

Ursodeoxycholate Reduces Ethinylestradiol Glucuronidation in the Rat: Role in Prevention of Estrogen-Induced Cholestasis

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ABSTRACT

Ethinylestradiol (EE) administration (5 mg/kg, s.c., daily for 5 days) to rats leads to cholestasis, and its derivative EE 17 β -glucuronide is a likely mediator of this effect. Coadministration of ursodeoxycholate (UDC) was shown to prevent ethinylestradiol-induced cholestasis. The aim of this study was to evaluate the inhibitory effect of UDC on EE glucuronidation in vivo and in vitro as a potential mechanism to explain UDC protection. UDC treatment (25 mg/kg, i.p., daily for 5 days) decreased the biliary excretion of EE 17 β -glucuronide in bile after administration of a trace dose of [³H]EE and reduced microsomal EE 17 β -glucuronidation activity by 20% and expression of UGT2B1, one of the enzymes involved in EE conjugation, by 30%. Glucuronidation kinetic studies were performed in vitro using normal microsomes and isolated hepatocytes in the presence of taurour-

sodeoxycholate (TUDC), the major endogenous derivative of UDC in the rat. Kinetic enzymatic studies in microsomes showed a noncompetitive inhibition of EE 17 β -glucuronidation by TUDC, which was unique for this bile salt since other endogenous bile salts such as taurocholate, taurochenodeoxycholate, or taurodeoxycholate did not affect the enzyme activity. Studies in isolated hepatocytes confirmed the inhibitory effect of TUDC on EE glucuronidation and indicated that TUDC can reach the enzyme active site in intact cells. In conclusion, both in vivo and in vitro experiments indicate that UDC decreased the metabolic pathways involved in EE glucuronidation, hence decreasing the formation of the cholestatic derivative EE 17 β -glucuronide.

Administration of ethinylestradiol (EE), a synthetic estrogen, is known to induce intrahepatic cholestasis in experimental animals (Gumucio and Valdivieso, 1971; Bouchard et al., 1993; Jacquemin et al., 1993; Crocenzi et al., 2001). The mechanism by which EE induces cholestasis is still unclear, however, although interference with bile salt-independent bile flow and reduced hepatic capacity to excrete bile salts and organic anions have been demonstrated to occur (Gumucio and Valdivieso, 1971; Bossart et al., 1993; Huang et al., 2000; Stieger et al., 2000).

Many of the cholestatic effects of naturally occurring estrogens have been suggested to be mediated by their D-ring

glucuronide conjugates, such as estradiol 17 β -glucuronide (Meyers et al., 1980; Vore, 1987). Conversely, A-ring (3-glucuronide) metabolites and the 3-sulfate conjugate of estradiol 17 β -glucuronide cause choleresis (Meyers et al., 1980; Sliker et al., 1983). Increased proportions of D/A-ring glucuronides have been found in the urine of patients with obstetric cholestasis (Adlercreutz et al., 1974), suggesting that D-ring glucuronides may be involved in its pathogenesis. Unlike nonconjugated estrogens, which require several hours to exert their cholestatic effect (Gumucio and Valdivieso, 1971), estradiol 17 β -glucuronide-induced cholestasis is very rapid, suggesting that a metabolic step, like glucuronide conjugation, may be required for estrogens to produce their cholestatic effect.

Ursodeoxycholate (UDC) and its taurine conjugate, tauroursodeoxycholate (TUDC), are known to improve liver tests in patients with cholestatic disorders such as primary biliary cirrhosis (Poupon et al., 1991) and cholestasis of pregnancy (Palma et al., 1997). Their beneficial effects on EE-induced cholestasis have been demonstrated in rats; e.g., UDC in-

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ABBREVIATIONS: EE, ethinylestradiol; UDC, ursodeoxycholate; TUDC, tauroursodeoxycholate; TC, taurocholate; TDC, taurodeoxycholate; TCDC, taurochenodeoxycholate; UDPGA, UDP-glucuronic acid; UDP-N-AG, UDP-N-acetylglucosamine; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; UGT, UDP-glucuronosyltransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase.

creases bile flow and bile salt output, leading to an improvement of the biliary secretory function impaired by EE (Bouchard et al., 1993; Jacquemin et al., 1993). TUDC also prevented the acute cholestasis induced by estradiol 17 β -glucuronide (Kinbara et al., 1997; Milkiewicz et al., 2001).

In previous studies, we demonstrated both *in vivo* and *in vitro* inhibitory effects of UDC on bilirubin glucuronidation (Sánchez Pozzi et al., 1994a,b). Since the cholestatic response of EE may be mediated by its 17 β -glucuronide derivative, we have tested here whether UDC decreases EE glucuronide formation. We evaluated glucuronidation *in vivo* in EE cholestatic rats coadministered with UDC and performed enzyme kinetic inhibition studies *in vitro*, using normal microsomes and isolated hepatocytes, in the presence of TUDC, the major endogenous derivative of UDC.

Materials and Methods

Chemicals

EE, UDC, taurocholate (TC), taurodeoxycholate (TDC), taurochenodeoxycholate (TCDC), UDP-glucuronic acid (UDPGA), D-saccharic acid 1,4-lactone, UDP-*N*-acetylglucosamine (UDP-*N*-AG), 3 α -hydroxysteroid dehydrogenase, sulfatase type H-1 from *Helix pomatia*, β -glucuronidase type B-1 from bovine liver, NADPH, and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). TUDC was a generous gift from Prodotti Chimici e Alimentari S.p.A. (Genoa, Italy). This bile salt was 99% pure when examined by HPLC. Collagenase type A from *Clostridium histolyticum* was purchased from Invitrogen (Paisley, UK). [³H]EE (44 Ci/mmol) was from PerkinElmer Life Sciences (Boston, MA). 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, given water *ad libitum*, and housed in a temperature- (21°C–23°C) and humidity-controlled (45–50%) room, under a constant 12-h light/dark cycle. All animals received humane care, according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the NIH (publication 86-23, revised 1985).

