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ENHANCED BILIARY EXCRETION OF CANALICULAR MEMBRANE ENZYMES IN ETHYNYLESTRADIOL-INDUCED CHOLESTASIS

EFFECTS OF URSODEOXYCHOLIC ACID ADMINISTRATION

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Abstract—Cholestasis is associated with a marked increase in the release of canalicular membrane enzymes into bile. This phenomenon has been related to an increased lability of these canalicular membrane integral proteins to the solubilizing effects of secreted bile salts. To further characterize the effects of oral ursodeoxycholic acid (UDCA) administration on ethynylestradiol (EE)-induced cholestasis, the influence of this bile acid on changes in biliary excretion of membrane-bound enzymes was investigated. Bile flow, basal bile salt and biliary lipid secretory rates, the maximum secretory rate of taurocholate (TC SRm), and the biliary excretion of the canalicular membrane-bound ectoenzymes alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (GGT) were measured in rats after EE and/or UDCA administration. The activities of ALP, GGT and Na^+,K^+-ATPase in purified isolated canalicular and sinusoidal membrane fractions and the ultrastructure of hepatic acinus, including histochemical studies of ALP distribution, were also examined. EE significantly reduced bile flow, bile salt and biliary lipid secretory rates, and TC SRm, and caused dilatation and loss of microvilli at the canalicular pole of hepatocytes. Biliary excretion of ALP increased 2-fold, whereas biliary excretion of GGT was unchanged. The relationship between biliary excretion of ALP or GGT and bile salt secretion (units of enzyme activity secreted per nanomole of bile salt) was greater in EE-treated rats compared with controls (2.1- and 1.5-fold greater for ALP and GGT, respectively), indicating that in EE-induced cholestasis more enzyme was released into bile per nanomole of bile salt. Na^+,K^+-ATPase activity in sinusoidal membrane fraction was reduced significantly, whereas ALP activity increased in both membrane fractions in EE-treated rats. The histochemical distribution of ALP in the acinus showed a strong reaction in acinar zone 3 and at both the canalicular and sinusoidal membranes. Oral administration of UDCA prevented EE-induced bile secretory failure by normalizing bile flow, bile salt and biliary phospholipid secretory rates, and TC SRm. UDCA also prevented the EE-induced changes in the biliary excretion of enzymes. On the contrary, UDCA did not modify either the enzyme activity in isolated membrane fractions or the morphological or ALP histochemical changes associated with EE administration. These data indicate that in EE-induced cholestasis changes occur at the canalicular membrane, enabling this portion of the plasma membrane to be more susceptible to the solubilizing effect of bile salt, and that oral administration of UDCA prevents bile secretory failure and changes in the biliary excretion of ALP and GGT in EE-treated rats.

Key words: cholestasis; bile acids; alkaline phosphatase; plasma membrane enzymes; ursodeoxycholic acid; γ-glutamyl transpeptidase

Recent studies from our laboratory performed in two different experimental models of cholestasis (bile duct-ligated rats and rats treated with EE§) have shown that these conditions are associated with a marked increase of canalicular membrane enzymes released into bile [1–3]. In addition, it was found that the biliary excretion of ALP and GGT is related, in part, to secreted bile salts and that more enzyme is secreted per nanomole of bile salt in cholestatic rats, particularly in EE-induced cholestasis, than in controls, suggesting an increased lability of these canalicular membrane integral proteins to the solubilizing effect of secreted bile salts [3]. Even though the meaning or importance of the excretion of canalicular membrane ectoenzymes into bile under normal conditions is unclear, it is likely that the observed increase in the excretion of ALP or GGT into the bile in cholestasis is an indication of membrane damage, particularly in EE-induced cholestasis in which this phenomenon may play a role in the pathogenesis and perpetuation of hepatic biliary secretory failure [1].

In recent years, UDCA has been used successfully in the treatment of cholestatic liver diseases, mainly primary biliary cirrhosis and sclerosing cholangitis [4, 5]. Its precise mechanism of action is unknown. However, it has been proposed that in cholestasis the accumulation of bile acids in the hepatocyte may have toxic effects, particularly on cellular membranes, and, since UDCA appears to lack the membrane toxicity of common bile acids, the replacement of the endogenous bile salt pool by UDCA could reduce the damage at this level. In addition, some experimental data suggest that UDCA has direct hepatoprotective properties [6–8].

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§ Abbreviations: EE, ethynylestradiol; ALP, alkaline phosphatase; GGT, γ-glutamyl transpeptidase; UDCA, ursodeoxycholic acid; and TC SRm, maximal secretory rate of taurocholate.

Experimental studies assessing the effects of UDCA in cholestasis have been carried out generally using models of cholestasis induced by infusion of hydrophobic bile acids, such as lithocholic acid or cholic acid [9, 10]. The preventive or therapeutic effects of UDCA on other experimental models of hepatocellular cholestasis and on obstructive cholestasis are controversial [11, 13]. Only a few studies have reported the effect of UDCA on bile secretory function in EE-induced cholestasis [14–16].

