Regulation of expression and activity of rat intestinal Mrp2 by cholestatic estrogens

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Abbreviations:
ALP, alkaline phosphatase; Bcrp, breast cancer resistance protein; Mdr1, multidrug resistance protein 1; Mrp2, Mrp3 and Mrp4, multidrug resistance-associated proteins 2, 3 and 4; BBM, brush border membrane; BDL, bile duct ligation; BSDF, bile salt-dependent bile flow; BSIF, bile salt-independent bile flow; CDNB, 1-cloro-2,4-dinitrobenzeno; DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; DNP-SG, dinitrophenyl-S-glutathione; E2-17G, estradiol 17β-D-glucuronide; EE, ethynylestradiol; GST, glutathione-S-transferase; PHIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; SL, spironolactone; TR(-), rats congenitally deficient in Mrp2; ZO-1, zonula occludens 1.
ABSTRACT

The effect of the cholestatic estrogens ethynylestradiol (EE) and estradiol 17β-d-glucuronide (E2-17G) on expression and activity of intestinal multidrug resistant-associated protein 2 (Mrp2, Abcc2) was studied in rats. Expression and localization of Mrp2 were evaluated by western blotting, Real-Time PCR, and confocal immunofluorescence microscopy. Mrp2 transport activity towards dinitrophenyl-S-glutathione (DNP-SG) was assessed in vitro in intestinal sacs. EE, administered s.c. at a 5 mg/kg b.w. dose, for 5 consecutive days, produced a marked decrease in Mrp2 expression at post-transcriptional level, without affecting its normal localization at the apical membrane of the enterocyte. This effect was selective, since expression of other ABC proteins such as Bcrp and Mrp3 were not affected, and that of Mdr1 only minimally impaired. Consistent with down-regulation of expression of Mrp2, a significant impairment in serosal to mucosal transport of DNP-SG, as well as in protection against absorption of this same compound, were registered. Simultaneous administration of EE with spironolactone (200 μmol/kg b.w./day for 3 days), an Mrp2 inducer, prevented these alterations, confirming downregulation of expression of Mrp2 by EE as a major component of functional changes. Incorporation of E2-17G (30 μM) in the serosal medium of intestinal sacs decreased serosal to mucosal transport of DNP-SG, likely due to competitive inhibition, without affecting normal Mrp2 expression or localization. Our data indicate impairment of function of intestinal Mrp2 by both cholestatic estrogens, though through a different mechanism. This represents an aggravation of deteriorated hepatic Mrp2 function that could further increase bioavailability of specific xenobiotics following oral exposure.
INTRODUCTION

The small intestine represents the principal site of absorption for any ingested compound, whether dietary, therapeutic, or contaminant. It has been shown that gastrointestinal absorption of these compounds is decreased by the presence of export pumps at the apical membrane of the enterocyte. Major intestinal drug export pumps are apical multidrug resistance protein 1 (Mdr1, Abcb1) or P-glycoprotein, breast cancer resistance protein (Bcrp, Abcg2) and multidrug resistance-associated protein 2 (Mrp2, Abcc2) (Mottino et al., 2000; van Herwaarden et al., 2003; Suzuki et al., 2003; Tanaka et al., 2005) and basolateral Mrp3 and Mrp4 (Rost et al., 2002; Johnson et al., 2006). While Bcrp and Mdr1 are preferentially expressed at the distal intestine, Mrp2 is preferentially expressed at the proximal intestine, gradually decreasing from the jejunum to the distal ileum, thus constituting a first barrier against absorption of specific dietary xenobiotics (Mottino et al., 2000). The importance of intestinal export pumps in this function is illustrated by experiments performed in TR(-) rats congenitally deficient in Mrp2. After oral administration of the abundant food-derived carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PHIP), its absorption was 2-fold higher in TR(-) rats than in wild-type rats (Dietrich et al., 2001). Consequently, 48 h later, PHIP tissue levels were 1.7- to 4-fold higher in several organs (liver, kidney, lung, and colon). In line with this, we have noted that induction of Mrp2 observed in post-partum lactating rats is likely an adaptive response to protect against absorption of food contaminants in response to a substantial (3-4 fold) increase in food intake (Mottino et al., 2001).

The fraction of xenobiotics of luminal origin escaping the small intestinal barrier reaches the liver via the portal vein, and is further metabolized and subjected to biliary excretion mediated by canalicular Mdr1, Bcrp or Mrp2. Efficient biliary elimination of xenobiotics is linked to normal bile flow formation. Impairment in bile flow formation under conditions of experimental cholestasis is accompanied by downregulation of canalicular Mrp2 and Bcrp in different models.
(Trauner et al., 1999; Lee et al., 2000; Villanueva et al., 2008). However, the impact on expression and activity of these same transporters at the intestine has been explored only under conditions of bile duct ligation (BDL), a well characterized model of obstructive cholestasis. Indeed, Dietrich et al. (2004) found a significant down-regulation of intestinal Mrp2 in BDL rats that was associated with increased bioavailability of PHIP, whereas Mdr1 and Bcrp remained normal. The influence of other forms of cholestasis such as drug-induced cholestasis remains unknown.