In Vivo Studies

Basal Bile Studies. Animals were randomly divided into five experimental groups, namely:

1. Control rats, receiving only propylene glycol (0.5 ml/kg b.wt., *s.c.*, and 0.5 ml/kg b.wt., *i.p.*), the vehicle of both EE and UDC.
2. EE-treated rats, which were administered daily with EE, at the dose of 5 mg/kg b.wt., *s.c.*, and propylene glycol 0.5 ml/kg b.wt., *i.p.*, for 5 consecutive days.
- 3 to 5. EE + UDC rats, which were daily coadministered with EE (5 mg/kg b.wt.) and different doses of UDC (15, 25, or 50 mg/kg b.wt., *i.p.*) for 5 consecutive days.

Surgical Procedures. Surgical procedures were made on the 6th day (*i.e.*, 1 day after the administration of EE last dose). Bile collection started between 9:00 and 11:00 AM to minimize influence of circadian variations. Animals were anesthetized with a single dose of sodium pentobarbital (50 mg/kg b.wt., *i.p.*) and maintained under this condition throughout the experiment. A middle abdominal incision was made, and the common bile duct was cannulated using a PE-10 polyethylene tubing (Intramedic, Clay Adams, Parsippany, NJ). Tracheal cannulation was systematically performed to remove bronchial secretion induced by the anesthetic. Body temperature was maintained at 37.0–38.5°C with a warming lamp to prevent hypo-

thermic alterations of bile flow. At the end of each experiment, animals were sacrificed by exsanguination, and the liver was removed and weighed. Liver microsomes were obtained from liver homogenates by differential centrifugation (Siekevitz, 1962), as previously described (Catania et al., 1995), and were kept at –70°C until used for studies of UDP-glucuronosyltransferase (UGT) and lipid composition (see below).

Analytical Procedures. Thirty minutes after bile duct cannulation, a 30-min period of bile collection was carried out. Bile flow was determined by gravimetry, assuming a bile density of 1.0 g/ml. The biliary excretion rate was calculated as the product between bile flow and biliary concentration. Basal bile was assayed for total and individual bile salt content. Total bile salt concentration was assayed using the 3 α -hydroxysteroid dehydrogenase procedure (Talalay, 1960). Individual bile salts were determined by HPLC (Waters, Milford, MA), as reported previously (Tietz et al., 1984), and identified using appropriate standards.

Activities of enzymes markers of hepatocellular damage, including aspartate aminotransferase (EC 2.6.1.1), alanine aminotransferase (ALT; EC 2.6.1.2), and alkaline phosphatase (ALP; EC 3.1.3.1) were determined in plasma. Aspartate aminotransferase and ALT activities were assessed spectrophotometrically by measuring NADH consumption at 340 nm. ALP was assessed using *p*-nitrophenyl phosphate as a substrate. In all cases, commercial kits were used (Wiener Lab, Rosario, Argentina).

Biliary Excretion of EE Metabolites. In this set of experiments, two groups of rats were injected with EE (5 mg/kg b.wt.) or with both EE and UDC (25 mg/kg b.wt.), as described above, but replacing the last, fifth administration of EE with an injection of [³H]EE (10 μ Ci/kg b.wt.); this allowed us to evaluate the fate of the last dose of estrogen by studying the appearance of labeled compounds in bile and the retention of [³H] in liver and plasma. The 5th day, after the EE group received the solvent of UDC and the EE + UDC group received UDC, animals were anesthetized, and the femoral vein (PE-50 polyethylene tubing) and the common bile duct were cannulated so that 2 h after the last UDC administration a trace dose of [³H]EE was injected via the femoral vein. Immediately, bile was collected in 10- to 30-min intervals for 120 min. At the end of the experiment, animals were sacrificed by exsanguination, and the liver was removed and weighed. Radioactivity of bile, serum, and liver homogenate (in saline) was measured in a liquid scintillation counter (RackBeta 1214; Pharmacia Wallac Oy, Finland), using OptiScint "Hi Safe" (LKB, Uppsala, Sweden). Samples of bile were deproteinized with isopropyl alcohol (1:9) and centrifuged at 8500g for 5 min. Supernatants were divided into three aliquots and evaporated under N₂. One aliquot remained untreated, and the others were incubated with either β -glucuronidase or sulfatase + β -glucuronidase, and then were deproteinized and evaporated. Treated and untreated samples were resuspended in running solvent and injected in HPLC to perform separation of EE derivatives, as described above. Glucuronides and sulfates were identified by comparing treated and untreated profiles, and EE 17 β -glucuronide was identified using the corresponding standard. To confirm the specificity of the glucuronide moiety cleavage by β -glucuronidase, a set of deproteinized bile samples were incubated with β -glucuronidase + D-saccharic acid 1,4-lactone, a specific inhibitor of the enzyme, and compared with the untreated samples; no difference was observed in the HPLC profile, confirming that the cleavage was due to the enzyme.

Expression and Activity of UGT Conjugating EE. *Enzyme activity determination.* Enzyme activity was evaluated in microsomes obtained after 5-day treatment of EE, UDC + EE, or solvent. The protein content of microsomal preparations was determined by the biuret method (Gornall et al., 1949). 17 β -Glucuronidation of EE was assessed according to Rao et al. (1977), except that microsomes were activated with UDP-*N*-AG (2.0 mM final concentration). A trace amount of [³H]EE was added along with unlabeled EE (1 mM). D-Saccharic acid 1,4-lactone (2 mM) was systematically included in every incubation medium to inhibit enzymatic hydrolysis of glu-

ronides. Reactions were initiated with the addition of UDPGA (4 mM, final concentration) and allowed to develop for 45 min at 37°C. Under these experimental conditions, enzyme activities varied linearly throughout as a function of both time and protein concentration. HPLC was performed to separate EE and its metabolites. The mobile phase consisting of methanol/0.1 M KH_2PO_4 , pH 4.5 (60:40, v/v) was as previously used for bile salts separation (Tietz et al., 1984). Detection of radioactive unconjugated and conjugated steroids was accomplished with an IN/US β RAM flow-through detector (Pine Brook, NJ). The peaks corresponding to glucuronides were confirmed by including a sample preincubated with β -glucuronidase. EE 17 β -glucuronide was identified using a standard, the generous gift of Dr. Burchell (Ninewells Hospital, Dundee, Scotland).