The present study was undertaken to further investigate whether oral UDCA administration affects the development of cholestasis in EE-treated rats and how this bile acid influences changes in biliary excretion of membrane-bound enzymes. In particular, we wanted to clarify how the potentially preventive or protective effects of UDCA on this model of cholestasis are related to changes in a specific bile secretory process, namely the biliary excretion of ALP and GGT, as representative membrane-bound enzymes. In addition, we examined (a) enzyme activities in isolated sinusoidal and canalicular membrane fractions, (b) the ultrastructure of hepatocytes using electron microscopy, and (c) the histochemical distribution of ALP in the hepatic acinus after UDCA and/or EE administration. Results indicate that UDCA prevented EE-induced bile secretory failure by normalizing bile flow, bile salt secretion, and TC SRm. Moreover, it prevented the increase in the biliary excretion of ALP and GGT per nanomole of bile salt observed after EE treatment. EE-induced changes in Na^+,K^+-ATPase and ALP activities in sinusoidal and canalicular membrane fractions, as well as morphological changes observed by electron microscopy, were not modified by UDCA administration.

MATERIALS AND METHODS

Materials

UDCA and taurocholic acid, sodium salt (both greater than 98% pure by HPLC), 3α-hydroxysteroid dehydrogenase and other organic chemicals were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. All inorganic products and solvents (from Merck, Darmstadt, Germany) were of the highest quality available.

Animals and treatments

Male Wistar rats (4–7 animals/group), weighing 200–250 g, were subjected to reverse light cycling for 2–3 weeks before use. The middle dark point was set at 10:00 a.m. During this period, the animals were fed ground food *ad lib.* that contained, in percentage (w/w), 64% carbohydrate, 18% protein, 5% lipid, 4% fiber and a vitamin–mineral mixture prepared according to the recommendations of the American Institute of Nutrition. Four experimental groups were studied: (1) "EE Group": received EE (3 mg/mL in propylene glycol) given subcutaneously in a single dose (9:00 a.m.) of 5 mg/kg daily for 3 days; (2) "UDCA Group": fed with UDCA in the diet (1%, w/w) for 4 days; (3) "UDCA-EE Group": received EE treatment and were fed with UDCA in the diet for 4 days, starting 1 day before the injection of EE. In this group, the diet was 1% (w/w) on day 1 and 3% (w/w) on days 2–4, during EE treatment, because this steroid induces marked anorexia and decrease of food intake by 50–60%; and (4) "Control group": treated with similar volumes of the solvent vehicle. UDCA was dissolved in chloroform, and then mixed with the diet; the solvent was evaporated at room temperature under a hood.

Experimental procedure

After treatment, rats were anesthetized with pentobarbital (4.8 mg/100 g body wt i.p.). Body temperature was then monitored continuously with a rectal probe and maintained at 37–37.5° with a heating lamp. The carotid artery was cannulated with PE-50 tubing (Clay Adams Inc., New York, NY, U.S.A.). Arterial blood samples were taken at 15-min intervals for 60 min and spun at 12,000 rpm for 4 min in an Eppendorf Microcentrifuge. The bile duct was cannulated with PE-10 tubing, and bile was collected in preweighed tubes for 90 min in 15-min aliquots. Serum and bile aliquots were stored at −20° until analyzed.

Bile flow was measured gravimetrically, and total biliary bile acids were quantitated by the 3α-hydroxysteroid dehydrogenase method [17, 18]. The biliary content of tauro-derivatives and glyco-derivatives of different bile acid species was estimated by HPLC, using appropriate standards as previously described [19].

Total biliary phospholipids were measured by the method of Baginski *et al.* [20]. Biliary cholesterol was quantitated by the enzymatic method of Allain *et al.* [21]. Enzymatic activity of ALP in serum and bile was measured by the method of Pekarthy *et al.* [22] modified by Keeffe *et al.* [23]. GGT activity was determined as described by Persijn and van der Slick [24].

TC SRm was determined as previously described [25, 26]. In brief, under pentobarbital anesthesia, a PE-10 cannula was placed into the proximal common bile duct. After two basal 10-min bile collection periods, sodium taurocholate dissolved in 0.9% NaCl was infused through the jugular vein catheter at stepwise-increasing rates: 2, 3, 4 and 6 μmol/min per rat, in 30-min periods, using a Braun™ pump. Bile was collected at 10-min intervals during the infusion periods. TC SRm was calculated as the mean of the three highest consecutive values of bile salt secretion and expressed as micromoles of bile salt per minute per gram of liver.

Morphological studies

The livers of different groups of rats treated identically to the groups previously described were excised and fixed for 90 min with 4% paraformaldehyde plus 1.5% glutaraldehyde in 0.1 M phosphate buffer. After washing overnight, the tissue was post-fixed for 90 min with 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.2), stained with 1% uranyl acetate, dehydrated in graded ethanol, and embedded in Epon [27].

To determine the location of hepatocytes in the liver acinus, semi-thin sections were stained with toluidine blue. Acinar zone 1 was estimated by the terminal portal vessels and zone 3 by the terminal hepatic vein [28]. Ultrathin sections were stained with 2% uranyl acetate and lead citrate and were examined with a Siemens™ Elmiskop 102 electron microscope at 80 kV.

Cell fractionation studies

Liver homogenate and canalicular and sinusoidal plasma membrane fractions were prepared from separate groups of rats (5–15 animals/group) by the method of Molitoris and Simon [29], modified by Rosario *et al.* [30]. In brief, liver slices were added to chilled buffer

(mannitol, 300 mmol/L; EGTA, 5 mmol/L; Tris-HCl, 18 mmol/L; and phenylmethylsulfonyl fluoride, 0.1 mmol/L; at pH 7.4). The slices were homogenized by use of a Polytron™ apparatus (Kinematica GmbH, Littau, Switzerland). The homogenate was centrifuged at 48,000 *g* for 30 min. The resulting pellet was resuspended in buffer and $MgCl_2$ (15 mmol/L); precipitation was followed by centrifugation for 15 min at 2445 *g*. The supernatant was centrifuged at 48,000 *g* for 30 min to obtain the canalicular membrane-enriched fraction. Sinusoidal membranes were isolated from the initial Mg^{2+} precipitation pellet on a discontinuous sucrose gradient using 41% (5 mL) and 37.5% (12 mL) as overlay layers in cellulose acetate tubes centrifuged at 88,000 *g* for 3 hr. The float layer was carefully harvested from the top of the gradient. Both fractions were washed and stored in 0.5 mL of 1 mM $NaHCO_3$ at −20°.

