al, 2006). This study was performed to ascertain whether eventual changes in apical or basolateral excretion of APAP-glu in IPL

could also result from differences among groups in endogenous synthesis of the common Mrp substrate.

Statistical analysis. Data are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA, followed by the Newman-Keuls test. Values of $p<0.05$ were considered to be statistically significant.

Results

Biliary secretory function.

Individual treatment with EE or SL as well as the co-administration 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; Crocenzi 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 EE protocol normalized bile flow through selective induction of glutathione and bicarbonate biliary output. Normalization in bile flow formation occurred in spite that bile salt output remained impaired. The hepatic content of glutathione was not different among groups (Table 2), in spite of the significant changes detected in its biliary excretion in rats treated with EE or SL alone. This could be tentatively explained by adaptive changes in same direction in its hepatic synthesis, or alternatively, by changes in opposite direction in its basolateral efflux. Alkaline phosphatase was increased by EE as expected from a cholestatic model and returned to control values in EE+SL group. Serum bile salt levels were significantly increased in EE-treated rats likely as a consequence of decreased biliary secretion (Crocenzi 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%) following EE treatment and increased (+60%) after SL administration, as was previously reported (Trauner et al., 1997; Lee et al., 2000; Ruiz et al., 2005; Ruiz et al., 2006). Co-administration of animals with both steroids up-regulated Mrp2 levels by 50% over controls, consistent with the effect registered for SL alone. Fig 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 EE+SL group it was observed a preferential induction of Mrp3 vs. Mrp2 when compared to controls. Bsep expression tended to decrease in response to EE though it did not reach statistical significance. SL administration neither affected Bsep levels. Fig 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 last injection of SL and/or EE. Because induction of mRNA levels may be transient, we selected 3, 6 and 12 h post-injection as time points. Data in Fig 2 are presented as percentages and were referred to the respective control, considered as 100%. In EE group, Mrp2 mRNA expression remained invariable, 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, though some differences in the magnitude of induction between individual vs. 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 vs. Mrp3 to preferential biliary vs. basolateral disposition of the common substrate APAP-glu was evaluated using the IPL model. Fig 3A shows that the content of APAP-glu in perfusate determined at 60 min, equivalent to the amount of the metabolite accumulated by this time, was increased by 60% in EE group, by 77% in SL group and by 130% in 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 EE+SL group, when compared with controls (Fig 3B). This latter result indicates that SL was able to restore EE-induced impairment in biliary secretory function associated with Mrp2. Fig 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, either when administered 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%), when compared to 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 towards APAP. Fig 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 isoform UGT1A7 (78% and 129%, respectively), whereas UGT1A6, another isoform involved in conjugation of APAP (Kessler et al., 2002), was not affected by the treatments (Fig 4B).

Discussion

EE administration to rats represents a well characterized model of drug-induced intrahepatic cholestasis, which has been largely tested in order 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 DNP-SG, 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 co-administration of the inducer with EE, for the last 3 days of EE protocol, completely prevented impairment in bile flow formation. This occurred despite bile salt secretion remained deteriorated, likely 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 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. In spite of the preserved levels of AE2, an activation of the exchanger by SL can not be ruled out due to 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, when compared to controls.

Consistent with restored biliary secretion of GSH described in *in vivo* experiments, administration of SL together with EE counteracted EE-induced impairment in biliary secretion of APAP-glu in IPL. This major APAP metabolite is a

common substrate for both Mrp2 and Mrp3, though it presents higher affinity for Mrp3 (Manautou et al., 2004). Thus, partial restoration of its biliary secretion in 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 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 vs. 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, likely reflecting a higher affinity towards APAP-glu reported for Mrp3 vs. Mrp2 (Manautou et al., 2004).

