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Research Article

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Ethinylestradiol Treatment Induces Multiple Canalicular Membrane Transport Alterations in Rat Liver

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Abstract

We investigated the effects of 17 α -ethinylestradiol treatment of rats on various transport functions in isolated basolateral and canalicular liver plasma membrane vesicles. Both membrane subfractions were purified to a similar degree from control and cholestatic livers. Although moderate membrane lipid alterations were predominantly observed in basolateral vesicles, no change in basolateral Na⁺/K⁺-ATPase activity was found. Furthermore, while Na⁺-dependent taurocholate uptake was decreased by ~40% in basolateral vesicles, the maximal velocity of ATP-dependent taurocholate transport was decreased by 63% in canalicular membranes. In contrast, only minimal changes or no changes at all were observed for electrogenic taurocholate transport in "cholestatic" canalicular membranes and total microsomes, respectively. However, canalicular vesicles from cholestatic livers also exhibited marked reductions in ATP-dependent transport of S-(2,4-dinitrophenyl)glutathione and in Na⁺-dependent uptake of adenosine, while in the same vesicles HCO₃⁻/SO₄²⁻ exchange and Na⁺/glycine cotransport activities were markedly stimulated. These data show that in addition to the previously demonstrated sinusoidal transport abnormalities ethinylestradiol-induced cholestasis is also associated with multiple canalicular membrane transport alterations in rat liver. Hence, functional transport alterations at both polar surface domains might ultimately be responsible for the inhibitory effects of estrogens on the organic anion excretory capacity and on bile formation in rat liver. (*J. Clin. Invest.* 1993. 91:2714-2720.) Key words: estrogens • cholestasis • membrane vesicles • bile acids • organic anions

Introduction

Estrogens are well known to cause reversible intrahepatic cholestasis in humans and animals. However, although estrogen-re-

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lated cholestasis has now been of major clinical and experimental interest for nearly four decades, it is still unclear by what pathogenetic mechanism(s) natural and synthetic (e.g., 17 α -ethinylestradiol [EE])¹ estrogenic compounds interfere with hepatic bile formation (1-3). Thus, based on more recent observations neither an increased permeability of the paracellular pathway (3, 4) nor a decreased activity of the sinusoidal (or basolateral) Na⁺K⁺-ATPase (3, 5, 6) appears to be primary event in EE-induced cholestasis. Similarly, the pathophysiological significance of the demonstrated decrease in sinusoidal membrane fluidity (6, 7) remains uncertain, since decreased liver plasma membrane fluidity has been associated with both reduced and increased bile flow (5, 8). On the other hand, there is general agreement in all studies that EE treatment of rats leads to a decreased bile excretory capacity of the liver for a variety of organic anions including conjugated bile salts, bilirubin and sulfobromophthalein (BSP) (1-3, 9, 10). Based on these observations it has been repeatedly suggested, but never directly tested, that an important cause of EE-induced cholestasis might be selective and/or multiple transport defects in the canalicular membrane of hepatocytes (1-3). This concept has been directly investigated in the present study using highly purified canalicular liver plasma membrane (cLPM) vesicles from control and EE-treated rats.

Methods

Animals and treatment. Male Sprague-Dawley rats (SUT:SDT, Süddeutsches Tierzuchtinstitut, Tutlingen, Germany) weighing 200-250 g were used throughout this study. The animals were housed in a constant temperature-humidity environment with alternating 12-h light (6:30 A.M. to 6:30 P.M.) and dark cycles. 17 α -Ethinylestradiol (Sigma Chemical Co., St. Louis, MO) was dissolved in 1,2-propanediol (10 mg/ml) and administered subcutaneously (5 mg/kg body wt.) between 7:30 and 9:30 A.M. for 5 consecutive d. Control animals received equivalent amounts of 1,2-propanediol only. The two groups of animals were pair-fed (690 diet; Nafag, Gossau, Switzerland) in order to adjust the weight gain of control animals to the decreased food intake of EE-treated rats. All animals had free access to water. On day 6 the animals were killed by decapitation between 7:30 and 8:30 A.M.

Cholestatic parameters. The degree of EE-induced cholestasis was estimated by the determinations in serum of conjugated primary bile acids (Becton-Dickinson, Orangeburg, NY), total cholesterol, total bilirubin, alkaline phosphatase, and leucine aminopeptidase (Department of Clinical Chemistry, University Hospital, Zürich). In addition, in some animals total bile flow was estimated by cannulation of the common bile duct (polyethylene 10 tubing) under pentobarbital (65 mg/kg body wt., i.p.) anesthesia. After 5 min bile was collected into tared

1. Abbreviations used in this paper: bLPM, basolateral (sinusoidal) liver plasma membrane vesicles; cLPM, canalicular liver plasma membrane vesicles; DIDS, 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid; DNP-SG, S-(2,4-dinitrophenyl) glutathione; EE, 17 α -ethinylestradiol.

tubes during two 5-min periods and the mean bile flow estimated gravimetrically assuming a bile solution density of 1.0.

