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Prevention of Mrp2 Activity Impairment in Ethinylestradiol-Induced Cholestasis by Ursodeoxycholate in the Rat[(#)]

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Running title: Ursodeoxycholate prevents estrogen-induced Mrp2 impairment.

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Abbreviations:

CDNB, 1-chloro-2,4-dinitrobenzene; DNP-SG, dinitrophenyl S-glutathione; EE, ethinylestradiol; MPM, mixed plasma membrane; Mrp2, multidrug resistance-associated protein 2; TLM, total liver membranes, TUDC, sodium tauroursodeoxycholate; UDC, sodium ursodeoxycholate

Abstract

EE induces cholestasis by affecting bile salt-dependent and -independent fractions of the bile flow. The decrease in bile salt-independent flow is thought to be due, in part, to a reduction in the expression of the canalicular transporter Mrp2. The impact of modulation of Mrp2 function by UDC in EE cholestasis is unknown. We evaluated the protective effect of UDC on EE-induced impairment of Mrp2 activity *in vivo* and in isolated hepatocytes, by using the substrate dinitrophenyl-S-glutathione (DNP-SG). EE was administered to male Wistar rats at a dose of 5 mg/Kg, i.p., for 5 days. UDC was coadministered with EE at a dose of 25 mg/kg body wt, i.p, for the same period. EE alone reduced DNP-SG biliary excretion by 55% when compared to controls. Coadministration with UDC partially restored the alteration. Secretion rate of DNP-SG was decreased by 30% in isolated hepatocytes from EE-treated rats, but, contrary to *in vivo* results, UDC coadministration did not restore DNP-SG transport, likely as a consequence of bile salt washout resulting from the isolation procedure. As a confirmation, tauroursodeoxycholate hepatocyte preloading significantly increased Mrp2 activity. Western blotting analysis of Mrp2 indicated that EE administration significantly reduced its level in total and plasma membranes and that UDC coadministration failed to revert this alteration. In conclusion, UDC improvement in Mrp2 transport activity *in vivo* likely derived from a direct enhancement of Mrp2 function rather than from a restoration of its expression levels. This provides a novel mechanism explaining the beneficial effects of UDC in EE-induced cholestasis.

EE, a synthetic estrogen, induces intrahepatic cholestasis in experimental animals (Gumucio and Valdivieso, 1971;Jacquemin et al., 1993;Crocenzi et al., 2001;Sanchez Pozzi et al., 2003) by reducing the liver's capacity to excrete bile salts and organic anions (Gumucio and Valdivieso, 1971;Bossard et al., 1993). Expression of Mrp2, a canalicular transporter involved in organic anion excretion and, hence, in the formation of the bile-salt independent fraction of bile flow (Crocenzi et al., 2004), is decreased in EE cholestasis (Trauner et al., 1997).

Ursodeoxycholate (UDC) is a bile salt commonly used in the treatment of cholestatic diseases (Paumgartner and Beuers, 2002) including cholestasis of pregnancy (Palma et al., 1997). Its beneficial effect on reversion of EE-induced cholestasis is based on the improvement of the biliary secretory function impaired by the estrogen (Jacquemin et al, 1993;Sanchez Pozzi et al, 2003). It was demonstrated that UDC up-regulates canalicular Mrp2 expression in normal mice (Fickert et al., 2001) and that its taurine derivative (tauroursodeoxycholate, TUDC) stimulates insertion of pre-existing pericanalicular vesicles containing Mrp2 into the canalicular domain thus accounting for prevention of taurolithocholate-induced cholestasis (Beuers et al., 2001). Whether the beneficial effect of UDC on EE cholestasis is associated with regulation of Mrp2 expression is not known. Activation/inactivation of canalicular transporters was proposed as an alternative explanation for the modulation of canalicular secretory function (Paumgartner and Beuers, 2002). Gerk et al (2003) recently demonstrated that UDC positively modulates the activity of human MRP2, expressed in Sf9 insect cells. The authors proposed that a direct activation of transport of MRP2 substrates by UDC may contribute significantly to the anticholestatic properties of the bile salt. This possibility has not been tested *in vivo*. The purpose of the current study was to analyze the potential protective effect of UDC on EE-induced impairment of Mrp2 transport activity and expression at the canalicular level. Transport activity

of the model substrate of Mrp2, dinitrophenyl-S-glutathione (DNP-SG), was evaluated *in vivo* and in isolated hepatocytes, in addition to assessment of Mrp2 expression by western blotting.