Western blot analysis. A polyclonal anti-peptide antibody that specifically recognizes UGT2B1, an isoenzyme involved in EE 17 β -glucuronidation (Mackenzie et al., 1997), was kindly provided by Dr. Ikushiro (Faculty of Science, Himeji Institute of Technology, Hyogo, Japan) and used in Western blot studies. Analyses were performed in microsomes from rats treated with solvent, EE, and EE + UDC (25 mg/kg b.wt.). The amount of microsomal protein used in the gels (15 μg) was found to give a densitometric signal in the linear range of the response curve for the antibody (data not shown). Preparations were loaded onto 10% SDS-polyacrylamide gel (Laemmli, 1970) and subjected to electrophoresis. After electrotransfer onto nitrocellulose membranes (Protran; Schleicher and Schuell, Keene, NH), the blots were blocked at least for 2 h at 4°C with Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk and then incubated overnight with the primary antibody (1:1000). The immune complex was detected by incubation with alkaline phosphatase-linked anti-rabbit secondary antibody (1:10,000; Sigma-Aldrich) for 1 h. Immunoreactive bands were detected by the alkaline phosphatase color reaction using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium and quantified by densitometry (Shimadzu CS-9000; Shimadzu, Kyoto, Japan).

Lipid analysis of microsomal membranes. The features of the lipid enzyme environment may affect glucuronidation (Zakim and Dannenberg, 1992); therefore, the cholesterol/phospholipid ratio and the composition in fatty acids of microsomal membranes were studied. Microsomal lipids were extracted by the procedure of Folch et al. (1957). Total cholesterol was determined by the cholesterol oxidase method (Omodeo-Salé et al., 1984). Lipid phosphorus was measured by the method of Chen et al. (1956). Fatty acid composition was determined by gas-liquid chromatography of the methyl esters (Metcalfe et al., 1966) in a GOW-MAC chromatograph model 580 (GOW-MAC Instrument Co., Bethlehem, PA). A Kromxpek column (Kromxpek Analítica SA, Barcelona, Spain) filled with 15% DEGS on 100-120 Chromosorb W-HP was used.

In Vitro Studies

Isolated Hepatocytes Study. In this set of experiments, we evaluated the effect of TUDC, the major endogenous metabolite of UDC, on EE glucuronidation in isolated hepatocytes from untreated rats. Rat hepatocytes were isolated by collagenase perfusion (Seglen, 1973). Cell viability was greater than 85% by a trypan blue exclusion. Hepatocytes were resuspended ($0.5 \times 10^6/\text{ml}$) in Hanks' medium (137 mM NaCl, 26 mM NaHCO_3 , 0.6 mM Na_2HPO_4 , 5.4 mM KCl, 0.4 mM KH_2PO_3 , 5 mM TRIS, 5.6 mM glucose, and 2.5 mM CaCl_2), containing different concentrations of TUDC (0.2 to 2 mM). Controls were incubated with TUDC vehicle (DMSO). After 5 min of preincubation at 37°C, unlabeled EE (1 mM) and a trace amount of [^3H]EE were added to the medium. Incubation continued for 30 min. The experiment was stopped by the addition of methanol (2:1). The cell suspension was centrifuged at 8500g for 5 min, and the supernatants were assayed for EE glucuronides by HPLC. Viability of hepatocytes was evaluated at the end of the experiment measuring the appearance of LDH and ALT in the medium. In preliminary studies, cell suspensions were centrifuged at 50g after a 30-min incubation without addition of methanol, and then they were washed

with Hanks' medium and recentrifuged at 50g. Radioactivity was measured in the sediment, and no differences was observed between DMSO- and TUDC-treated cells, suggesting that the bile salt did not alter EE uptake.

In a different set of experiments, the inhibitory effect of TUDC on EE glucuronidation by hepatocytes was compared with that of the major endogenous bile salts TC, TCDC, and TDC, at a concentration of 0.5 mM.

Inhibition Kinetic Studies. Microsomes from normal adult male rats were used as a source of UGT to test direct inhibition of EE glucuronidation by TUDC. To allow freely access of substrates and inhibitor to the enzyme active site, microsomes were preincubated with digitonin at a detergent/protein ratio of 0.5 (w/w), at 0°C for 30 min. The reaction mixtures comprised digitonin-activated microsomes (about 0.5 mg of protein), MgCl_2 (9.6 mM), UDPGA (4.2 mM), variable amounts of EE + [^3H]EE (0.05 to 2 mM) and TUDC (0 to 2 mM), D-saccharic acid 1,4-lactone (2 mM), and HEPES-NaOH buffer (pH 6.5), final volume 300 μl . The reaction was initiated by addition of UDPGA and allowed to develop for 45 min at 37°C; during this time period, a linear function with time was obtained throughout. The reaction was stopped by addition of methanol (1:2). The mixtures were centrifuged at 8500g for 5 min, and the supernatants subjected to HPLC analyses. The kinetic constant $K_{m,app}$ and $V_{max,app}$ for EE as well as $K_{i,app}$ for TUDC were estimated by fitting the data with a nonlinear regression computing program.

In a different set of experiments, the inhibitory effect of TUDC on EE glucuronidating activity was compared with that of the major endogenous bile salts TC, TCDC, and TDC. This set of experiments was performed in digitonin-activated microsomes, as described above, but using fixed concentrations of EE (1 mM) and endogenous bile salts (0.9 mM).

Statistical Analysis

Results were expressed as mean \pm S.E. One-way analysis of variance, followed by Newman-Keuls test, were performed for multiple comparison among groups. The paired Student's *t* test was used for comparison between groups in [^3H]EE excretion studies. Values of $p < 0.05$ were considered statistically significant.

Results

In Vivo Experiments

Bile Flow and Bile Salt Output. The effect of different doses of UDC on the impairment of bile flow and bile salt output induced by EE is depicted in Table 1. While EE-induced reduction in bile flow was only partially prevented by administration of 15 mg of UDC/kg b.wt., impairment in bile salt secretion was fully prevented by UDC, indicating that the bile salt independent fraction of the bile flow remained partially impaired. Administration of higher doses of UDC did not further prevent these alterations.