Estrogens contribute to the pathogenesis of both oral contraceptive-induced cholestasis and cholestasis of pregnancy (Vore 1987; Reyes and Simon 1993). Ethynylestradiol (EE), a synthetic estrogen, decreases bile flow formation in experimental animals, thus representing a useful model to study both estrogen- and drug-induced cholestasis (Crocenzi et al., 2001). In the rat, EE-induced decreases in both bile salt-dependent (BSDF) and –independent (BSIF) bile flow are attributed to decreased expression and activity of canalicular bile salt export pump (Bsep; Abcb11) and Mrp2 (Trauner et al., 1997; Lee et al., 2000), and concomitant impairment in biliary excretion of bile salts and Mrp2 substrates such as glutathione species. Acute effects of cholestatic estrogen metabolites are well characterized in the liver by the single i.v. administration of estradiol 17β-d-glucuronide (E2-17G), an endogenous metabolite of estradiol, to rats (Stieger et al., 2000; Mottino et al., 2002; Crocenzi et al., 2003). The effect consists of a dose-dependent and reversible decrease in bile flow formation (Meyers et al., 1980) mainly due to inhibition of bile salt transport (Stieger et al., 2000) and endocytic retrieval of Bsep and Mrp2 (Mottino et al., 2002; Crocenzi et al., 2003), leading to decreased canalicular excretory function. Whether E2-17G affects normal localization or function of Mrp2 in extrahepatic tissues such as the intestine has not been explored.

We here evaluated the effect of repeated EE administration on expression of major intestinal apical and basolateral transporters. As a main finding we demonstrated significant downregulation of Mrp2 expression at post-
transcriptional level, with concomitant deterioration of its function to prevent xenobiotics absorption. In view of these results, we further evaluated the possibility of reversing EE effects by co-administration of the therapeutic agent spironolactone (SL). SL, an Mrp2 inducer, has been shown to prevent the impairment in bile flow formation and biliary excretion of glutathione associated with down-regulation of canalicular Mrp2 induced by EE (Ruiz et al., 2007). The data confirmed such a protective effect. Additional studies in intestinal sacs incubated in the presence of E2-17G demonstrated inhibition of Mrp2 transport activity, whereas Mrp2 expression and localization at the apical membrane was not affected.
MATERIALS AND METHODS

Chemicals. EE, E2-17G, SL, probenecid, leupeptin, phenylmethylsulfonyl fluoride, pepstatin A, 1-cloro-2,4-dinitrobenceno (CDNB), dimethyl sulfoxide (DMSO), 4-aminoantipyrine, and glutathione were obtained from Sigma Chemical Co (St. Louis, MI). [3H] E2-17G (39.8 Ci/mmol) was from Perkin Elmer Life and Analytical Sciences (Boston, MA). Dinitrophenyl-S-glutathione (DNP-SG) was prepared as described (Mottino et al., 2001). All other chemicals were of analytical grade purity and used as supplied.

Animals and treatments. Adult male Wistar rats (300-350 g), 90-120 days old, were used throughout. They were maintained ad libitum on a standard laboratory pellet diet and were allowed free access to water (and saline in SL treatment protocols), during treatment. Animals were randomly divided in two experimental groups. EE-treated rats were administered EE dissolved in propylene glycol (33.7 mM), at a daily dose of 5 mg/kg body wt. s.c., for 5 consecutive days (Crocenzi et al., 2001). Control rats were injected with vehicle (propylene glycol; 0.5 ml/kg body wt. s.c.) according to the same schedule described for EE. In order to confirm development of cholestasis, basal bile flow was measured in all animals from both groups as described (Ruiz et al., 2006). Additional experiments were performed to evaluate if decreased food intake, as occurs in response to EE administration, may influence expression of main drug transporters. For this purpose, a group of rats was subject to food restriction (25% of daily food intake) for 5 days that led to a 8.5% total body weight loss, matching that seen in EE treated rats after 5 days (see Table 1), and then used to evaluate drug transporter expression by comparison with normal rats.

We also evaluated the effect of co-administration of SL with EE on intestinal Mrp2 expression and function. 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 wt. for 3 days (Ruiz et al., 2005). 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. As a control group, rats were injected with vehicle (propylene glycol) both i.p. for 3 days (3.3 ml/kg body wt) and s.c. for 5 days (0.5 ml/kg body wt.).

Basal bile and serum were collected in all these groups as described (Ruiz et al., 2007) to confirm induction of cholestasis by EE and its prevention by SL. Bile flow rate and the serum markers of cholestasis alkaline phosphatase (ALP) and bile salts were assessed using commercial kits as described (Ruiz et al., 2007).

A different set of normal rats was used to test the acute effect of E2-17G on intestinal localization and activity of Mrp2 as described below.

All procedures involving animals were conducted in accordance with NIH guidelines for the Care and Use of Laboratory Animals.

**Specimen collection.**

**EE studies:** Except otherwise stated, animals were sacrificed 18 h after the last EE or SL injection. Proximal jejunum and distal ileum were used in the studies. For collection of the proximal segment, the first 15 cm starting from the pylorus valve and corresponding to duodenum were excluded, and the following 30 cm were considered as the proximal jejunum. The last 30 cm of small intestine, proximal to the ileo-cecal valve, were considered as the distal ileum. These segments were carefully rinsed with ice-cold saline. For western blot studies they were immediately opened lengthwise, the mucus layer was carefully removed, and the mucosa was obtained by scraping, weighed, and used for total homogenate or brush border membrane (BBM) preparation. For Mrp2 transport studies, either efflux or absorption, 3-cm segments were isolated from proximal jejunum and carefully rinsed with ice-cold saline and immediately used in the everted and non-everted sac preparation. For confocal microscopy analysis of Mrp2 localization, small rings (5 mm length) were cut from this same region of intestine, gently frozen in liquid nitrogen-cooled isopentane, and kept at −70 °C.
before use in slice preparation. For Real-Time PCR studies, animals were sacrificed 6 h after the last EE injection and total RNA was prepared from the proximal jejunum using Trizol reagent (Invitrogen, CA), following the manufacturer’s protocol.