Materials and Methods

Chemicals: EE, UDC, DMSO, phenylmethylsulfonyl fluoride (PMSF), leupeptin and pepstatin A were purchased from Sigma Chemical Co (St Louis, MO). Collagenase type A from *Clostridium histolyticum* was purchased from Invitrogen (Paisley, UK). 1-chloro-2,4-dinitrobenzene (CDNB) was from Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan). All other reagents were of the highest analytical grade and used as supplied.

Animals: Adult male Wistar rats weighing 300 to 350 g were used throughout. Before the experiments, animals were maintained on a standard diet and water ad lib., and housed in a temperature- (21ºC-23ºC) and humidity- (45%-50%) controlled room, under a constant 12-hour light, 12-hour dark cycle. All animals received humane care, according to the criteria of the "Guide for the Care and Use of Laboratory Animals" published by the NIH (publication 25-28, revised 1996).

Animals were randomly divided into 3 experimental groups, namely: 1) Control rats, receiving only propylene glycol, vehicle of EE and UDC; 2) EE-treated rats, which were administered daily with EE (5 mg/kg body wt, s.c.) and propylene glycol (0.5 ml/kg body wt, i.p.), for 5 consecutive days; 3) EE+UDC rats, which were daily co-administered with EE (5 mg/kg body wt, s.c.) and UDC (25 mg/kg body wt, i.p.), for 5 consecutive days.

Surgical Procedures: Surgical procedures were performed on the sixth day. Bile collection started between 9:00 a.m. and 11:00 a.m. to minimize influence of circadian variations. Animals

were anaesthetized with sodium pentobarbital (50 mg/kg body wt, i.p.), and thus maintained throughout. The femoral vein and the common bile duct were cannulated with polyethylene tubing (PE50 and PE10, respectively, Intramedic, Clay Adams, Parsippany, NJ). Body temperature was maintained at 37.0°C-38.0°C with a warming lamp. After a 30-min stabilization period, bile was collected for 30 min. Bile flow was determined by gravimetry, assuming a bile density of 1.0 g/ml.

Transport of DNP-SG in vivo: CDNB is conjugated in liver by glutathione S- transferases (GST) to its derivative DNP-SG (Habig et al., 1974). To evaluate the excretion rate of DNP-SG (an exogenous substrate of Mrp2) into bile, a set of animals from every group was subjected to a single dose injection of CDNB (10 μmol/kg body wt) through the femoral vein. Bile samples were collected every 10 min for 60 min. At the end of each experiment, animals were sacrificed by exsanguination, and the livers were removed and weighed. A portion of the major lobe was homogenized in saline (2 ml per g of liver). DNP-SG content was assessed in bile and liver homogenate by HPLC (Hinchman et al., 1991) using an external standard. Biliary excretion rate of DNP-SG was calculated as the product between bile flow and its biliary concentration.

Membrane preparations. Another set of rats was sacrificed and the livers were washed *in situ* with iced saline, the major lobe was removed, a portion was snap frozen in liquid nitrogen, and preserved at -70 ºC until use. Total liver membrane fraction (TLM), microsomal fraction (internal membranes, IM) and plasma membrane enriched fraction (mixed plasma membrane, MPM), were prepared as described (Carreras et al., 2003) and stored at –70°C for Mrp2 western blot studies. Protein concentration was measured in the different membrane preparations (Lowry et al., 1951).

Western blot studies of Mrp2: Either TLM, IM and MPM proteins were separated in 8 % SDS-polyacrylamide gels. After electrotransfer onto nitrocellulose membranes (Protran; Schleicher and Schuell, Keene, NH), the blots were incubated for 2 h with the primary antibody to human MRP2 (1:2,000; M_2 III-6, Alexis Biochemicals, Carlsbad, CA). The immune complex was detected by incubation with alkaline phosphatase-linked secondary antibody (1:2,000; Sigma Chemical Co) for 1h. Immunoreactive bands were detected using 5–bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium, quantified by densitometry (Shimadzu CS-9000, Shimadzu Corporation, Japan), and expressed in arbitrary units.