The decrease in total bile salt output induced by EE was primarily accounted for by a decrease in the excretion of deoxycholate, cholate, muricholate, and UDC (-53, -69, -43, and -56%, respectively). Instead, output of chenodeoxycholate and hyodeoxycholate remained virtually unchanged. Table 1 also shows that UDC prevented the diminution in the output of the different endogenous bile salts at different degrees. As expected, biliary excretion of UDC was clearly increased in UDC groups when compared with EE and control groups.

Serum Markers. EE treatment led to a 37% increase in ALP serum activity, a biochemical marker of cholestasis (Table 1). None of the other serum biochemical parameters studied were modified by EE in agreement with results reported

TABLE 1

Effect of UDC administration on alterations of bile flow, bile salt output and serum markers induced by EE

All parameters were assessed after a 5-day treatment with EE (5 mg/kg b.wt.), EE + UDC (15, 25, or 50 mg/kg b.wt.), or vehicle in controls. Bile salts were separated by HPLC, as described under *Materials and Methods* in bile collected for 30 min. Results are expressed as mean \pm S.E. for 8 to 12 animals/group.

	Control	EE	EE + UDC15	EE + UDC25	EE + UDC50
Bile Flow (μ l/min/g liver)	1.8 \pm 0.1	0.9 \pm 0.1 ^a	1.3 \pm 0.1 ^b	1.4 \pm 0.1 ^b	1.3 \pm 0.2 ^b
Bile salt output (nmol/min/g liver)	29 \pm 1	16 \pm 1 ^a	29 \pm 1 ^b	27 \pm 2 ^b	28 \pm 1 ^b
Individual bile salt output					
MC	12.4 \pm 0.4	7.3 \pm 0.4 ^a	12.6 \pm 0.6 ^b	12.1 \pm 0.5 ^b	12.0 \pm 0.5 ^b
UDC	2.5 \pm 0.3	1.1 \pm 0.1 ^a	4.3 \pm 0.1 ^{a,b}	4.0 \pm 0.4 ^{a,b}	7.0 \pm 0.6 ^{a,b,c,d}
HDC	1.9 \pm 0.1	2.3 \pm 0.3	3.7 \pm 0.4	2.7 \pm 0.6	2.2 \pm 0.4
C	8.2 \pm 0.4	2.6 \pm 0.2 ^a	4.1 \pm 0.1 ^{a,b}	3.8 \pm 0.3 ^a	3.4 \pm 0.2 ^a
CDC	1.8 \pm 0.1	1.0 \pm 0.1	2.4 \pm 0.3 ^b	2.3 \pm 0.4 ^b	1.5 \pm 0.3
DC	2.3 \pm 0.1	1.2 \pm 0.4 ^a	1.8 \pm 0.2	1.8 \pm 0.2	1.6 \pm 0.1
Serum biochemical parameters					
AST (U/l)	151 \pm 6	161 \pm 4	162 \pm 6	149 \pm 4	155 \pm 3
ALT (U/l)	139 \pm 4	123 \pm 5	121 \pm 3	125 \pm 6	130 \pm 4
ALP (U/l)	284 \pm 10	389 \pm 6 ^a	370 \pm 5 ^a	370 \pm 7 ^a	335 \pm 11 ^{a,b,c,d}

MC, muricholate; HDC, hyodeoxycholate; C, cholate; CDC, chenodeoxycholate; DC, deoxycholate; AST, aspartate aminotransferase.

^a Different from control group ($p < 0.05$).^b Different from EE group ($p < 0.05$).^c Different from EE-UDC15 group ($p < 0.05$).^d Different from EE-UDC25 group ($p < 0.05$).

elsewhere (Bouchard et al., 1993; Crocenzi et al., 2001). Only the highest dose of UDC decreased ALP serum activity but did not reach control values.

Biliary Excretion of EE Metabolites. We observed differences in the hepatic handling of a trace dose of [³H]EE between EE and EE + UDC-treated rats (see Table 2). Two hours after injection of [³H]EE, cumulative biliary excretion of total [³H] was significantly greater in EE-treated rats receiving UDC than in rats receiving EE alone. The label retained in liver was decreased by UDC, whereas plasma labeling did not differ among groups.

The analysis of bile samples by HPLC showed three main ³H peaks (data not shown). Preincubation with β -glucuronidase or sulfatase + β -glucuronidase, followed by HPLC, led to identification of one of the peaks as a sulfate derivative, whereas the two other peaks were identified as glucuronides. One of the glucuronide peaks presented a retention time consistent with that of EE 17 β -glucuronide and was assumed as such. Figure 1 shows biliary excretion of the radiolabeled sulfate and glucuronide derivatives of EE and of total [³H] in EE and EE-UDC-treated rats. Whereas UDC pretreatment did not modify the shape of the profiles of biliary excretion of the EE metabolites, biliary excretion of sulfated EE tended to be enhanced by UDC, while that of EE 17 β -glucuronide was lower compared with EE alone. As a consequence, after 2 h of bile collection, cumulative excretion of the sulfate derivative was enhanced by UDC, whereas cumulative excretion of EE 17 β -glucuronide was significantly decreased by UDC; the excretion of the unidentified glucuronide instead was not affected by the bile salt. An important fraction of the dose

TABLE 2

Disposition of [³H]EE in vivo

Animals receiving a 5-day administration protocol of either EE (5 mg/kg b.wt.) or EE + UDC (25 mg/kg b.wt.) were injected with [³H]EE (10 μ Ci/kg b.wt.) in lieu of the last administration of unlabelled EE. Immediately, bile was collected for 120 min. At the end of the experiment, radioactivity of serum and liver homogenate was measured and biliary secretion of total ³H was evaluated in the whole 120-min period.

	EE	EE + UDC
[³ H]Plasma concentration (nCi/ml)	2.1 \pm 0.2	2.2 \pm 0.2
Cumulative [³ H]biliary excretion (% of injected dose)	72.7 \pm 1.8	81.1 \pm 1.4 ^a
³ H in liver (% of injected dose)	5.87 \pm 0.20	4.49 \pm 0.29 ^a

^a Different from EE group ($p < 0.05$).

excreted in bile was not identified (40% in EE; 48% in EE + UDC).