Expression of hepatobiliary transporters are regulated by nuclear receptors, as also 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 (FXR) and constitutive androstrane receptor (CAR) 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 (RXR). SL is a well known ligand of PXR, as also demonstrated for the steroids PCN, 5β pregnane-3,20-dione, dexamethasone, etc. (Schuetz et al., 1998; Kliewer et al., 2002). Thus, our data on increased expression of Mrp2 at transcriptional level is consistent with SL-PXR interaction followed by heterodimer formation. Interestingly, this protocol of administration with SL also leads to up-regulation of PXR mRNA and protein levels, either when administered alone or in combination with EE (unpublished results), reinforcing a role for this nuclear receptor as a modulator of the action of SL. In contrast, down-regulation of Mrp2 by EE is likely at post-transcriptional level since mRNA levels were not affected at any time studied. This is unlikely restricted to EE since post-transcriptional regulation of Mrp2 has been also observed for other drugs (Gerk and Vore, 2002).

The regulation of *MRP3/Mrp3* gene is still unclear and seems to be dependent on the species analyzed. C/EBPs and Sp1/Sp3 can also cooperatively regulate the basal and inducible promoter activity of rat *Mrp3* gene as demonstrated by Tzeng et al. (2005). MRP3/Mrp3 mRNA levels were increased in human hepatoma cell lines HuH7 and HepG2 as well as in mice treated with PXR ligands different from SL (Teng et al., 2003). In the current study, we demonstrated transcriptional up-regulation of Mrp3 by EE as revealed by western blot and real time PCR studies. EE was previously shown to produce down-regulation 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 was demonstrated in mice that the effect on Bsep was mediated by binding of EE to the estrogen receptor alpha (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. Miguet et al. (1980) demonstrated that the administration of SL to humans with alcoholic cirrhosis, accelerates the clearance of co-administered 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. Fehér 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 (Ruiz 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, 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 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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Footnotes

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Figure Legends

Fig 1. Immunodetection of Mrp2, Mrp3, Bsep, and AE2.

These transporters were detected in mixed plasma membranes 18 h after the last dose of EE and/or SL. Equal amounts of total protein (15 µg for Mrp2 and Bsep and 30 µg for AE2 and Mrp3) were loaded in the gels. These amounts of protein gave a densitometric signal in the linear range of the response curve for both antibodies. Uniformity of loading and transfer from gel to nitrocellulose membrane was controlled with Ponceau S. Data on densitometric analysis represent means ± SD of 3 rats per group.

[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 2. Mrp2 and Mrp3 mRNA expression.

Changes in mRNA levels with time were detected by Real Time PCR. Livers were removed at 3, 6 or 12 h after the last injection of EE and/or SL. Data are presented as percentages and were referred to the respective controls, considered as 100%, and were expressed as means ± SD of 3 rats per group. 18S mRNA was used as an internal control.

[a] Significantly different from the respective controls, $p<0.05$.

[b] Significantly different from livers removed at 3 h, $p<0.05$.

Fig 3. APAP-glu excretion in the isolated perfused liver.

The hepatic disposition of APAP-glu, a common substrate for Mrp2 and Mrp3, was evaluated in the isolated perfused liver for a 60-min period. The panels represent the excretion of APAP-glu into perfusate (A) and bile (B), the APAP-glu hepatic content (C), and the total amount of this glucuronide in bile + perfusate + liver tissue (D), by the end of the experiment. Data are means ± SD of 3-6 rats per group.

[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 ± SD of 3 rats per group.

[a] Significantly different from controls, $p<0.05$.

[b] Significantly different from EE, $p<0.05$.

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Table 1. Nucleotide sequences of PCR primers.

Gene		Primer sequence (5´-3´)	denaturation	annealing	elongation
Mrp2	F	accttccacgtagtgatcct	95 ºC (1 s)	57 ºC (30 s)	72 ºC (30 s)
	R	acctgctaagatggacggtc			
Mrp3	F	gtgctgaagaatttgactctg	95 ºC (1 s)	60 ºC (10 s)	72 ºC (40 s)
	R	gaccaggacccggttgtagtc			
Bsep	F	cactgggtacatgtggtgtctcat	95ºC (10 s)	60ºC (30 s)	72ºC (30 s)
	R	atggccaatattcatagctgctaat			
18S	F	gtaacccgttgaaccccatt	95 ºC (1 s)	55 ºC (30 s)	72 ºC (30 s)
	R	ccatccaatcggtagtagcg			

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Table 2. *In vivo* biliary secretory function.