**E2-17G studies.** Three-cm segments from the proximal jejunum were prepared as described above and used to evaluate Mrp2 activity in the presence or absence of E2-17G at a 30 µM concentration. Similar segments were alternatively used to study Mrp2 expression and localization by western blotting of BBM or confocal microscopy. Specimen collection for either of these studies was as described above. A liver sample from normal rats was also collected to prepare 1-mm width slices and used to confirm *in vitro* the effect of E2-17G as promoter of endocytic retrieval of Mrp2, already demonstrated *in vivo* (Mottino et al., 2002).

**Western blot studies.** Total homogenates were prepared from mucosa samples as previously described (Mottino et al., 2000). BBMs were prepared from total homogenates by using the divalent cation precipitation method (Kessler et al., 1978) with some modifications (Mottino et al., 2000). Aliquots of the homogenates and BBM preparations were kept on ice and used the same day in western blot studies of GST and transport proteins, as previously described (Ruiz et al., 2005; Villanueva et al., 2008). Apical Mrp2, Mdr1, Bcrp, and villin were detected in BBMs using a mouse monoclonal antibody to human MRP2 (M2 III-6, Alexis Biochemicals, Carlsbad, CA), and rabbit polyclonal antibodies to human MDR1 (Santa Cruz Biotechnology, Santa Cruz, CA), to rat Bcrp (Santa Cruz Biotechnology), and to human villin (Santa Cruz Biotechnology). Because of its basolateral localization, Mrp3 and Mrp4 content was assessed in homogenates, which were previously solubilized with Triton X-100 as described (Cao et al., 2002). These proteins were detected using goat polyclonal antibodies to human MRP3 (Santa Cruz Biotechnology) and MRP4 (Abcam Inc, Cambridge, UK).
Equal loading and transference of protein was checked by detection of β-actin using a monoclonal antibody to rat β-actin (Sigma Chemical Co), and by Ponceau S staining of the membranes. The immunoreactive bands were quantified with the Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD) software.

**Quantitative Real-Time PCR.** 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 Mrp2 and 18S were designed to optimally detect the respective mRNAs (Ruiz et al., 2007). 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 referred to the amount in the control group (ΔCt\_treated - ΔCt\_control = ΔΔCt). The sequences of the primers used for amplification of Mrp2 were, forward: 5'-ACCTTCCACGTAGTGATCCT-3'; reverse: 5'-ACCTGCTAAGATGGACGGTC-3'; and for 18S, forward: 5'-GTAACCCGTTGAACCCCATT-3; reverse: 5'-CCATCCAATCGGTAGTAGCG-3'.

**Confocal immunofluorescence studies.** For *in situ* immunodetection of Mrp2, the frozen intestinal rings from jejunum were sectioned (thickness: 5 µm), air dried, and fixed for 10 min with cold methanol (-20 °C). Mrp2 and zonula occludens 1 (ZO-1) proteins were labeled with anti-MRP2 (indicated above) and with a polyclonal antibody directed to human ZO-1 (Zymed Laboratories Inc., San Francisco, CA), followed by treatment with appropriate Cy2- or Cy3-conjugated donkey anti-IgGs (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA). For detection of the nucleus, the slices were incubated with 1.5 µM 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes, CA) for 5 min, just before the last washing. All confocal studies were performed in a Nikon C1 Plus microscope.
(Tokyo, Japan). To ensure comparable staining and image capture performance for the different groups belonging to the same experimental protocol, intestinal slices were prepared on the same day, mounted on the same glass slide in a single well, and subjected to the staining procedure and confocal microscopy analysis simultaneously. False color of Mrp2 positive images were generated by using the confocal microscope software.

**Transport activity in intestinal sacs.**

**EE studies:** Two different strategies were used to characterize the effect of EE on intestinal Mrp2 activity, either associated with efflux of DNP-SG from the intracellular compartment to the lumen, or with protection against its absorption from the lumen to the serosal compartment. While the model of non-everted intestinal sacs was used in the former studies (Ghanem et al., 2006), everted sacs were used in the latter (Mottino et al., 2001).

For assessment of efflux activity, 3-cm segments from proximal jejunum were filled with Krebs-Henseleit buffer (40 mM glucose, pH 7.4) previously gassed with O₂/CO₂ (95:5) (corresponding to the mucosal compartment) and incubated in 30 ml of the same buffer containing 100 µM CDNB (serosal compartment) for 0, 5, 10, 20, and 30 min. The serosal compartment was continuously gassed with O₂/CO₂ (95:5). All experiments were performed in the presence or absence of 1.0 mM probenecid in both the mucosal and serosal sides to inhibit Mrp2 activity (Yokooji et al., 2007). After incubation, the exterior of the intestinal sac was carefully washed with drug-free buffer and the entire mucosal solution collected. Intestinal sacs were weighed and homogenized in twice the mass of ice-cold saline. Aliquots of mucosal, serosal and homogenate samples were treated with 70% (w/w) HClO₄ (50 µl per ml of sample) and centrifuged at 3,500 g for 5 min. DNP-SG content was determined in the supernatants by HPLC as previously described (Mottino et al., 2001).