Microsomal UGT Activity. Figure 2 shows that administration of EE significantly decreased its own 17 β -glucuronidation, whereas UDC decreased even more this activity when compared with EE values. This magnitude of the latter effect was similar (approximately -20%) among the different UDC doses.

Western blot analysis of UGT2B1, an isoenzyme involved in EE 17 β -glucuronidation, revealed that the estrogen decreased isoenzyme expression (Fig. 3). Administration of UDC (25 mg/kg b.wt.) reduced UGT2B1 expression in a magnitude (-28%) consistent with the decrease in enzyme activity.

Lipid Composition of Microsomal Membranes. EE administration significantly increased the microsomal cholesterol/phospholipid ratio and decreased the unsaturation index of fatty acids (see Table 3). These alterations were not reversed by UDC.

In Vitro Experiments

Isolated Hepatocytes. Analysis of the effect of TUDC on EE metabolism in the intact cell revealed that the bile salt decreased 17 β -glucuronide formation in a dose-dependent manner (Fig. 4). Viability of hepatocytes, measured by release of LDH and ALT into the medium, was not modified by TUDC treatment (data not shown). TCDC and TDC were toxic to hepatocytes since incubation with these bile salt produced a 3-fold increase in the release of LDH and ALT compared with control preparations; hence, their effect on EE glucuronidation could not be evaluated. TC incubation, which did not modify enzyme release, did not alter EE 17 β -glucuronidation and differed from TUDC at the same concentration (TC: 91 \pm 10% of control activity; TUDC: 67 \pm 5%, $n = 3$, $p < 0.05$).

Inhibition Kinetic Studies. Kinetic studies performed in disrupted microsomes are shown in Fig. 5. TUDC inhibited 17 β -glucuronidation of EE. Kinetic analysis demonstrated a noncompetitive inhibition (K_i : 1.4 \pm 0.2 mM). Figure 6 shows the inhibitory effect of a fixed concentration of TC, TCDC, or TDC, in comparison with that of TUDC. None of the endogenous bile salt affected EE 17 β -glucuronidation.

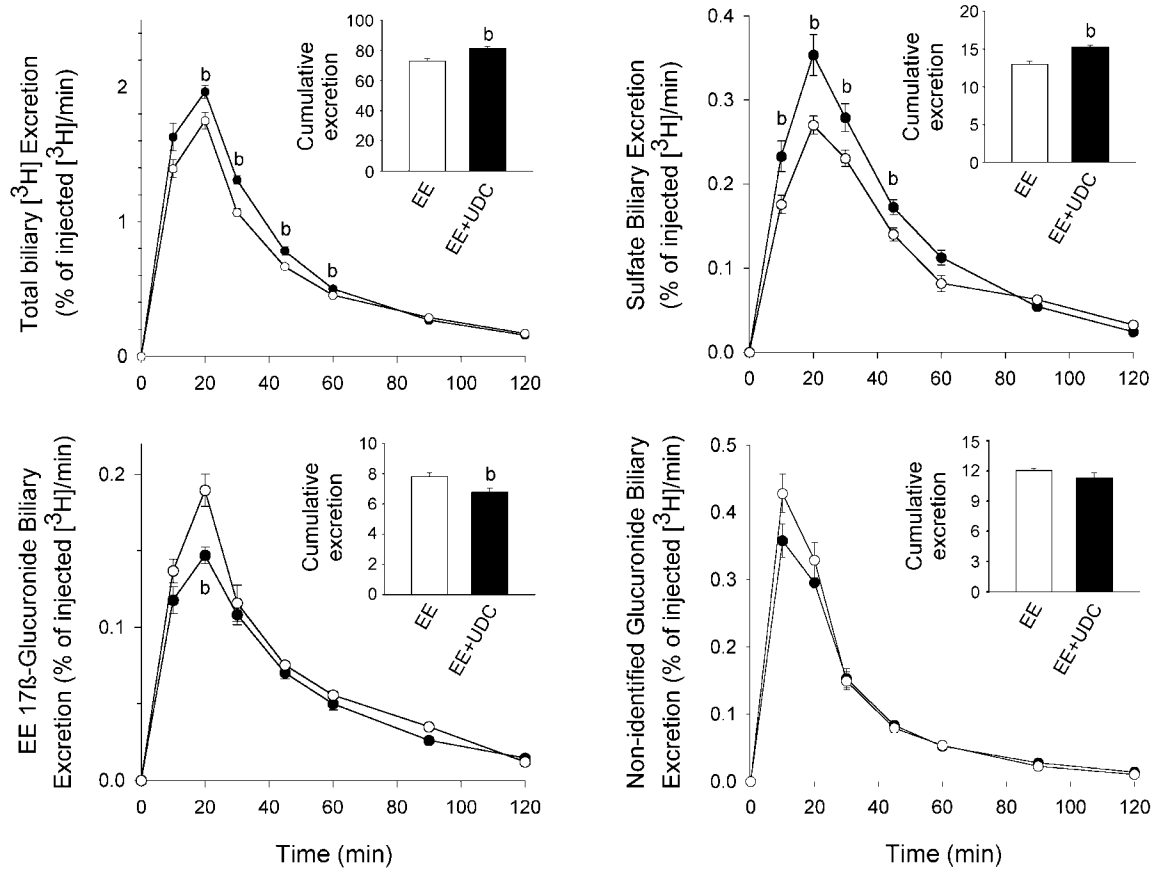


Fig. 1. Biliary excretion of EE metabolites after a trace dose injection of [³H]EE (10 μCi/kg b.wt.) in rats pretreated with either 5 mg/kg b.wt. of EE (open symbol) or EE and 25 mg/kg b.wt. of UDC (filled symbol). EE derivatives were identified by HPLC. Glucuronides and sulfates were identified by comparison with samples pretreated with β-glucuronidase or sulfatase plus β-glucuronidase; EE 17β-glucuronide was identified using a standard. HPLC profiles presented three main peaks of [³H]. One peak was identified as a sulfate and the other peaks as EE 17β-glucuronide and another unidentified glucuronide. Results are expressed as mean ± S.E. for four animals per group. The inset shows the cumulative biliary excretion of total ³H or the respective metabolite over the 120-min period expressed as the percentage of the injected dose. b, different from EE (*p* < 0.05)

