Short Communication

PREVENTION OF MRP2 ACTIVITY IMPAIRMENT IN ETHINYLESTRADIOL-INDUCED CHOLESTASIS BY URSODEOXYCHOLATE IN THE RAT

Received December 30, 2004; accepted April 19, 2005

ABSTRACT:

Ethinylestradiol (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 sodium ursodeoxycholate (UDC) in EE-induced 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 s.c. for 5 days. UDC was coadministered with EE at a dose of 25 mg/kg i.p. for the same period. EE alone reduced DNP-SG biliary excretion by 55% when compared with 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, taurosodeoxycholate 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 taursine derivative [taurosodeoxycholate (TUDC)] stimulates insertion of preexisting pericanalicular vesicles containing Mrp2 into the canalicular domain, thus accounting for prevention of tauroliothicholate-induced cholestasis (Beuers et al., 2001). Whether the beneficial effect of UDC on EE cholestasis is associated with modulation 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, dimethyl sulfoxide, phenylmethylsulfonyl fluoride, leupeptin, and pepstatin A were purchased from Sigma-Aldrich (St. Louis, MO). Collagenase type A from Clostridium histolyticum was purchased from InVitrogen (Paisley, UK). 1-Chloro-2,4-dinitrobenzene (CDNB) was obtained 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 through-
out. Before the experiments, animals were maintained on a standard diet and water ad libitum and housed in a temperature (21–23°C)- and humidity (45–50%)- controlled room, under a constant 12-h light, 12-h 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 National Institutes of Health (publication 25-28, revised 1996).

Animals were randomly divided into three 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 b.wt. s.c.) and propylene glycol (0.5 ml/kg b.wt. i.p.) for 5 consecutive days; and 3) EE + UDC rats, which were daily coadministered with EE (5 mg/kg b.wt. s.c.) and UDC (25 mg/kg b.wt. i.p.) for 5 consecutive days.

**Surgical Procedures.** Surgical procedures were performed on the 6th day. Bile collection started between 9:00 AM and 11:00 AM to minimize the influence of circadian variations. Animals were anesthetized with sodium pentobarbital (50 mg/kg b.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 37.0–38.0°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 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 b.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 liver was 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 high-performance liquid chromatography (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, and a portion was snap-frozen in liquid nitrogen and preserved at −70°C until use. Total liver membrane (TLM) fraction, microsomal fraction [internal membranes (IMs)], and plasma membrane-enriched fraction [mixed plasma membranes (MPMs)] 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, or MPM proteins were separated in 8% SDS-polyacrylamide gels. After electrophoresis onto nitrocellulose membranes (Protran; Schleicher & Schull, Keene, NH), the blots were incubated for 2 h with the primary antibody to human MRP2 (1:2000; M2 III-6; Alexis Biochemicals, Carlsbad, CA). The immune complex was detected by incubation with alkaline phosphatase-linked secondary antibody (1:2000; Sigma-Aldrich) for 1 h. Immunoreactive bands were detected using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium, quantified by densitometry (Shimadzu CS-9000; Shimadzu, Kyoto, 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°C. Cells were washed twice with the buffer without CDNB at 10°C and cooled to 0°C. 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°C for 0, 30, 60, and 90 s. Then, they were centrifuged at 12,000 rpm (Microfuge; Beckman Coulter, Fullerton, 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 HClO4) and cells (after their lysis by incubation at room temperature for 24 h) by high-performance liquid chromatography (Hinchman et al., 1991). Transport rate was estimated by the slope of the regression of nanomoles of DNP-SG excreted per milligram of hepatocyte protein versus time.

To evaluate the direct effect of 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 37°C 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°C. Finally, cells were cooled to 0°C, and the rest of the procedure was as described above.

**Statistical Analysis.** Results were expressed as mean ± S.E.M. One-way analysis of variance, followed by the Newman-Keuls test, was 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% and 55% respectively) compared with rats treated only with the estrogen (169 nmol/kg b.wt.; 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).

**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 versus 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).
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 (Gumucio and Valdivieso, 1971; Crocenzi et al., 2001). 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 down-regulation 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 the cell isolation procedure.

The current data on Mrp2 expression correlate well with the findings in isolated hepatocytes, since UDC did not prevent EE down-regulation 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. In this regard, it was reported that protein kinase C 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 tauroliothocholate-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

**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 three different hepatocyte preparations. *, significantly different from 0 μM TUDC (p < 0.05).
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 [3H]estradiol 3-glucuronide in membrane vesicles from Mrp2-transfected Sf9 insect cells, with a peak effect of 950%. According to this finding, 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.

Instituto de Fisiología Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

References


**Address correspondence to:** Dr. Enrique J. Sánchez Pozzi, Instituto de Fisiología Experimental, Facultad de Ciencias Bioquímicas y Farmacéuticas, CONICET–Universidad Nacional de Rosario, Suipacha 570 (2000) Rosario, Argentina. E-mail: esanchez@unr.edu.ar