Mrp2 transport activity in isolated hepatocytes: Hepatocytes were isolated by collagenase perfusion and mechanical dissociation (Seglen, 1973). Cell viability was determined by trypan blue exclusion and was always greater than 87%. Protein concentration in the suspensions was determined using bovine serum albumin as a standard (Lowry et al, 1951). To evaluate Mrp2 activity, the transport rate of DNP-SG was measured as described (Elferink et al., 1989). Briefly, hepatocytes from the three experimental groups were loaded with DNP-SG by incubation with its precursor CDNB (100 µM) in Krebs-Henseleit Ringer-Hepes buffer, pH = 7.4 for 20 min at 10 ºC. Cells were washed twice with the buffer without CDNB at 10 ºC and cooled to 0 ºC. Aliquots were taken, loaded in the test tubes (0.4 ml polyethylene conical tubes) containing a lysis solution (3 M NaCl, 0.1% Triton X-100) and a silicone layer (Wacker-Chemie GmbH, Munich, Germany) and incubated at 37 ºC for 0, 30, 60 and 90 s. Then they were centrifuged at 12000 rpm (Beckman Microfuge, Palo Alto, CA) for 20 s causing the cells to filter through the silicone layer down to the lysis medium. DNP-SG was determined in supernatants (previous deproteinization with 70% v/v $HClO_4$) and cells (after their lysis by incubation at room

temperature for 24 h) by HPLC (Hinchman et al, 1991). Transport rate was estimated by the slope of the regression of nmol of DNP-SG excreted per mg of hepatocyte protein vs. time.

To evaluate the direct effect of tauroursodeoxycholate (TUDC), the main endogenous metabolite of UDC, on Mrp2 activity, isolated hepatocytes from untreated rats were preincubated with different concentrations of the bile salt (0, 5, 10 and 100 µM) for 10 min at 37ºC in Krebs-Henseleit Ringer-Hepes buffer, pH = 7.4. Then, CDNB was added to a final concentration of 100 µM and cells were incubated for 20 min at 10 ºC. Finally, cells were cooled to 0 ºC and the rest of the procedure was as described above.

Statistical Analysis: Results were expressed as mean ± S.E.M. One way ANOVA, followed by Newman-Keuls test, were performed for multiple comparison among groups. Values of $p < 0.05$ were considered statistically significant.

Results

Biliary excretion of DNP-SG: In all groups, excretion of DNP-SG followed a similar pattern with time and reached a maximum 10 min after injection (Fig 1). EE reduced DNP-SG cumulative excretion by 55 % and UDC partially restored the alteration (Fig 1 inset). In liver homogenates, DNP-SG content decreased in rats treated with both UDC and EE (92±18 nmol/kg body wt; n = 3) compared with rats treated only with the estrogen (169±12 nmol/kg body wt; n = 3); controls (150±20 nmol/kg body wt; n = 3) did not differ from the other groups.

Western blot studies of Mrp2: Fig 2 shows that EE administration reduced significantly Mrp2 protein content in all membrane preparations. The data also show that UDC coadministration reverted this alteration only in the microsomal fraction.

Transport of DNP-SG in isolated hepatocytes: The secretion rate of DNP-SG was decreased in hepatocytes in response to treatment with EE (Control: 0.77±0.04 vs. EE: 0.53±0.04 nmol/min mg protein, n = 3, $p<0.05$). UDC coadministration did not restore DNP-SG transport (EE+UDC: 0.53±0.05 nmol/min mg protein, n = 3). Intracellular levels of DNP-SG did not vary among groups (Control: 55±3; EE: 45±3; EE+UDC: 52±3 nmol/mg protein, n = 3).

Modulatory effect of TUDC on Mrp2 activity in isolated hepatocytes: Preloading of normal hepatocytes with TUDC increased DNP-SG transport with a maximal rise of 90 % at a TUDC concentration of 10 µM in the medium (Fig 3).

Discussion

EE administration results in a marked decrease in both bile-salt dependent and bile salt independent fractions of bile flow (Crocenzi et al, 2001;Gumucio and Valdivieso, 1971). Expression of canalicular Mrp2, a key determinant of the bile salt independent flow, is reduced in EE cholestasis (Trauner et al, 1997). A stimulated insertion of Mrp2 in the canalicular membrane or alternatively, an increased synthesis of the transporter by UDC, may tentatively explain its protective effect in EE cholestasis. Our study suggests that the mechanism by which the bile salt protects against EE-induced downregulation of Mrp2 differed from these possibilities. Clearly, UDC restored the biliary excretion of the model substrate of Mrp2, DNP-SG, and reduced hepatic accumulation of the compound, indicating an improvement of the transport capacity of Mrp2. Interestingly, EE-induced alteration in Mrp2 transport activity was not restored by the bile salt in isolated hepatocytes. Dissociation between *in vivo* and *in vitro* studies likely resulted from cells isolation procedure.