Na^+,K^+-ATPase activity was measured after freeze–thawing by the method of Ismail-Beigi and Edelman [31]. ALP and GGT were determined as described above. *N*-Acetylglucosaminidase and succinic dehydrogenase were measured by the methods of Scalera *et al.* [32] and Seubert [33], respectively. NADH-dehydrogenase was determined as described by Simon *et al.* [34]. Proteins were measured according to Lowry *et al.* [35], with bovine albumin as standard.

Histochemical studies

Liver slices (1–2 mm thick) were fixed in 4% paraformaldehyde and 1.5% glutaraldehyde in 0.2 M buffer phosphate (pH 7.4) at 2° for 4 hr. Frozen sections (20 mm thick) were obtained in a cryostat and histochemically stained for ALP activity according the calcium cobalt method of Gomori [36].

Statistics

All results are expressed as means ± SD. A two-tailed non-paired Student's *t*-test was used to compare differences between groups. Values were considered significantly different when the *P* value was equal to or less than 0.05.

RESULTS

Table 1 compares body and liver weight and various serum and bile secretion parameters in the different groups. EE administration for 3 days resulted in a significant decrease (9%) of body weight, while no significant changes in body and liver weight were observed in animals from UDCA or UDCA–EE groups. There were no significant differences in serum ALP activity between different groups, and serum GGT activity (not shown) was not detectable. The cholestatic effect of EE was characterized by a significant increase in serum bile acids and a significant reduction in basal bile flow and bile salt secretory rate. TC SRm decreased significantly (by 38%) compared with that of control rats, and biliary secretory rates of cholesterol and phospholipids were also reduced significantly. In the UDCA-EE group, some of the EE-induced changes were prevented. In fact, in this group bile flow, bile salt secretory rate, TC SRm and biliary secretion of phospholipids were not significantly different from those in control animals.

In rats treated with UDCA alone, serum bile acids, bile salt secretion and biliary secretory rate of phospholipids increased significantly, while serum activities of ALP and GGT, bile flow and biliary secretory rate of cholesterol were unchanged. Interestingly, TC SRm was increased significantly (by 39%) in this experimental group.

The biliary bile acid composition analysis confirmed that tauro-conjugated UDCA became the major bile acid in bile from both UDCA (70%) and UDCA-EE groups (73%), compared with 4.2 and 5.4% in controls and the EE group, respectively.

Table 1. Characterization of control and experimental groups

Parameter	Group			
	Control	EE	UDCA	UDCA-EE
Body weight (initial/final) (g)	220 ± 10.5/ 222 ± 13.2	225 ± 8.6/ 205.4 ± 6.2*	203 ± 6.5/ 203 ± 10.1	228 ± 12.5/ 214 ± 11.4
Liver weight (g)	7.9 ± 0.7	8.8 ± 1.1	7.8 ± 1.0	8.9 ± 0.7
Serum ALP ($U \cdot mL^{-1}$)	13.6 ± 3.0	17.7 ± 5.0	17.3 ± 4.2	14.8 ± 4.2
Serum bile acids ($\mu mol \cdot L^{-1}$)	2.3 ± 0.3	10.3 ± 1.0†	11.7 ± 4.5†	15.8 ± 8.7†
Bile flow ($\mu L \cdot min^{-1} \cdot g\ liver^{-1}$)	2.1 ± 0.4	1.36 ± 0.2†‡	2.16 ± 0.1	1.80 ± 0.08
Bile salt secretory rate ($\mu mol \cdot min^{-1} \cdot g\ liver^{-1}$)	0.091 ± 0.02	0.046 ± 0.01†	0.146 ± 0.02†	0.094 ± 0.01‡
TC SRm ($\mu mol \cdot min^{-1} \cdot g\ liver^{-1}$)	0.37 ± 0.06	0.23 ± 0.02†	0.52 ± 0.09†	0.33 ± 0.03‡
Biliary cholesterol secretory rate ($\mu mol \cdot min^{-1} \cdot g\ liver^{-1}$)	0.99 ± 0.5	0.22 ± 0.2†	0.76 ± 0.2	0.40 ± 0.1†
Biliary phospholipid secretory rate ($\mu mol \cdot min^{-1} \cdot g\ liver^{-1}$)	8.9 ± 1.9	6.4 ± 1.9†	11.1 ± 1.3†	8.8 ± 1.4

Values are means ± SD, N = 4–7 animals per group. Abbreviations: EE, ethynylestradiol; UDCA, ursodeoxycholic acid; ALP, alkaline phosphatase; and TC SRm, maximum secretory rate of taurocholate.

*·†·‡ Significant differences ($P < 0.05$) compared with initial body weight (*), with the control group (†) and with the UDCA group (‡).