For assessment of absorption activity, 3-cm segments from jejunum were everted and filled with Krebs Henseleit buffer as describe above. DNP-SG (100
µM) was added to the external (mucosal) compartment, and its content determined in the serosal compartment after 30 min of incubation.

**E2-17G studies:** To evaluate the acute effect of E2-17G on Mrp2 activity, efflux experiments were performed as described above using 3-cm segments from proximal jejunum obtained from normal rats. Either E2-17G (30 µM) or its solvent (DMSO, 1.0 µl per ml of buffer) was added to the serosal side, in addition to CDNB (25 or 100 µM). Content of DNP-SG was determined in the mucosal side at 30 min of incubation.

**Uptake of E2-17G by liver slices and intestinal sacs.** In some experiments we compared the uptake of E2-17G in liver slices and intestinal sacs after 30 min of incubation. A trace of \(^{3}\)H-E2-17G (0.5 µCi/l; 13 pmol/l) was added to the incubations containing 30 µM of non-labeled E2-17G. Aliquots of the tissues were homogenized in 2 volumes of saline and the amount of \(^{3}\)H-E2-17G accumulated was determined in the homogenates using a liquid scintillation counter (Rack Beta 1214, Pharmacia, Piscataway, NJ).

**GST expression and activity.** CDNB is highly hydrophobic and therefore diffuses into the intracellular compartment where it is efficiently converted to DNP-SG through both spontaneous and Glutathione-S-transferase (GST)-mediated reactions. Because EE may affect GST function, and thus endogenous generation of DNP-SG, we evaluated its activity as well as expression of the major GST classes present in intestine. Glutathione conjugating activity towards CDNB was assayed in cytosol from proximal jejunum as described (Catania et al., 2000). Western blot studies of the different GST classes were performed using goat antisera against rat alpha and mu GSTs (GS9 and GS23, respectively, Oxford Biomedical, MI) and rabbit antiserum against human pi GST (Immunotech, Marseille, France) as described (Catania et al., 2000; Villanueva et al., 2008).
**Statistical analysis.** Data are presented as the means ± SD. Comparison between groups was performed using the Student's *t* test or one way ANOVA followed by Bonferroni’s test (when more than two groups were compared). Values of *p*<0.05 were considered to be statistically significant.
RESULTS

**Effect of EE on body and tissue weights and on markers of cholestasis.** Basal bile flow was reduced by 51% by EE, confirming establishment of cholestasis (Table 1). Consistently, the serum markers of cholestasis ALP and bile acids were increased by 32 and 460%, respectively. We have also confirmed decreased expression of liver Mrp2 by western blotting (image not shown), as a major component of cholestasis. While the weight of the proximal fragments of intestine was not affected, treatment with EE slightly increased liver weight (7%) and decreased body weight (10%) with respect to controls, in agreement with previous findings (Crocenzi et al., 2001).

**Effect of EE and E2-17G on expression and localization of Mrp2.** The effect of repeated administration with EE on expression of Mrp2 was analyzed in the proximal jejunum whereas the effect on Mdr1, Bcrp and Mrp3 was analyzed in distal ileum, according to their sites of maximal expression (Mottino et al., 2000; Rost et al., 2002; Lindell et al., 2003; Tanaka et al., 2005). Fig 1A shows that expression of Mrp2 in the EE group was substantially decreased (92%) with respect to controls, whereas that of Mdr1 was only slightly decreased (27%) and that of Mrp3 and Bcrp was preserved. Additional experiments on food restriction showed no differences in Mrp2 or Mdr1 expression, as detected by western blotting, between normal and food restriction groups (data not shown). Thus, down-regulation of Mrp2 and Mdr1 are not secondary to weight loss but rather result from a direct action of EE. We analyzed the effect of EE on Mrp4 expression in both proximal jejunum and distal ileum and did not detect any signal in any group, consistent with the very low expression reported previously in these regions (Johnson et al., 2006). The acute effect of the potent cholestatic E2-17G on Mrp2 localization was also evaluated but in the *in vitro*, non-everted intestinal sac model. Western blot studies performed in BBM from sacs incubated
with 30 µM E2-17G or its solvent for 30 min show that E2-17G did not affect Mrp2 content (Fig 1B).

The effect of EE on expression of Mrp2 was also evaluated in situ in the proximal jejunum by immunofluorescence confocal microscopy by co-staining of Mrp2 with ZO-1, a tight junction marker. Mrp2 (red fluorescence) was preferentially expressed at the tip of the villi and decreased towards the crypt region, whereas ZO-1 (green fluorescence) was more uniformly distributed along the villus (Fig 2, panel A, image 1). Also, Mrp2 was localized to the luminal surface of the enterocyte, mostly outside the region delimited by ZO-1 (arrows in image 2). A tissue section providing a frontal view clearly showed that Mrp2 is uniformly distributed on the apical surface of the enterocyte, with ZO-1 marking the region of cell to cell contact (see inset in image 2). By comparing images 3 (controls) and 4 (EE) in panel A, in which Mrp2 was co-stained with the nuclei of the enterocytes (DAPI blue fluorescence), it is clear that EE treatment substantially decreased expression of Mrp2. This marked difference between groups is better illustrated in the false color replica (images 5 and 6 for control and EE group, respectively). Normal localization of Mrp2 at the luminal surface of the enterocyte was maintained however, as shown in the insets of images 3 and 4, representative from control and EE groups, respectively.