	Control	EE	SL	EE+SL
Liver weight/body weight (%)	3.25 ± 0.14	3.83 ± 0.21 [a]	3.7 ± 0.1 [a]	4.4 ± 0.2 [a,b,c]
Bile flow (µl/min/g liver)	2.00 ± 0.17	0.98 ± 0.41 [a]	3.43 ± 0.35 [a,b]	1.84 ± 0.17 [b,c]
Bile salt output (nmol/min/g liver)	58.2 ± 9.7	36.7 ± 15.1 [a]	39.7 ± 7.6 [a]	29.2 ± 7.5 [a]
Biliary excretion of bicarbonate (nmol/min/g liver)	51.2 ± 4.0	23.8 ±7.9 [a]	90.1 ± 10.5 [a,b]	41.2 ± 4.1 [b,c]
Biliary excretion of total glutathione (nmol/min/g liver)	2.53 ± 0.46	0.38 ± 0.26 [a]	9.79 ± 0.71 [a,b]	2.11 ± 0.16 [b,c]
Hepatic content of total glutathione (nmol/g liver)	5984 ± 562	5391 ± 773	5623 ± 1466	4344 ± 977
Alkaline Phosphatase (U/L)	260 ± 47	333 ± 24 [a]	143 ± 12 [a,b]	199 ± 25 [b]
Serum bile acids (µM)	213 ±79	1171 ± 489 [a]	145 ± 96 [b]	272 ±77 [b]

Results are expressed as mean ± SD (n = 3-6).

[a] Significantly different from controls ($p<0.05$).

[b] Significantly different from EE ($p<0.05$).

[c] Significantly different from SL ($p<0.05$).

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Table 3. Biliary and perfusate excretion of APAP-glu relative to its total amount in the IPL system.

	Control	EE	SL	EE+SL
Perfusate (%)	64 ± 4	93 ± 1 [a]	56 ± 5 [b]	80 ± 8 [a, b, c]
Biliary (%)	36 ± 4	6 ± 1 [a]	43 ± 5 [a,b]	20 ± 8 [a,b,c]
Hepatic content (%)	0.057 ± 0.013	0.033 ± 0.008 [a]	0.066 ± 0.015 [b]	0.028 ± 0.012 [a,b,c]
Biliary/ Perfusate ratio	0.57 ± 0.10	0.07 ± 0.02 [a]	0.78 ± 0.16 [a,b]	0.27 ± 0.12 [a,b,c]

Excretory rates and liver content of APAP-glu represent the amounts accumulated by the end of the experiment and were referred to the total amount of this metabolite synthesized by IPL by that time. Results are expressed as mean ± SD (n = 3-6).