Biliary excretion of canalicular enzymes

Table 2 shows the changes observed in biliary secretion of ALP and GGT in the different experimental groups. The biliary secretory rate of ALP was increased significantly after EE administration (83% increase), while GGT secretion was not affected, compared with the control group. When we analyzed the relationship between ALP and GGT secretion and bile salt secretion, the ratio (units of ALP or GGT secreted per nanomole of bile salts) for both enzymes was significantly higher in the EE group (115 and 58% increase for ALP and GGT, respectively). The ALP and GGT activity secreted into bile per nanomole of bile salt was used as an indicator of the lability of canalicular membrane to the solubilizing effect of secreted bile acids [3].

The increased biliary excretion of ALP observed in the EE group was not apparent in the UDCA–EE group, while GGT excretion was not different from the control value. On the other hand, the ratios between ALP and GGT and bile salt secretion, which were higher in cholestatic rats, decreased in the UDCA–EE group to values similar to those of controls.

The biliary excretion of canalicular enzymes was not significantly different in rats treated with UDCA alone compared with controls. Remarkably, the relationship between biliary excretion of either ALP or GGT and bile salt secretion was reduced significantly by 33 and 36%, respectively, in this experimental group (Table 2), compared with the control group.

Enzyme activities in liver membrane subfractions, histochemical distribution of ALP, and liver morphology in experimental groups

Table 3 shows the specific activities of enzymes located preferentially at the canalicular or sinusoidal domains of hepatocyte plasma membrane. EE treatment was followed by a significant reduction of Na^+,K^+-ATPase activity in both homogenate (27% decrease) and sinusoidal membrane fraction (47% decrease), whereas ALP activity increased in all fractions. No changes were observed in GGT activity in this group. UDCA administration did not prevent the EE-induced decrease of Na^+,K^+-ATPase activity observed in homogenate and sinusoidal membrane fraction. Moreover, the increased ALP activity observed in liver membrane fractions after EE treatment was not modified in the UDCA–EE group. In rats receiving UDCA alone, the activity of ALP in the canalicular membrane fraction was reduced significantly compared with control rats, while Na^+,K^+-ATPase activity was not modified. On the other hand, GGT activity was increased significantly in liver homogenate, as well as in both sinusoidal and canalicular membrane fractions, from UDCA and UDCA–EE groups. Enzyme histochemistry of EE-treated rat livers revealed a marked increase of the bile canalicular staining of ALP mainly in zone 1 compared with controls in which only few periportal canaliculi revealed a light positivity for ALP activity (Fig. 1). In addition, ALP activity was also increased in the basolateral area of zone 1 hepatocytes from the EE group. The histochemical distribution of ALP in hepatic acinus in the UDCA–EE group (Fig. 2) was similar to that observed in the EE group. In livers from the UDCA group (Fig. 2), ALP staining was similar to that observed in control rats but with a less intense staining of canaliculi, which is concordant with the reduced activity of this enzyme observed in the isolated canalicular membrane fraction.

Transmission electron microscopy of livers from experimental groups showed changes at the canalicular level in EE-treated rats characterized by dilatation and irregular shape of luminal surface with partial loss of microvilli (Fig. 3). The hepatocyte organelles appeared normal but a disarrangement of the tight junctional pattern with elongation and loss of sites of membrane fusion was observed (Fig. 3, inset). The changes were more pronounced in acinar zone 3 hepatocytes.

Livers from rats of the UDCA group (Fig. 4) showed some distortion of canaliculi in hepatocytes located at acinar zone 1, characterized by a dilated and tortuous lumen. The junctional complexes, as well as hepatocyte organelles, appeared normal.

Morphological studies of livers from the UDCA–EE group showed changes at the canalicular surface of hepatocytes similar to those observed in the EE group (Fig. 4). However, tight junctions were consistently more conserved, with several sites of focal membrane fusion in the former group.

DISCUSSION

Data reported in this paper confirm and expand results of previous reports regarding EE-induced cholestasis

Table 2. Biliary secretory rates of canalicular enzymes and their relationship with bile salt secretory rates in control, EE-, UDCA- and UDCA-EE-treated rats

Parameter	Group			
	Control	EE	UDCA	UDCA–EE
Biliary ALP secretory rate ($mU \cdot min^{-1} \cdot g\ liver^{-1}$)	2.24 ± 0.5	4.12 ± 0.9*	2.6 ± 0.1	2.58 ± 0.7
Biliary ALP/Bile salt secretory rate ($U \cdot nmol\ bile\ salt^{-1}$)	38.2 ± 12.3	82.4 ± 23.8*†	25.5 ± 4.8*	43.0 ± 17.4†
Biliary GGT secretory rate ($\mu U \cdot min^{-1} \cdot g\ liver^{-1}$)	10.5 ± 3.5	10 ± 3.0	11 ± 2.6	7.72 ± 1.1
Biliary GGT/Bile salt secretory rate ($mU \cdot nmol\ bile\ salt^{-1}$)	0.17 ± 0.05	0.27 ± 0.06*	0.11 ± 0.04*	0.13 ± 0.03

Values are means ± SD, N = 5–7 animals per group. Abbreviations: EE, ethynylestradiol; UDCA, ursodeoxycholic acid; ALP, alkaline phosphatase; and GGT, γ-glutamyl transpeptidase.

*·† Significant differences ($P < 0.05$) compared with the control group (*) and with the UDCA group (†).