Isolation of basolateral (bLPM) and canalicular (cLPM) liver plasma membrane subfractions. To be able to isolate highly purified bLPM and cLPM vesicles in parallel from control and EE-treated rats, a combination of two previously described subfractionation procedures (11, 12) was applied. All steps were done at 4°C. Three livers (25–40 g) from each group of animals were homogenized in ice-cold 1 mM NaHCO₃ adjusted to pH 7.4 with HCl and supplemented with 1 mM PMSF. After centrifugation at 1500 g for 15 min the “crude nuclear pellet” was resuspended in 30 ml of 0.25 M STM (0.25 M sucrose, 1 mM MgCl₂, 5 mM Tris HCl, pH 7.4). Subsequently 120 ml of 2.0 M STM was added and the suspension adjusted to a density of 1.18 g/cm³ (1.42 M, $n_D = 1.4016$) with 0.25 M STM. 30-ml aliquots of the sample were added to cellulose nitrate tubes and overlaid with 5 ml 0.25 M sucrose. After centrifugation for 60 min at 82,600 g (TST 28.38 rotor; Kontron Elektronik GmbH, Zürich, Switzerland), the membranes at the interface were collected with a plastic pasteur pipette, resuspended in 200 ml ice-cold 1 mM NaHCO₃, pH 7.4, and pelleted at 7500 g for 15 min. These “mixed” liver plasma membranes were washed once more in 200 ml of 1 mM NaHCO₃, pH 7.4, resedimented at 2700 g for 15 min, and finally resuspended in 8.0 ml of 0.25 M sucrose. The subsequent separation of bLPM and cLPM was performed as described (12). Microsomes were isolated according to Meier et al. (13). The isolated membrane subfractions were resuspended in the appropriate buffer media by repeated passages through a 25-gauge (0.5 mm) needle and the samples (> 5 mg protein/ml) stored in liquid nitrogen for up to 1 mo. without loss of transport activities.

Marker enzyme analysis. The degree of purification of bLPM and cLPM subfractions was estimated by the determination of marker enzyme activities such as Na⁺,K⁺ATPase (14) for basolateral plasma membranes; Mg⁺⁺-ATPase (14), alkaline phosphatase (15) and leucine aminopeptidase (16) for canalicular plasma membranes; succinate cytochrome *c* reductase (17) for mitochondria; NADPH cytochrome *c* reductase (17) for microsomes; glucosaminidase (18) for lysosomes; and galactosyltransferase (19) for Golgi membranes.

Chemical analysis. Total protein was determined by a modification of the Lowry procedure (20) using bovine serum albumin as a standard. DNA was measured in homogenates according to Labarca and

Paigen (21). Membrane lipids were extracted (22) and individual phospholipid species separated by a modification (23) of the high-performance thin-layer chromatography (HPTLC) method of Schmitz et al. (24) using an automatic Camag ATS III sample applicator and a Camag TLC scanner II (Muttentz, Switzerland). Free and esterified cholesterol were determined by an enzymatic fluorometric assay as described (25).

Vesicle transport studies. Tracer uptake studies were performed by routine rapid filtration assays (26). The following transport activities were determined according to established procedures. In bLPM: Na⁺-gradient dependent uptake of [³H]taurocholic acid (2.1–6.6 Ci/mmol; Du Pont-New England Nuclear, Boston, MA) (26). In cLPM: ATP-dependent uptake of [³H]taurocholate (27); 4,4-diisothiocyanato-2,2'-stilbenedisulfonic acid (DIDS)-sensitive electrogenic [³H]taurocholate uptake in the presence of an intravesicular positive K⁺-diffusion potential (26); HCO₃⁻/³⁵SO₄⁻-exchange (carrier-free [³⁵S]-sulfate was obtained from DuPont-New England Nuclear, Boston, MA) (28); Na⁺-dependent uptake of [2-³H]adenosine (27 Ci/mmol; Amersham International, Amersham, United Kingdom) (29); Na⁺-dependent uptake of [2-³H]glycine (43 Ci/mmol; DuPont-New England Nuclear) (30); and ATP-dependent uptake of the glutathione-conjugate S-(2,4-dinitrophenyl)-glutathione (DNP-SG) (31). The latter compound was synthesized in vitro from [¹⁴C(U)]-1-chloro-2,4-dinitrobenzene (CDNB; 10 mM Ci/mmol; Amersham International) as described (32).