[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 1

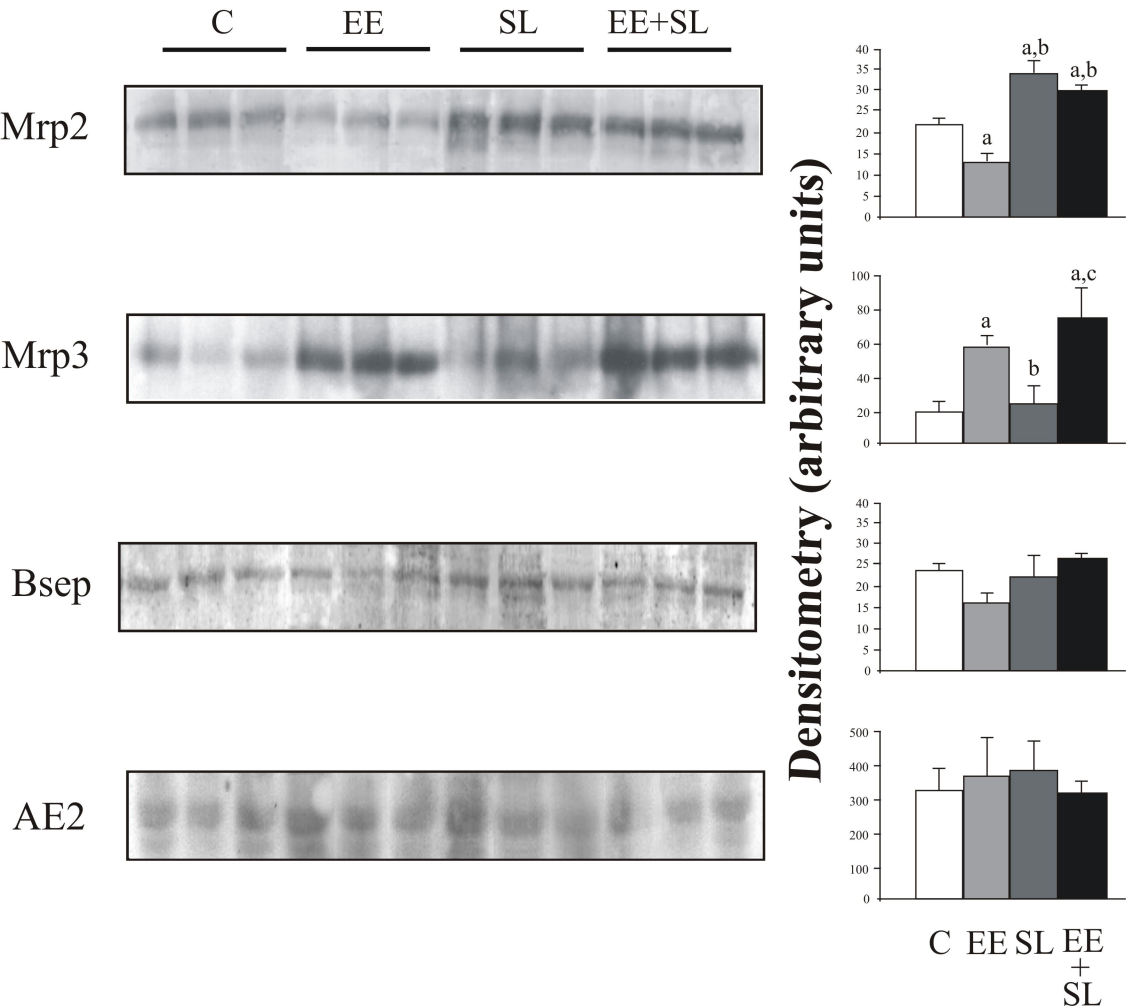

C
EE
SL
EE+SL
Mrp2
Mrp3
Bsep
AE2
Densitometry (arbitrary units)
a
a,b
a,b
a
b
a,c
C
EE
SL
EE + SL