Table 3. Enzymatic specific activities in homogenate and liver membrane fractions (sinusoidal and canalicular) from control and EE-, UDCA- or UDCA-EE-treated rats

	Na^+, K^+-ATPase (U/mg protein)			Alkaline phosphatase (U/mg protein)			γ-Glutamyl transpeptidase (U/mg protein)		
Group	Homogenate	Sinusoidal	Canalicular	Homogenate	Sinusoidal	Canalicular	Homogenate	Sinusoidal	Canalicular
Control	0.97 ± 0.23	18.8 ± 5.9 (19×)†	ND*	0.19 ± 0.04	1.8 ± 0.5 (9×)	4.8 ± 1.7 (25×)	6.6 ± 3.7	35.1 ± 19.1 (5×)	112.5 ± 68.7 (17×)
EE	0.71 ± 0.19‡	10.1 ± 3.4‡ (14×)	ND	0.42 ± 0.06‡	5.7 ± 1.2‡ (13×)	11.2 ± 4.1‡ (26×)	5.48 ± 0.9	36.9 ± 21.8 (0.4×)	90.8 ± 29 (16×)
UDCA	1.22 ± 0.5	15.5 ± 4.8 (14×)	6.7 ± 4.2 (5×)	0.21 ± 0.04	1.4 ± 0.6 (7×)	2.0 ± 0.9‡ (9×)	23.5 ± 8.1‡	99.7 ± 71‡ (8×)	207.3 ± 89.1‡ (13×)
UDCA-EE	0.63 ± 0.07‡	8.8 ± 1.0‡ (14×)	ND	0.43 ± 0.07‡	3.0 ± 0.4‡ (7×)	8.2 ± 1.0‡ (19×)	29.2 ± 0.9‡	72.9 ± 17.8‡ (2.5×)	223.6 ± 42.5‡ (7×)

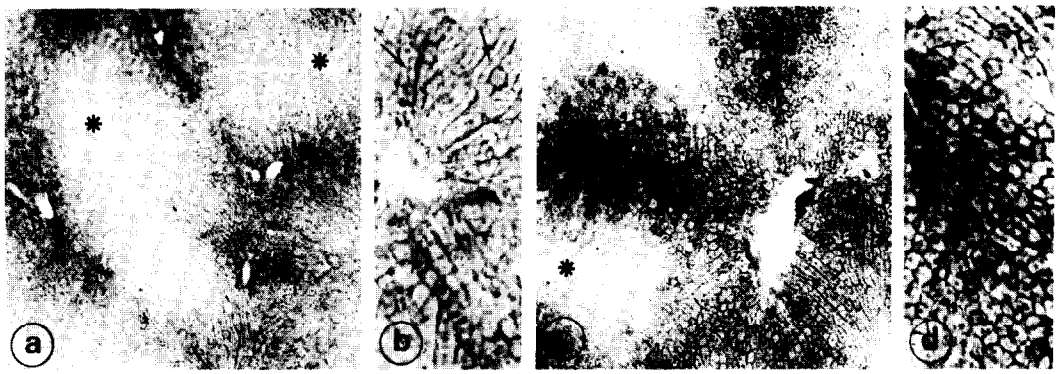

Fig. 1. Histochemical localization of alkaline phosphatase in frozen sections of liver from control and EE-treated rats. In the control rat (a), alkaline phosphatase reaction product was localized just at zone 1 of the hepatic acini; no reaction was observed in acinar zone 3 (*). At high magnification (b), the sinusoidal sides of acinar zone 1 hepatocytes appeared clearly outlined by the reaction product. Enzyme activity was also detected along the bile canaliculi (arrows) at the acinar zone 1. In the EE-treated rat (c), a marked increase in the intensity of reaction was observed extending from zone 1 toward the central vein (*). At high magnification (d), a strong staining reaction outlining both the sinusoidal face and the bile canaliculi (arrows) of the hepatocytes was clearly observed. Magnification: a and c: 260×; b and d: 875×.

and the effects of UDCA on the development of bile secretory failure [14–16]. Well-established alterations induced by EE such as a reduction of basal bile flow, bile salt secretory rate and TC SRm, as well as an increase of serum bile acid concentration and, marked reduction of the activity of sinusoidal membrane enzyme Na^+,K^+-ATPase [30, 37–39], were apparent in EE-treated animals. In addition, we observed a significant increase of the activity of ALP in sinusoidal and canalicular hepatocyte membrane fractions. Moreover, in contrast with previous reports, we found significant morphological changes after EE treatment characterized by dilatation of canalicular lumen and a decrease in the number and blunting and distortion of microvilli and changes in the histochemical distribution pattern of ALP in the hepatic acinus. These changes are similar to those described in obstructive cholestasis [40].

To have further insights into the characteristics of EE-induced cholestasis and assess the effects of UDCA in this condition, the biliary excretion of canalicular membrane-bound enzymes was studied. The mechanisms of release of these enzymes into bile, in normal and cholestatic conditions, are not clearly understood. The involvement of bile salts in the appearance of plasma membrane enzymes in bile, probably resulting from bile salt action on the canalicular membrane, is supported by studies performed in normal and cholestatic rats [3, 41], as well as by morphological and biochemical studies of human and rat bile in which canalicular membrane enzymes are preferentially associated with bile acid micelles in bile [42]. After EE administration, biliary excretion of ALP increased and GGT excretion into bile was unchanged. Using the ratio between biliary excretion of ALP or GGT and bile salt secretion, we found that it was significantly higher for both enzymes in cholestatic rats and that more enzyme was secreted per nanomole of bile acid. Despite a 50% decrease of bile salt secretory rate, the cholestatic phenomenon was associated with a 115 and 58% increase of ALP and GGT excretion per nanomole of bile salt, respectively. Similar findings were documented previously in studies performed in isolated perfused livers from normal and cholestatic rats [1, 2]. Collectively, these results support the hypothesis that increased lability of specific canalicular membrane proteins to the solubilizing effect of secreted bile acids may be a common alteration in cholestatic conditions.