Because of the significant down-regulation of Mrp2 in response to EE administration, it was of interest to further explore the basis for this effect. Detection of mRNA levels at 6 h after the last dose of EE showed no change in the treated vs. control group (222 ± 128% and 100 ± 33%, respectively, n = 3).

The acute effect of E2-17G on in situ localization of Mrp2 is shown in images 7 and 8 (Fig 2, panel B), corresponding to control sacs and to sacs incubated with 30 µM E2-17G, respectively. The patterns of staining of Mrp2 and ZO-1 are essentially the same, with no apparent internalization of Mrp2 into the cytosol in the presence of E2-17G. To confirm the effect of E2-17G as a promoter of Mrp2 endocytic retrieval from the apical membrane in the hepatocyte, we also incubated normal liver slices under the same conditions as for intestinal sacs.
Images 9 and 10 illustrate the effect of E2-17G on canalicular localization of Mrp2. In the control group, Mrp2 is mainly localized in the canalicular space as delimited by ZO-1 staining (image 9), whereas in slices incubated in the presence of E2-17G, a significant proportion of Mrp2 was internalized and detected outside the canalicular compartment (see arrows in image 10). We measured the accumulation of $^3$H-E2-17G to determine if differential intracellular accumulation of E2-17G might explain the lack of effect in the enterocyte, but found that retained $^3$H-E2-17G did not differ between liver slices and intestinal sacs incubated in the presence of 30 µM E2-17G for 30 min (7.8 ± 1.9 and 6.1 ± 0.8 nmol/g tissue for liver and intestine, respectively, n = 3).

**Effect of EE and E2-17G on intestinal transport of DNP-SG.** In order to characterize the relationship between Mrp2 expression and its transport activity, we evaluated secretion of DNP-SG, a typical substrate for Mrp2, from the serosal to the mucosal compartment, in non-everted intestinal sacs. HPLC analysis confirmed that DNP-SG always represented more than 85% of the absorption at 365 nm, both in control and EE groups, indicating that conversion of the glutathione conjugate to further metabolites (e.g. mediated by γ-glutamyltransferase) was minimal under the current experimental conditions, and was not affected by EE. Panel A in Fig 3 shows that transport of DNP-SG was substantially decreased by 80% at 30 min in the EE group when compared to controls, agreeing well with western blot studies in Fig 1 and confocal microscopy studies in Fig 2. Consistent with impairment in Mrp2-mediated excretion, retention of DNP-SG was increased (p<0.01, n = 3) in the intestinal tissue in the EE group (423 ± 24 nmol/g of tissue) with respect to controls (280 ± 38 nmol/g of tissue) by the end of the incubation period. Panel A in Fig 3 also shows that probenecid, an Mrp2 inhibitor, decreased transport activity in both the EE and control groups to the same basal value.

Absorption of DNP-SG in everted sacs, inversely associated with Mrp2 function, was substantially increased by EE treatment (panel B in Fig 3). At the
30 min period, detection of DNP-SG on the serosal side was 5-6 fold higher in the EE group with respect to controls. Incubations in the presence of probenecid increased DNP-SG absorption in controls and EE groups to the same level as in the EE-treated group.

We also evaluated GST activity towards CDNB and its expression in cytosolic preparations to exclude the possibility that a reduction in endogenous formation of DNP-SG could influence the decreased DNP-SG efflux shown in panel A, Fig 3. GST activity was increased 27% rather than decreased by EE treatment (panel A in Fig 4), likely due to a 59% induction of the alpha class of GST (panel B in Fig 4).

Finally, we examined the effect of E2-17G on DNP-SG efflux (Fig 5). E2-17G significantly inhibited DNP-SG efflux by 68% only when 25 µM CDNB was added to the serosal medium, but not when the effect of a 100 µM concentration was examined.

**Preventive effect of SL on EE-induced down-regulation of expression and function of Mrp2.** Table 2 shows that basal bile flow was reduced by 52% by EE, whereas SL increased this measure by 59%, and conjoint treatment did not produce any change. The serum markers of cholestasis ALP and bile acids were increased by EE by 26 and 330%, respectively. SL alone decreased ALP by 48% and bile salts to the same extent. EE+SL group exhibited no changes in either of these measures. Table 2 also shows that the body weight and the weight of the proximal fragments of intestine were not affected by any treatment. Liver weight was affected by EE and SL either administered alone or in combination (15, 17, and 30% increase over controls). Panel A in Fig 6 shows the content of Mrp2 in BBM from jejunum isolated from rats treated with EE and SL, either alone or in combination. As expected, EE treatment significantly decreased Mrp2 protein expression (90%) relative to controls receiving propylene glycol s.c. and i.p. In contrast, SL significantly induced Mrp2 expression by 409%, consistent with results demonstrating SL-mediated increased expression of Mrp2 in rat liver.
(Ruiz et al., 2005). When administered in combination with EE, SL prevented the decrease in Mrp2 expression so that no differences were detected with respect to control rats. However, when compared to the group receiving SL alone, Mrp2 levels in EE+SL group represented only a 22% of those in the single group. All these effects reported for EE and/or SL are likely specific, since expression of villin, a constitutive component of the BBM, was not affected by any treatment (see Fig 6A).