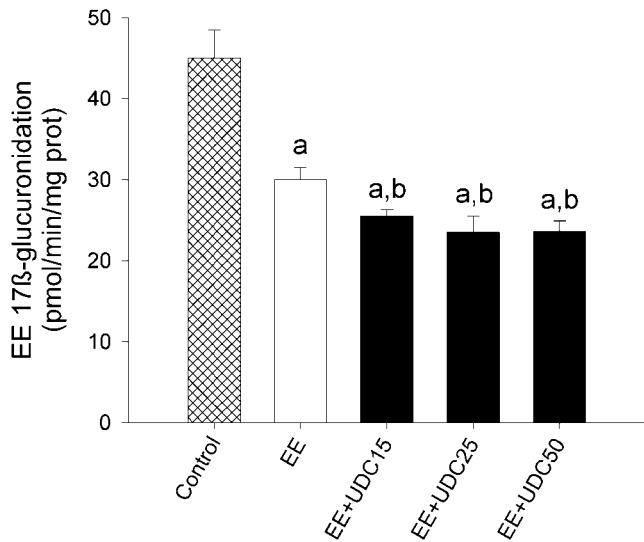


Fig. 2. Ethinylestradiol glucuronidation in hepatic microsomes from rats treated during 5 days with EE (5 mg/kg b.wt.), EE + UDC (15, 25, or 50 mg/kg b.wt.) or solvent (control). Enzyme activities were determined as described under *Materials and Methods* in the presence of the physiological activator UDP-*N*-AG (2 mM). Results are expressed as mean ± S.E. for 8 to 12 animals per group. a, different from control (*p* < 0.05); b, different from EE (*p* < 0.05).

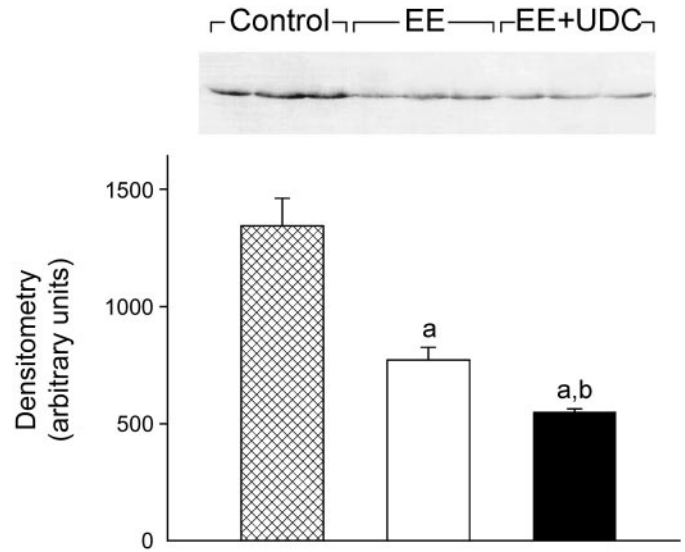


Fig. 3. UGT2B1 levels in hepatic microsomes from control rats (lanes 1–3), rats treated with EE (5 mg/kg b.wt., for 5 days) (lanes 4–6), and rats receiving EE and UDC (25 mg/kg b.wt., for 5 days) (lanes 7–9). Equal amounts of microsomal protein (15 μg) were loaded in all lanes. The densitometric analysis is presented as mean values ± S.E. of relative areas expressed in arbitrary units (*n* = 3). a, significantly different from control rats (*p* < 0.05); b, significantly different from EE rats (*p* < 0.05).

TABLE 3

Microsomal lipid composition

Lipids were analyzed in hepatic microsomes from rats receiving a 5-day treatment of EE (5 mg/kg b.wt.), EE + UDC (15, 25, or 50 mg/kg b.wt.), or vehicle in controls. Cholesterol and phospholipids were measured by spectrophotometry, as described under *Materials and Methods*. Methyl esters of the fatty acids were analyzed by gas liquid chromatography. Values indicate the relative content (percentage) of each individual fatty acid. Results are expressed as mean \pm S.E. of 4 animals/group. U.I. is the unsaturation index = $\sum n_i x_i / \text{FA}$, where n_i = number of double bonds in each fatty acid, x_i = moles of each fatty acid, and FA = total moles of fatty acid.

	Control	EE	EE + UDC15	EE + UDC25	EE + UDC50
Cholesterol/phospholipid ratio (mol/mol)	0.10 \pm 0.01	0.14 \pm 0.01 ^a	0.14 \pm 0.01 ^a	0.14 \pm 0.01 ^a	0.14 \pm 0.01 ^a
16:0	18.9 \pm 0.9	24.2 \pm 1.1	26.8 \pm 0.6	23.5 \pm 0.8	24.5 \pm 0.6
16:1	0.40 \pm 0.05	0.37 \pm 0.04	0.20 \pm 0.05	0.25 \pm 0.05	0.25 \pm 0.05
16:2	0.70 \pm 0.11	0.50 \pm 0.06	0.60 \pm 0.08	0.45 \pm 0.03	0.55 \pm 0.06
18:0	31.0 \pm 1.1	26.8 \pm 1.0	25.5 \pm 0.9	28.3 \pm 2.1	24.8 \pm 0.8
18:1	7.9 \pm 0.6	11.1 \pm 1.1	10.5 \pm 0.9	10.0 \pm 1.6	10.9 \pm 0.5
18:2	11.5 \pm 1.1	13.9 \pm 0.9	12.5 \pm 0.3	12.5 \pm 1.4	12.9 \pm 0.9
18:3	1.3 \pm 0.1	1.0 \pm 0.2	1.2 \pm 0.1	1.2 \pm 0.1	1.0 \pm 0.1
20:3	0.30 \pm 0.05	0.50 \pm 0.05	0.25 \pm 0.05	0.20 \pm 0.04	0.40 \pm 0.02
20:4	26.2 \pm 0.7	20.7 \pm 0.9	21.3 \pm 1.3	22.5 \pm 0.9	23.6 \pm 1.3
22:6	1.8 \pm 0.2	1.0 \pm 0.1	1.2 \pm 0.1	1.1 \pm 0.1	1.1 \pm 0.1
U.I.	1.53 \pm 0.04	1.33 \pm 0.04 ^a	1.34 \pm 0.03 ^a	1.37 \pm 0.05	1.43 \pm 0.03

^a Different from control group ($p < 0.05$).