The augmented biliary excretion of ALP after EE treatment could be related to an increase of ALP content at the canalicular domain of hepatocyte cell membrane. In addition to a previously described increase of ALP activity in homogenate and sinusoidal membrane fraction [30], we found a significant increase at the canalicular membrane in EE-induced cholestasis (Table 3). From our results we cannot clarify the mechanism involved in the increased ALP activity in membranes in EE-treated rats, but it can be hypothesized that increased synthesis of the enzyme can occur, similar to obstructive cholestasis. In this latter model, the increase of intracellular content of bile acids probably induces the synthesis of ALP [43, 44]. Increased ALP activity in the sinusoidal membrane fraction could also represent redistribution of the enzyme as it had been described in bile duct-ligated rats [45].

Changes in the biliary excretion of GGT in EE-induced cholestasis were apparent only when using the ratio GGT/bile salt secreted, and were quantitatively smaller than changes observed for biliary excretion of ALP. These differences could be explained by the different membrane attachment of GGT (hydrophobic transmembrane sequence) and ALP (phosphatidylinositol-glycan anchor) and the unchanged GGT activity in membrane fractions from EE-treated rats (Table 3). The increased excretion of GGT per nanomole of bile salt, like ALP changes, is also consistent with the hypothesis that a disturbance of canalicular membrane occurs in cholestasis, which makes that domain of hepatocyte plasma membrane more susceptible to the solubilizing effects of secreted bile acids.

Published information regarding the effects of UDCA on EE-induced cholestasis is sparse. Hillstrom *et al.* [14] reported a lack of benefit from UDCA administration in drug-induced cholestasis including EE, α-naphthyl-isothiocyanate and cyclosporine A. In the latter report, studies were carried out using 14 days of EE treatment, with high mortality of animals and using only Bromsul-

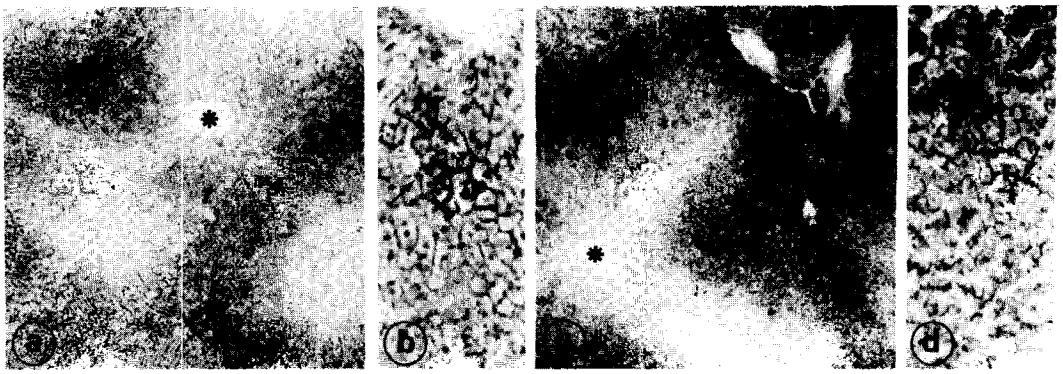

Fig. 2. Histochemical localization of alkaline phosphatase in frozen sections of liver from rats of the UDCA and UDCA–EE groups. The distribution of reaction product in the UDCA-treated rat (a) at the hepatic acini was similar to that of the control rat, but the acinar zone 1 showed a less intense staining (*, central vein). At high magnification (b), the reaction product was concentrated at the luminal surface of the bile canaliculi (arrows), and the sinusoidal face of the hepatocytes was poorly stained. In the rat from the Na^+,K^+–EE group (c), the distribution of the reaction product was similar to that observed in the EE-treated rat (*, central vein). At high magnification (d), the reaction product was highly concentrated at the bile canaliculi. Magnification: a and c: 260×; b and d: 875×.

phalein clearance as an indicator of bile secretory function. This approach might not be sufficient to conclude that UDCA administration did not prevent EE-induced cholestasis. More recently, Bouchard *et al.* [15] and Jacquemin *et al.* [16] reported that UDCA given orally to rats improved EE-induced cholestasis by amelioration of bile flow and bile salt secretion, in agreement with present data. Moreover, we found that the TC SRm was also normalized after UDCA administration, a previously unreported finding.