In order to establish if protection by SL was also evident for Mrp2 transport activity, we evaluated DNP-SG efflux in non-everted intestinal sacs from control, EE- SL- and EE+SL-treated rats. While EE treatment decreased (42%) and SL treatment increased (56%) mucosal accumulation of DNP-SG at 30 min, the combined treatment of EE and SL showed no effect on DNP-SG efflux when compared to controls (panel B in Fig 6), correlating well with western blot studies.
DISCUSSION

ABC transporters at the apical level of the enterocyte play a crucial role in prevention of absorption of potentially toxic compounds, including drugs, toxins, carcinogens and other xenobiotics that are orally introduced into the intestine. We assessed the influence of the synthetic cholestatic estrogen EE, of therapeutic use, and of the endogenous estrogen metabolite E2-17G, highly produced during pregnancy, on major ABC intestinal transporters. The data demonstrates that only expression of Mrp2 was significantly affected by EE administration. Because of this selective impairment, a significant alteration in the fate and bioavailability of specific xenobiotics introduced into the intestine can be expected. We further focused our attention to investigate the basis for Mrp2 down-regulation and its consequences on Mrp2 function to prevent xenobiotics absorption.

Western blot and Real-Time PCR studies clearly demonstrated down-regulation of expression of Mrp2, likely at post-transcriptional level. Immunofluorescence microscopy studies confirmed decreased expression of Mrp2, and further demonstrated no changes in its localization, which was preserved at the apical membrane of the enterocyte. To estimate the functional impact of the significant impairment in Mrp2 expression, we performed additional studies in intestinal sacs. The data demonstrated a substantial decrease in serosal to mucosal transport of the model Mrp2 substrate DNP-SG and increase in its mucosal to serosal absorption in EE group. Similar experiments using probenecid, an Mrp2 inhibitor (Yokooji et al., 2007), suggested that deterioration of Mrp2 activity was indeed involved.

The impact of EE treatment on expression of other ABC members was found to be minimal. EE impaired the expression of Mdr1, though to a much lesser extent than for Mrp2. The functional impact of Mdr1 down-regulation was not assessed, though we predict an increase in the availability of orally administered Mdr1 substrates. In contrast to Mrp2 and Mdr1, expression of Bcrp
was preserved. Sulphate derivatives of estrogens are choleretic (Vore, 1987; Krishnamurthy and Schuetz, 2006) and more selectively transported by Bcrp than by Mrp2 (Tanaka et al., 2005). It is known that estrogens undergo significant enterohepatic recirculation (Shenfield, 1993). In consequence, impairment of secretory function associated to Mrp2 but not to Bcrp may result in preferential retention in the entero-hepatic circuit of biliary excreted cholestatic vs. choleretic derivatives of estrogens, thus exacerbating their cholestatic effects. Expression of Mrp3 was also preserved in EE group, which would indicate an intact capability for transport into blood of common Mrp2 and Mrp3 substrates internalized in the enterocytes.

Biotransformation reactions and transport of drugs have been postulated to act coordinately to eliminate potentially toxic endo- and xenobiotics (Elias and Mills, 2007; Iyanagi, 2007). We explored the possibility that glutathione conjugation of CDNB, a requisite for conversion of CDNB to the Mrp2 substrate DNP-SG, is also impaired by EE. However, we found that EE increased GST activity through up-regulation of expression of the alpha class, one of the major forms of GST mediating conjugation of CDNB. Impaired intestinal efflux of DNP-SG in the EE group, in spite of increased GST-mediated conjugation of CDNB, is consistent with the assumption that Mrp2 transport represents the rate-limiting step in overall elimination of their substrates (Mottino et al., 2000; Cao et al., 2001; Mottino et al., 2001). We do not know the reason for the differential response to EE between GST and Mrp2 expression, but the data clearly indicate distinct regulatory mechanisms.

Endogenous metabolism of estrogens to specific derivatives, such as those glucuronidated at the D-ring, has been assigned a role in estrogen induced cholestasis (Vore et al., 1997). This was demonstrated in EE-induced cholestasis in rats, in which inhibition of formation of the 17β-d-glucuronide in vivo, led to partial prevention of cholestasis (Crocenzi et al., 2006). The glucuronidated derivative of estradiol, E2-17G, acutely induces cholestasis in a dose-dependent manner in rats (Meyers et al., 1980), and may play a role in estrogen cholestasis
occurring in pregnancy. We recently observed that a significant component of the cholestatic effect of E2-17G is associated with internalization of Mrp2 and bile salt export pump from the canalicular membrane to intracellular vesicles. It is possible that formation of a cholestatic glucuronide derivative during EE administration also constitutes a component of the effects observed under condition of its repeated administration (Vore et al., 1983). We thus tested the acute effect of E2-17G on localization and activity of intestinal Mrp2 in the non-everted sac model. The data indicate decreased efflux of DNP-SG in the presence of 30 µM E2-17G, particularly evident at 25 µM CDNB, but not at 100 µM CDNB in the serosal medium. Together with the finding of preserved expression and localization of Mrp2 at the BBM level in E2-17G treated intestinal sacs, these data indicate that decreased activity results from competitive inhibition of DNP-SG transport by E2-17G, consistent with both E2-17G and DNP-SG as model substrates for Mrp2 with similar affinities (Keppler, 1999).