The current data on Mrp2 expression correlate well with the findings in isolated hepatocytes, since UDC did not prevent EE downregulation of Mrp2 in preparations enriched in plasma membrane. In contrast, the bile salt prevented the decrease in Mrp2 observed in intracellular membranes. This latter effect could result from decreased lysosomal degradation. On this regard, it was reported that PKC inhibits lysosomal protein degradation (Larocca et al., 2002) and that UDC activates this kinase (Rao et al., 1997) and uses this signaling pathway to exert its beneficial effect in taurolithocholate-induced cholestasis (Beuers et al, 2001). Thus, it is possible that UDC inhibits protein degradation as was seen for taurocholate (Larocca et al., 1999).

Because the restoration in Mrp2 activity cannot be attributed to an increase in transporter content at the canalicular level, it is possible to rule out stimulation of reinsertion of Mrp2 after retrieval, or synthesis followed by insertion of newly synthesized molecules, as mechanisms for UDC protection. Hence, what emerges is that UDC likely improves transporter function. In fact, incubation of isolated hepatocytes with TUDC, a major endogenous metabolite of UDC in rats, significantly increased DNP-SG transport agreeing well with the findings by Gerk et al. (2003), who demonstrated that UDC activates Mrp2 transport of [^{3}H]-estradiol 3-glucuronide in membrane vesicles from Mrp2-transfected Sf9 insect cells, with a peak effect of 950%. According to this, the absence of UDC effect in the transport function of Mrp2 in isolated hepatocytes is predictable since bile salts are washed out during the isolation procedure and probably no UDC or TUDC is present in the medium to exert their effects.

In summary, UDC positively modulates Mrp2 function without affecting its content at the canalicular level in EE cholestasis. This may tentatively explain part of the protective effects of UDC in liver pathologies.

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Footnotes

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Figure legends

Fig 1. *Biliary excretion of DNP-SG.*

Biliary excretion rate of the prototypical substrate of Mrp2 in rats pretreated with either 5 mg/kg body wt of EE (open circles), EE and 25 mg/kg body wt. of UDC (filled triangles) or solvent (Control, filled circles). Insets depict cumulative excretion of DNP-SG by 1 hr. Data are means ± S.E.M. of 3 animals per group.

[a] significantly different from control group ($p < 0.05$).

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

Fig 2. *Detection of Mrp2 by western blotting.*

Canalicular transporters were detected in total liver membranes (TLM), mixed plasma membranes (MPM) and intracellular membranes (IM). Equal amounts of total protein (15 µg for TLM and MPM and 30 µg for IM) were loaded in all lanes. Data on densitometric analysis represent means ± S.E.M. of 3 rats per group.

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

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

Fig 3. *Effect of TUDC on DNP-SG transport in isolated hepatocytes.*

DNP-SG transport was analyzed in normal hepatocytes preincubated with different concentrations of TUDC. Data represent means ± S.E.M. of 3 different hepatocyte preparations.

* Significantly different from TUDC 0 µM ($p<0.05$)