^b Different from EE group ($p < 0.05$).

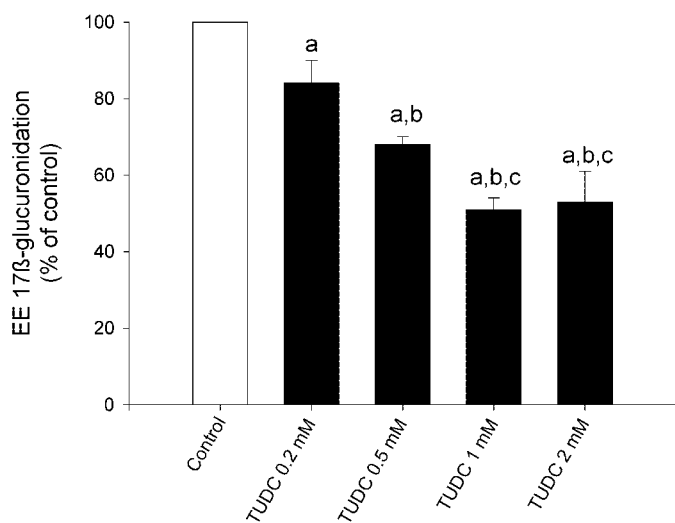


Fig. 4. Effect of different concentrations of tauroursodeoxycholate (TUDC) in ethinylestradiol (EE) 17 β -glucuronidation in isolated rat hepatocytes. Glucuronidation was measured according to the *Materials and Methods* in the presence of fixed concentration of EE (1 mM) and increasing concentrations of TUDC (0.2 to 2 mM). Each bar represents the mean value \pm S.E. in three to four preparations per group. a, different from control ($p < 0.05$); b, different from TUDC 0.2 mM ($p < 0.05$); c, different from TUDC 0.5 mM ($p < 0.05$).

Discussion

UDC is a bile salt commonly used in the treatment of cholestatic diseases (Poupon and Poupon, 1995). Several potential mechanisms of action have been proposed: protection against injury by hydrophobic bile salts, stimulation of secretion of hydrophobic bile salt and other hepatotoxins, immune modulation, stabilization of hepatocellular membranes, and hypercholerisis (Poupon and Poupon, 1995; Beuers et al., 1998). In this study, we present evidence that UDC decreases ethinylestradiol glucuronidation as a potential mechanism of protection against EE-induced cholestasis at a dosage that was also instrumental in preventing both the bile flow decrease and the bile salt excretion failure induced by the cholestatic agent.

The effect of UDC on EE glucuronidation was first evaluated in vivo by administering a trace dose of [³H]EE to EE and UDC-EE treated rats. UDC enhanced biliary excretion of

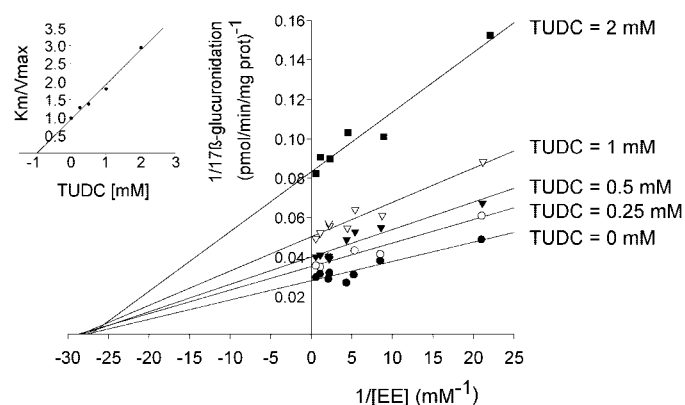


Fig. 5. Kinetic of inhibition of rat liver microsomal EE 17 β -conjugating activity by tauroursodeoxycholate (TUDC). Enzyme activity was assayed as described under *Materials and Methods* using digitonin-activated microsomes and increasing amounts of TUDC (0 to 2 mM) and EE (0.05 to 2 mM), whereas UDPGA concentration was kept constant (4.2 mM). Inset shows a secondary plot that illustrates the dependence of the slope (K_m/V_{max}) on inhibitor concentration. The data depicted represent a typical experiment and indicate the mean value of duplicate measurements in normal microsomes. Data of all experiments fitted with a nonlinear regression program resulted in V_{max} : 34 \pm 2 pmol/min/mg of protein; K_m : 35 \pm 7 μ M; K_i : 1.4 \pm 0.2 mM.

total EE derivatives, with an increase in sulfate conjugates and a decrease in EE 17 β -glucuronide, a potent cholestatic metabolite of EE (Vore, 1987). The increased excretion of sulfate compounds may be directly related with the improvement in the transport function of Mrp2, which transfers glutathione, glucuronide, and sulfate derivatives to bile. Indeed, it was reported that EE down-regulates Mrp2 expression at the canalicular level (Trauner et al., 1997), and that UDC up-regulates canalicular Mrp2 expression (Fickert et al., 2001) and its taurine derivative stimulates insertion of pre-existing pericanalicular vesicles containing Mrp2 into the canalicular domain (Beuers et al., 2001), which may help to increase the density of functionally competent Mrp2 in this membrane domain. Another explanation for the increased excretion of sulfate metabolites could result from the effect of UDC as a ligand for pregnane X receptor, the xenobiotic receptor that induces a number of cytochromes (Schuetz et al., 2001). This would result, sequentially, in an increase hydroxylation of compounds, an enhanced phase II metabolism, and an improved excretion by canalicular Mrp2.