Because of the significant down-regulation of Mrp2 expression following EE treatment, we performed additional studies co-administering SL and EE. SL induces the expression of Mrp2 in rat liver (Ruiz et al., 2005) and prevents its down-regulation following EE treatment (Ruiz et al., 2007). Western blot studies clearly demonstrated significant up-regulation of intestinal Mrp2 by SL when administered alone; further, SL prevented Mrp2 down-regulation induced by EE following their co-administration. Prevention was particularly evidenced when data on densitometry of Mrp2 levels were compared between EE+SL and control groups. However, when expression in EE+SL group is compared with that in the group receiving SL alone, it is evident a significant decrease. It is possible that EE counteracted the inducer effect of SL, by acting through common or independent pathways finally leading to opposite effects on Mrp2 regulation. The exact mechanism is currently unknown. Data demonstrating decreased efflux activity of DNP-SG in intestinal sacs were consistent with western blot studies showing decreased Mrp2 expression in the EE group. SL, when administered alone, increased Mrp2 transport activity and, when co-administered with EE, led
to same values as controls. These results confirm that EE-induced down-regulation of Mrp2 is the main cause for decreased intestinal function in EE group. SL is a diuretic used therapeutically for the treatment of edema or ascites and was found to induce hepatic phase I biotransformation enzymes in humans (Miguet et al., 1980). Whether SL, under the usual therapeutic protocol, induces MRP2 either in liver or intestine in humans remains unknown.

There is significant evidence on drug interactions with oral contraceptive steroids, EE being particularly involved (Back and Orme, 1990; Shenfield, 1993). These interactions have been ascribed to interference with common metabolic pathways such as those involving cytochrome P450, UDP-glucuronosyltransferase, and sulfotransferase, either at the liver or intestine. The possibility arises that drug interactions may also occur at the level of membrane transport. Our data on downregulation of expression and function of intestinal Mrp2 under repeated treatment with EE, together with competitive inhibition of secretion of DNP-SG by E2-17G, support this possibility. Although the dose of estrogens in contraceptive pills is much lower than that administered to rats to induce cholestasis, in contrast to the current protocol of administration, women consume contraceptive estrogens chronically.

In summary, we here report selective and significant down-regulation of expression and activity of intestinal Mrp2 in EE-induced cholestasis that resulted in impairment of its function to prevent absorption of the model substrate DNP-SG. The potent cholestatic E2-17G inhibited intestinal secretion of DNP-SG, most likely competitively, without affecting expression and normal localization of Mrp2 at the apical membrane of the enterocyte.
ACKNOWLEDGEMENTS

We want to express our gratitude to Dr. Enrique J. Sánchez Pozzi for his advice and valuable suggestions.


FOOTNOTES

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FIGURE LEGENDS

Figure 1: Effect of cholestatic estrogens on expression of Mrp2, Mrp3, Mdr1 and Bcrp.
Effect of EE in vivo (panel A) and E2-17G in vitro (panel B) treatments. Equal amounts of total protein (35 μg for Mrp2 and Mdr1 detection, 20 μg for Bcrp and 50 μg for Mrp3 detection) were loaded in all lanes. Uniformity of protein loading and transfer from gel to nitrocellulose membrane was controlled with Ponceau S and detection of β-actin. Data on densitometric analysis represent means ± SD of 6 (panel A) or 3 (panel B) rats per group.
* Significantly different from controls (C), p<0.05.

Figure 2: Effect of cholestatic estrogens on in situ detection of Mrp2.
Panel A (treatment with EE): Mrp2 (red fluorescence) is normally expressed at the apical membrane of the enterocyte in jejunum, with the highest expression at the villus tip, whereas ZO-1 (green fluorescence) is homogeneously distributed along the villus (image 1). Mrp2 localization is mostly external to the region delimited by ZO-1 (arrows in image 2). In a frontal view of the surface of the villus, Mrp2 appears uniformly distributed on the apical surface of the enterocyte, with ZO-1 delimiting the region of cell to cell contact (inset in image 2). Mrp2 (red) detection was significantly reduced in response to EE treatment (image 4) when compared to controls (image 3), whereas nuclear staining (blue) remained constant. This is better illustrated in false color representations in image 5 (controls) and 6 (EE). Localization of Mrp2 (red) relative to ZO-1 (green) was preserved in the EE group (inset in image 4), as compared to controls (inset in image 3).
Panel B (treatment with E2-17G): Incubation of intestinal sacs with E2-17G (30 μM) did not affect the localization of Mrp2 relative to ZO-1 (image 8) as compared to the solvent group (image 7). In contrast, Mrp2 was found in part outside the limits of the canaliculus, as delimited by ZO-1, in liver slices.
incubated with E2-17G (arrows in image 10), whereas it remained mostly inside the canaliculus in solvent group (image 9). All pictures are representative images from at least 3 independent experiments per group. Scale bars are indicated in white and correspond to 50 μm.

**Figure 3: Effect of EE treatment on intestinal transport of DNP-SG.** The time-course of secretion of DNP-SG into the mucosal side, studied in non-everted sacs, is shown in panel A. CDNB (100 μM) was added to the serosal side. The effect of the inhibitor probenecid (1 mM) is shown for the 30-min period in the corresponding bar graphic. The time-course of absorption of DNP-SG from the mucosal to the serosal side, studied in everted sacs, is shown in panel B. DNP-SG (100 μM) was added to the mucosal side. The effect of the inhibitor probenecid (1 mM) is shown for the 30 min period in the corresponding bar graphic. The data represent means ± SD of 3 rats per group. * Significantly different from controls, p<0.05.