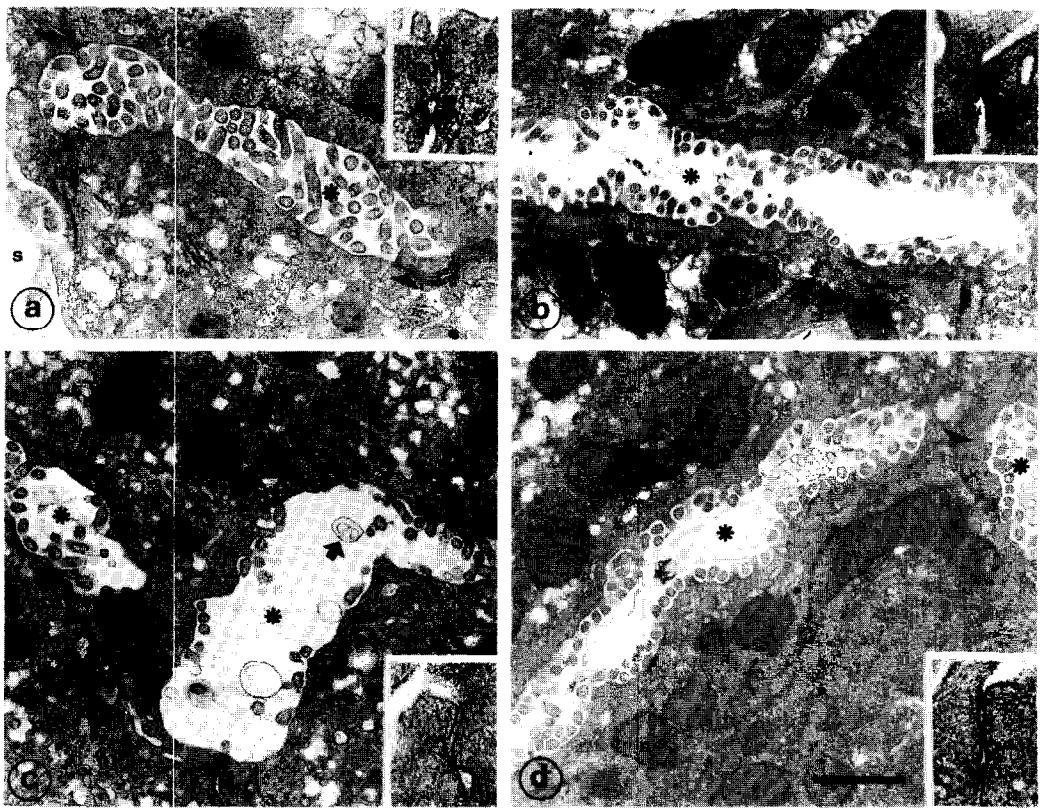

Fig. 3. Electron micrograph of bile canaliculi and pericanalicular cytoplasm in hepatocytes at acinar zones 3 and 1 from a control rat (a and b) and an EE-treated rat (c and d). The intercellular limits (arrowheads) and the structure of tight junctions (insets) are shown. At acinar zone 3, bile canaliculus (*) of the cholestatic rat exhibited a very dilated lumen and an irregular shape of the luminal surface with partial loss of microvilli; in addition, the tight junction did not present sites of membrane fusion (arrow: intercellular space). The changes at zone 1 were less pronounced. Scanty membranous material was found within the bile canaliculi lumen (arrow). Magnification: 15,000×. Bar = 1 μm; insets: 60,000×.