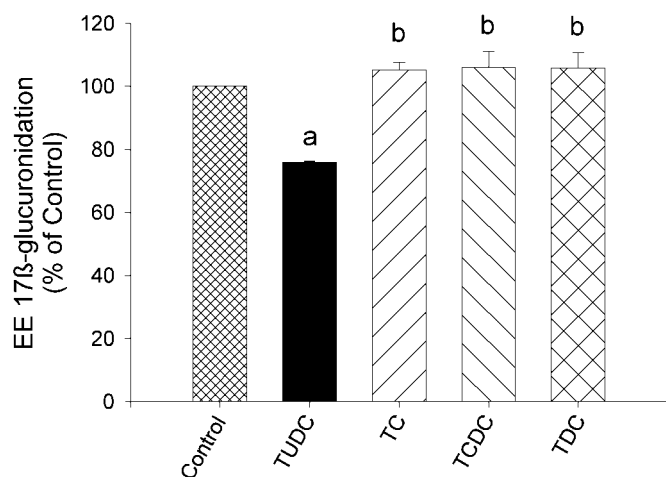


Fig. 6. Effect of a fixed concentration (0.9 mM) of TUDC, TC, TCDC, and TDC on ethinylestradiol 17 β -glucuronidation. Enzyme activity was assessed in digitonin-activated microsomes, the bile salts being added to the incubation mixtures just before ethinylestradiol. Each bar represents the mean value \pm S.E. in three preparations per group. a, different from control ($p < 0.05$); b, different from TUDC ($p < 0.05$).

Contrarily to sulfate, excretion of EE 17 β -glucuronide, which is also an Mrp2 substrate, was reduced rather than enhanced by UDC. This is indicative of a decreased availability of 17 β -glucuronide, due, in turn, to decreased synthesis. The reduction in the formation of glucurono-conjugates of the estrogen leaves more EE available for alternative metabolic pathways like sulfation, which in addition to an enhanced biliary transport, may help to detoxify EE. Indeed, sulfate conjugation leads to formation of noncholestatic derivatives (Slikker et al., 1983), and it is in line with increased biliary secretion of these kind of metabolites in response to UDC administration. The alteration in the excretion of other EE metabolites by UDC that could be relevant to the mechanism of toxicity of EE could not be ruled out since our results on biliary excretion of [3 H]EE left an important fraction of non-identified compounds.

Our results provide some evidences on the mechanism by which UDC impaired EE glucuronidation. UDC administration decreased microsomal EE 17 β -glucuronidating activity (about 20%) and decreased expression of UGT isoenzyme, UGT2B1 (28%). Since glucuronidation depends not only on the expression of the enzymes involved in the process but also on the features of the enzyme environment (Zakim and Dannenberg, 1992), lipid composition was also analyzed. Whereas EE increased cholesterol/phospholipid ratio and decreased the unsaturation index, UDC administration did not prevent these alterations, in accordance with a previous report from Bouchard et al. (1993). The lack of modification in the lipid composition indicates that the decrease in microsomal EE-glucuronidating activity mediated by UDC is most likely associated with decreased enzyme synthesis.

The possibility that TUDC, the major metabolite of UDC, exerts an acute and direct effect on EE glucuronidation, in addition to the chronically induced decrease in enzyme expression, was analyzed in microsomes. When TUDC was incubated with control microsomes, a noncompetitive inhibitory effect on EE 17 β -glucuronidation was evident; this effect was specific, as it was not shared by major endogenous bile salts. For TUDC to cause a direct inhibitory effect on microsomal UGT, the bile salt needs to reach the inhibition

site, which is likely located in the luminal side of the endoplasmic reticulum, like the catalytic site of the enzyme. This fact casts some doubts on whether this inhibitory effect can also be exerted in vivo. To overcome this question, we performed studies in isolated hepatocytes, which preserve the restrictive membrane barrier of the endoplasmic reticulum. Using this model, TUDC was able to inhibit EE glucuronidation, indicating that the bile salt can reach the inhibition site of UGT in the intact cell. Taken together, the evidence suggests that a direct inhibitory action of TUDC may be responsible, at least in part, for the decreased biliary secretion of EE 17 β -glucuronide observed in vivo. Unfortunately, from the current data it is not possible to establish to what extent UDC-induced decrease in expression of UGT or, alternatively, a direct inhibitory effect of its major metabolite, participate in decreasing the synthesis of the cholestatic metabolite.

It has been demonstrated that the excretion of the 17 β -glucuronide of estradiol, the major natural estrogen, is mediated by Mrp2 (Vore et al., 1997), and its interaction with this transporter is necessary to exert the cholestatic effect (Huang et al., 2000). Assuming that a similar interaction may occur between Mrp2 and EE 17 β -glucuronide, our finding that a reduced excretion of the cholestatic metabolite occurs in UDC-treated rats may explain the beneficial effect of this bile salt. These results are in accordance with prevention of estrogen-induced cholestasis by *S*-adenosylmethionine, which increases the formation of sulfate metabolites (Stramentinoli et al., 1981; Milkiewicz et al., 2001). These mechanisms of UDC and *S*-adenosylmethionine give additional support to the hypothesis that EE 17 β -glucuronide is the mediator of the cholestatic effect of EE. Meyers et al. (1980) demonstrated that estradiol 17 β -glucuronide-induced cholestasis in the intact rat is a dose-dependent process. In consequence, reduction in the synthesis of the cholestatic derivative of EE, although only partial, may attenuate cholestasis. The participation of other toxic metabolite of EE whose levels could be reduced by UDC cannot be ruled out in the light of our findings. Nevertheless, UDC prevention of EE cholestasis is not limited to decreased glucuronide formation since this bile salt has shown protective effects on acute cholestasis induced by estradiol 17 β -glucuronide in the rat (Kinbara et al., 1997; Milkiewicz et al., 2001).

In summary, UDC administration decreases biliary secretion of EE 17 β -glucuronide, possibly by reducing its intracellular availability, as a consequence of a decreased expression of UGT and/or a noncompetitive inhibition of the enzyme. These may explain, at least partially, the beneficial effect of UDC on EE-induced cholestasis.

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