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Fig 1

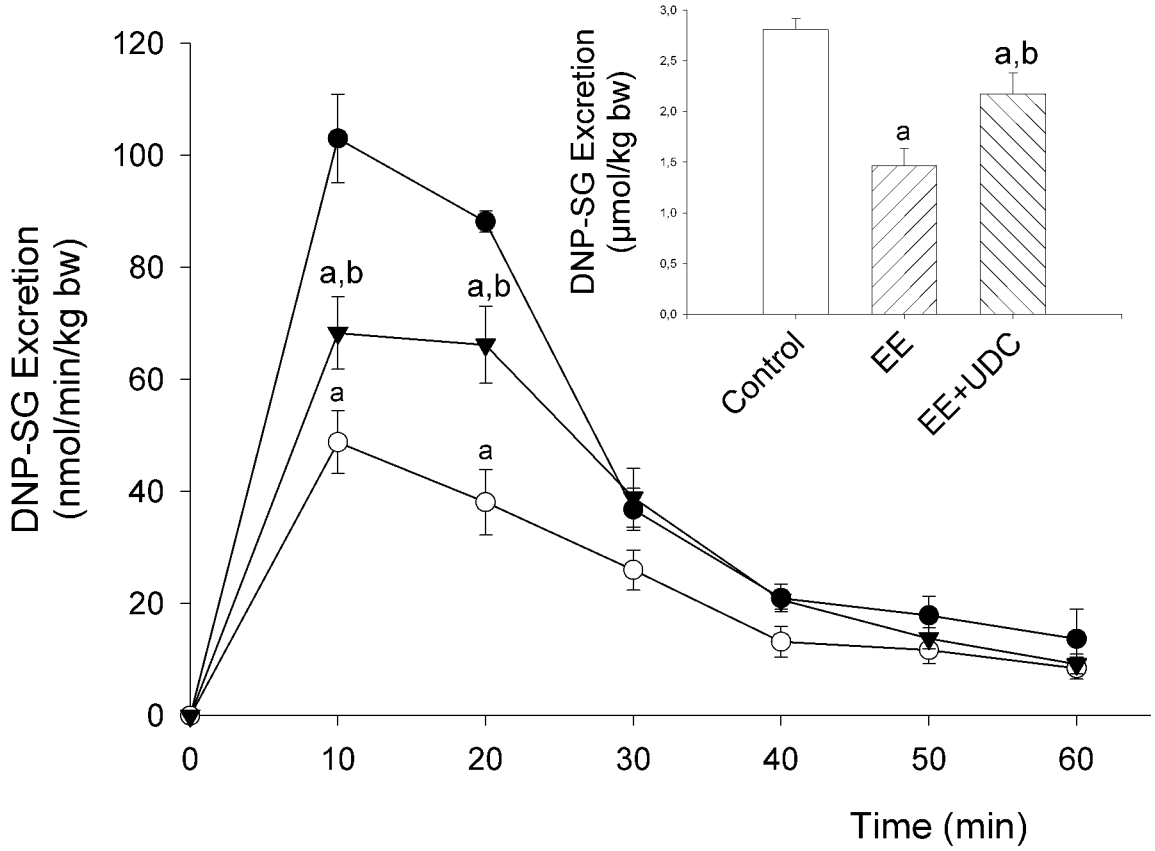
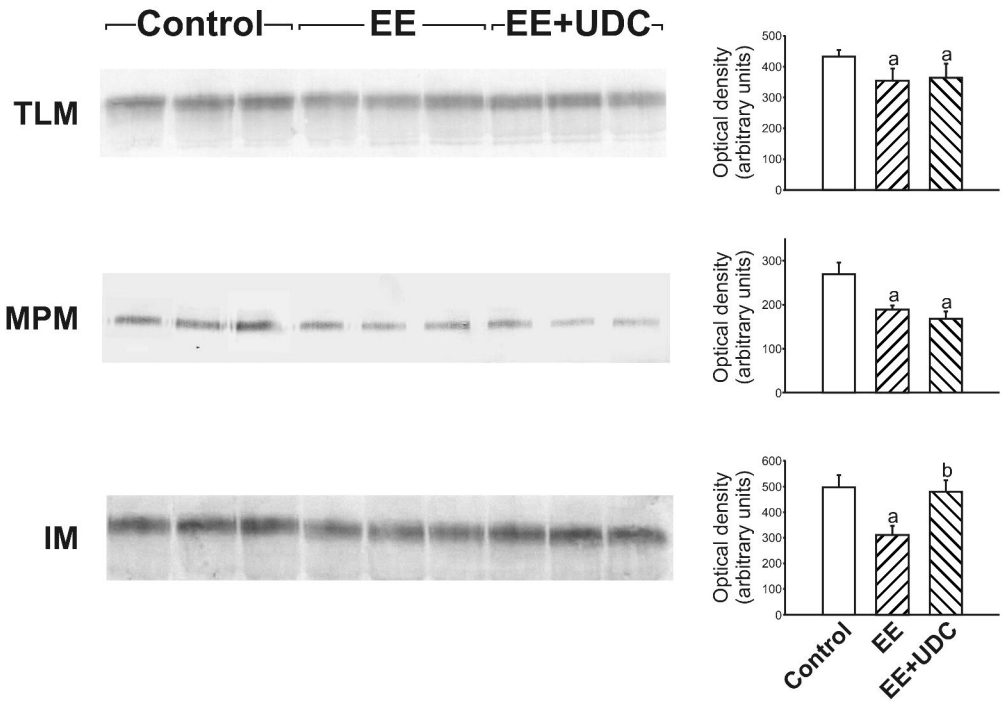


Fig 2


Control
EE
EE+UDC
TLM
MPM
IM
Optical density (arbitrary units)
500
400
300
200
100
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Optical density (arbitrary units)
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200
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a
a
Optical density (arbitrary units)
600
500
400
300
200
100
0
a
b
Control
EE
EE+UDC

Fig 3

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