**Figure 4: Effect of EE on expression of GST classes.** GST activity towards CDNB is shown in panel A, whereas western blot studies of main GST classes is shown in panel B. These studies were performed in cytosol isolated from jejunal enterocytes. For western blot study, equal amounts of protein from cytosol (10 μg) were loaded in all lanes. Uniformity of protein 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. * Significantly different from controls, p<0.05.

**Figure 5: Effect of E2-17G on intestinal secretion of DNP-SG.** The secretion of DNP-SG into the mucosal side was studied in non-everted sacs after 30 min of incubation, in the presence of E2-17G (30 μM) or its solvent in the serosal side. CDNB (25 or 100 μM) was added to the serosal side. The data represent means ± SD of 4 rats per group.
Figure 6: Effect of EE and/or SL treatments on expression and function of Mrp2.

Panel A: Western blot studies of Mrp2 and villin were performed with BBM protein (35 μg) from jejunum. Uniformity of protein loading and transfer from gel to nitrocellulose membrane was controlled with Ponceau S and detection of β-actin. Data on densitometric analysis represent means ± SD of 3 rats per group.

- Significantly different from control group, p<0.05.
- Significantly different from EE group, p<0.05.
- Significantly different from SL group, p<0.05.

Panel B: Time-course of secretion of DNP-SG into the mucosal side, studied in non-everted sacs. CDNB was added to the serosal side at a 100 μM concentration. The data represent means ± SD of 3 rats per group.

- Significantly different from control group, p<0.05.
- Significantly different from EE group, p<0.05.
- Significantly different from SL group, p<0.05.
Table 1: Effect of EE on body and tissue masses and on markers of cholestasis.

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>EE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile flow (µl/min/g liver)</td>
<td>1.76 ± 0.42</td>
<td>0.90 ± 0.23 *</td>
</tr>
<tr>
<td>Serum alkaline phosphatase (U/l)</td>
<td>296 ± 47</td>
<td>391 ± 24 *</td>
</tr>
<tr>
<td>Serum bile salts content (µM)</td>
<td>243 ± 79</td>
<td>1376 ± 489 *</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>327 ± 7</td>
<td>294 ± 5 *</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>12.20 ± 0.40</td>
<td>13.10 ± 0.10 *</td>
</tr>
<tr>
<td>Proximal jejunum weight (g)</td>
<td>5.15 ± 0.70</td>
<td>4.85 ± 0.50</td>
</tr>
</tbody>
</table>

The proximal jejunum corresponds to the 30-cm segment following the duodenum. Data are means ± SD of 3 animals per group.

* significantly different from controls (p< 0.05).
Table 2: Effect of EE and/or SL on body and tissue masses and on markers of cholestasis.

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>EE</th>
<th>SL</th>
<th>EE+SL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile flow (μl/min/g liver)</td>
<td>2.10 ± 0.13</td>
<td>1.00 ± 0.6 (^a)</td>
<td>3.33 ± 0.34 (^{a,b})</td>
<td>1.94 ± 0.11 (^{b,c})</td>
</tr>
<tr>
<td>Serum alkaline phosphatase (U/l)</td>
<td>262 ± 37</td>
<td>330 ± 34 (^a)</td>
<td>135 ± 22 (^{a,b})</td>
<td>200 ± 19 (^b)</td>
</tr>
<tr>
<td>Serum bile salts content (μM)</td>
<td>269 ± 40</td>
<td>1155 ± 310 (^a)</td>
<td>140 ± 100 (^b)</td>
<td>262 ± 57 (^b)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>333 ± 11</td>
<td>325 ± 18</td>
<td>322 ± 14</td>
<td>333 ± 12</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>11.60 ± 0.49</td>
<td>13.40 ± 0.49 (^a)</td>
<td>13.60 ± 0.74 (^a)</td>
<td>15.12 ± 1.72 (^{a,b,c})</td>
</tr>
<tr>
<td>Proximal jejunum weight (g)</td>
<td>4.40 ± 0.30</td>
<td>4.85 ± 0.05</td>
<td>4.10 ± 0.40</td>
<td>4.60 ± 0.20</td>
</tr>
</tbody>
</table>

The proximal jejunum corresponds to the 30-cm segment following the duodenum. Data are means ± SD of 3 (bile flow, ALP and bile salts) or 6 (body and tissue masses) animals per group.

\(^a\) Significantly different from control group, p<0.05.

\(^b\) Significantly different from EE group, p<0.05.

\(^c\) Significantly different from SL group, p<0.05.
Figure 3

A

DNP-SG efflux (nmol/g intestine)

Time (min)

C

EE

* * *

Cumulative efflux (nmol/g intestine)

C

EE

C+Probenecid

EE+Probenecid

* * *

B

DNP-SG absorption (nmol/g intestine)

Time (min)

C

EE

C+Probenecid

EE+Probenecid

* * *

Cumulative absorption (nmol/g intestine)

* * *
Figure 5

Cumulative efflux (nmol/g intestine)

<table>
<thead>
<tr>
<th>CDNB</th>
<th>Solvent</th>
<th>E2-17G</th>
</tr>
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<tbody>
<tr>
<td>25μM</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>100μM</td>
<td></td>
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