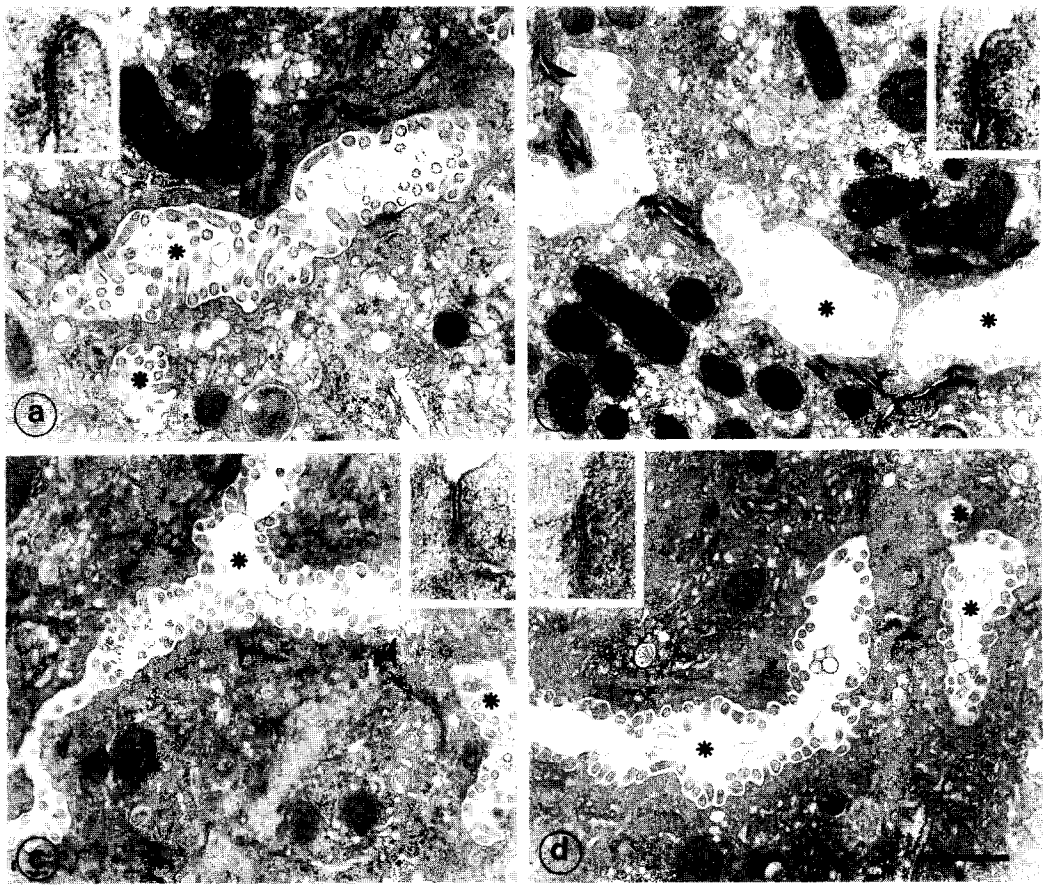

Fig. 4. Electron micrograph of bile canaliculi and pericanalicular cytoplasm in hepatocytes from rats of the UDCA and UDCA–EE groups. The intercellular limits (arrowheads) and the structure of tight junctions (insets) are shown. Sections through acinar zones 3 (a) and 1 (b) of a UDCA-treated rat showed a tortuous and slightly dilated lumen (*) with an irregular shape of the luminal surface and partial loss of microvilli, which appeared rather long. The tight junctions (arrow: intercellular space) showed several sites of membrane fusion. In a rat receiving EE and UDCA simultaneously, both acinar zone 3 (c) and 1 (d) showed bile canaliculi with a similar shape; however, few sites of fusion were observed in the tight junction of acinar zone 3 hepatocytes (c, inset). Magnification: 15,000×. Bar = 1 μm; insets: 60,000×.

The mechanism of improvement of EE-induced cholestasis by UDCA cannot be exactly determined from this study. However, our results raise some interesting points with regard to the protective effect of UDCA in this model of hepatocellular cholestasis. An improvement of canalicular excretory function, which is altered in EE-induced cholestasis [46], could be postulated since the most important effects of UDCA were the normalization of bile acid secretion and TC SRm. These effects could represent a protective action of UDCA against liver injury induced by bile acids, as previously described in acute experiments using the infusion of different bile acids [9]. A physicochemical mechanism resulting in stabilization of liver cell membranes by UDCA, as well as a direct effect within the canalicular lumen preventing membrane disruption, could explain the protective effect of UDCA [6, 47].

On the other hand, UDCA prevented the higher biliary excretion of ALP and GGT per nanomole of bile acids in cholestatic rats, which is also consistent with changes at the canalicular membrane [6]. In addition, because of its lower hydrophobicity, UDCA secretion is associated with less protein mobilization from membrane [1, 48]. Thus, replacement of the endogenous bile acid pool by UDCA could reduce the amount of enzyme being removed from the canalicular membrane. This interpretation agrees with previous evidence on bile acid-dependent secretion of ALP into bile, UDCA being weaker than more hydrophobic bile acids in removing ALP from membrane [41].

Morphological (ultrastructural and histochemical) alterations and changes in enzyme activity in liver plasma membrane fractions were not modified by UDCA treatment, indicating that not all EE-induced alterations are influenced similarly by this bile acid. Unchanged ALP histochemistry raises the possibility that intracellular events (i.e., delivery of ALP to the canalicular membrane) are not modified and that a direct effect of UDCA on hepatocyte plasma membrane occurs, as previously suggested [47]. On the other hand, improvement of bile flow and bile salt secretion observed after UDCA administration in the EE group did not correlate with the changes in plasma membrane enzyme activity. On these bases, and as previously suggested [48], it is likely that enzyme activity alterations do not have pathogenic significance in cholestasis. Indeed, the activity of Na^+,K^+-ATPase during EE cholestasis has been reported to be either decreased or normal [49, 50].

The effects of UDCA in normal rats are in agreement with findings from other authors [51]. UDCA became the major bile acid in bile, and its administration resulted in an increased basal bile salt secretory rate. Additionally, we found a significant increase of TC SRm after UDCA feeding, suggesting that bile acid pool expansion with UDCA also results in up-regulation of hepatic bile acid transport as it has been described after cholate (but not chenodeoxycholate or secondary bile acids) pool expansion [52]. With regard to ALP, its biliary excretion, its activity in canalicular membranes, and the intensity of histochemical staining at the canaliculi were decreased by UDCA. These effects could hypothetically be explained by UDCA-induced changes in physicochemical characteristics of canalicular membrane that modify lipid environment and enzyme activity.

UDCA administration increased GGT specific activity in homogenate and sinusoidal and canalicular liver plasma membrane fractions in both normal and EE-treated rats without a parallel increase in bile excretion of the enzyme. The mechanism of this effect is not clear, but it may be related to an increased synthesis of the enzyme by UDCA as well as to activation of GGT present at the canaliculi [53].

In the present study, we found some previously undescribed ultrastructural changes at the secretory pole of hepatocytes after UDCA treatment, namely distortion of canaliculi in hepatocytes located at the periportal zone, characterized by a dilated and tortuous lumen. It is generally accepted that UDCA is not hepatotoxic in rodents. Japanese studies (cited in Ref. 50) using larger doses of the bile acid failed to demonstrate toxicity. In one study [54], only proliferation of endoplasmic reticulum and peroxisomes was noted after a 1-week administration of UDCA. The importance and functional implications of the present findings remain to be elucidated.

In conclusion, this study demonstrated that in EE-induced cholestasis the biliary excretion of specific canalicular membrane-bound enzymes per nanomole of bile salts was increased significantly, suggesting that changes occur at the canalicular membrane making this portion of plasma membrane more susceptible to the solubilizing effect of bile salts. Oral administration of UDCA prevented EE-induced bile secretory failure and changes in the biliary excretion of ALP and GGT, suggesting an action of this bile acid at the canalicular membrane of the hepatocyte. Na^+,K^+-ATPase and ALP activities in sinusoidal and canalicular membrane fractions, as well as morphological changes observed by electron microscopy, remained unchanged after UDCA administration, indicating that not all EE-induced alterations are influenced similarly by UDCA.

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