Fig 2

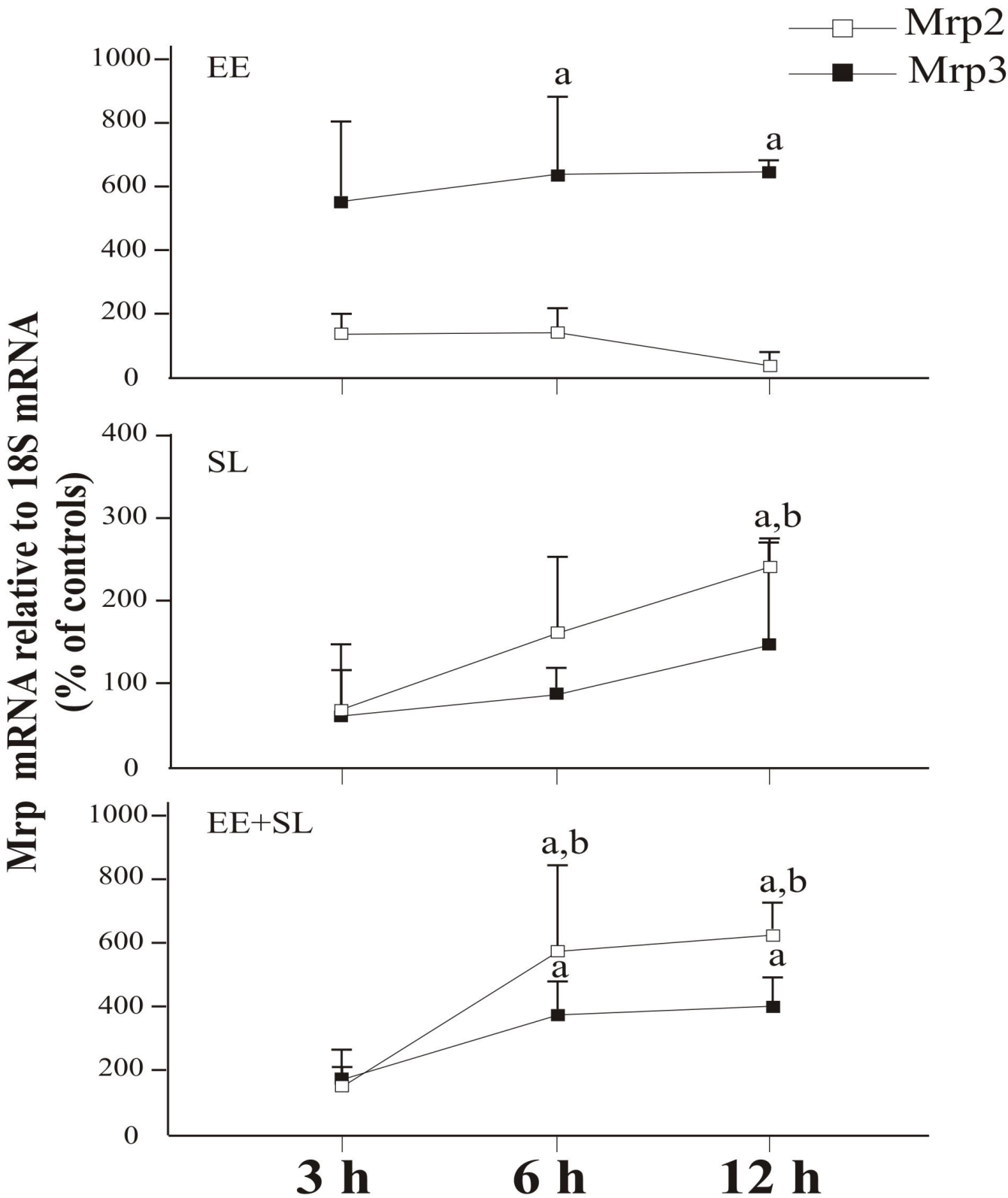
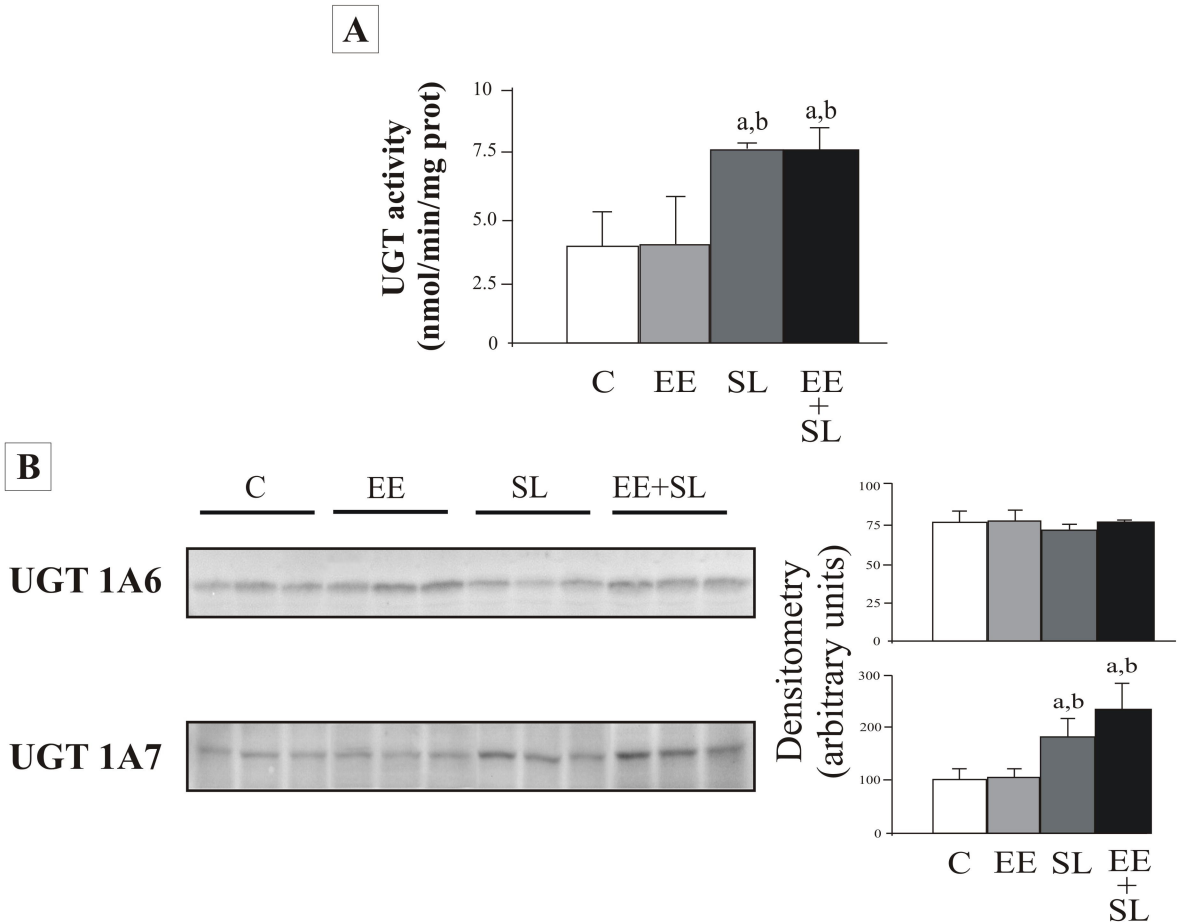

Mrp2
Mrp3
EE
SL
EE+SL
Mrp mRNA relative to 18S mRNA
(% of controls)
1000
800
600
400
200
0
400
300
200
100
0
a
a
a,b
a,b
a
a
3 h
6 h
12 h

Fig 3

A
Perfusate excretion (nmol/g liver)
4000
3000
2000
1000
0
a
a
a,b,c
B
Cumulative biliary excretion (nmol/g liver)
2000
1500
1000
500
0
a
a,b
b,c
C
Hepatic content (nmol/g liver)
3
2
1
0
a,b
c
D
Bile+Perfusate+Liver Content (nmol/g liver)
5000
4000
3000
2000
1000
0
a,b
a,b
C
EE
SL
EE+SL

Fig 4


A
UGT activity
(nmol/min/mg prot)
10
7.5
5.0
2.5
0
a,b
a,b
C
EE
SL
EE
+
SL
B
C
EE
SL
EE+SL
UGT 1A6
UGT 1A7
Densitometry
(arbitrary units)
100
75
50
25
0
300
200
100
0
a,b
a,b
C
EE
SL
EE
+
SL