In microsomes: Electrogenic [³H]taurocholate uptake in the presence of an intravesicular positive K⁺-diffusion potential (26). Further details and modifications of the indicated vesicle transport assays are given in the corresponding figure legends.

Results

The effects of EE treatment on various hepatobiliary parameters are summarized in Table I. While body wts were similar in control and treated animals, the liver weight increased in the treated group by 67%. Since at the same time the DNA content per unit liver wt remained unaltered, these data confirm induction of liver growth by EE (parenchymal hyperplasia) (33).

Table I. Basic Parameters of EE-induced Cholestasis in Male Sprague-Dawley Rats

	Day 0	Day 5	
	(before treatment)	Controls / EE treated	
Weights (g)	(n = 10)	(n = 11)/(n = 11)	
Body (body wt)	230±20	231±26/223±26	NS
Liver	11.0±1.4	8.3±1.2/13.9±2.3	P < 0.001
Bile flow	(n = 10)	(n = 10)/(n = 11)	
(μl/min × g liver)	2.0±0.3	2.1±0.4/0.7±0.2	P < 0.001
(μl/min × 100 g body wt.)	9.5±1.5	7.6±1.7/4.6±1.4	P < 0.001
Serum parameters	(n = 10)	(n = 13)/(n = 14)	
Bile acids (μmol/liter)	3.4±1.7	1.4±0.6/15.7±10.1	P < 0.001
Cholesterol (mmol/liter)	2.3±0.4	1.9±0.6/0.6±0.2	P < 0.001
Bilirubin (μmol/liter)	2.8±1.3	3.0±1.3/3.6±1.9	NS
Alkaline phosphatase (U/liter)	316±78	284±80/684±180	P < 0.001
Leucineaminopeptidase (U/liter)	50±8	50±8/47±7	NS
Liver homogenates	(n = 7)	(n = 13)/(n = 13)	
Protein (mg/g liver)	124±4	143±9/128±11	P < 0.001
DNA (mg/g liver)	2.5±0.4	2.7±0.3/2.5±0.3	NS

All data represent the means±SD with the number of measurements given in parenthesis. Statistical analysis was performed by the unpaired Student's *t* test.

The cholestatic effect of EE was documented by a 40% decrease of total bile flow (expressed per unit body wt.), an 11-fold increase of the serum bile acid concentration, a 2.4-fold increase of serum alkaline phosphatase activity, and a 70% decrease in serum cholesterol (Table I). No significant changes were observed in serum bilirubin and leucineaminopeptidase activity.

The degree of purification and the enzyme recoveries in the bLPM and cLPM subfractions isolated from control and cholestatic rat livers are compared in Table II. No significant differences in membrane associated Na⁺K⁺-ATPase activity were found between homogenates and LPM subfractions of normal and cholestatic livers, respectively. Among the canalicular marker enzyme activities alkaline phosphatase was increased eightfold in "cholestatic" homogenates while leucineaminopeptidase did not change. Although protein and canalicular enzyme recoveries were 30–50% lower in cLPM isolated from cholestatic as compared with control livers, the lower enzyme activities were not due to an increased contamination of "cholestatic" cLPM with bLPM or with intracellular organelles as documented by similar enrichment factors for Na⁺/K⁺-ATPase and for mitochondrial, endoplasmic reticulum, lysosomal, and Golgi marker enzyme activities (Table II). Thus, the cLPM subfraction could be purified to a similar degree from control and cholestatic rat livers.

With respect to the membrane lipid composition the greatest changes were found in bLPM vesicles, where EE treatment induced a fivefold increase in cholesteryl esters (control/cholestatic = 9±6/46±11 nmol per mg protein; means±SD; n = 8), but no changes in either free cholesterol (control/cholestatic = 0.17±0.03/0.18±0.05 μmol/mg protein) or total phospholipids (control/cholestatic = 0.67±0.10/0.62±0.08 μmol/mg protein). Among selective phospholipid species, phos-

phatidylethanolamine (PE) decreased by 16% and phosphatidylserine (PS) increased by 22%, while the relative contents of sphingomyelin (SPH), phosphatidylcholine (PC) and phosphatidylinositol (PI) remained unchanged. Thus, the ratios of PE/PC decreased by 15% while the SPH/PC ratio was not significantly altered. In cLPM, EE administration had no significant effects on either cholesteryl esters (control/cholestatic = 19±11/24±8 nmol/mg protein), free cholesterol (control/cholestatic = 0.59±0.06/0.63±0.08 μmol/mg protein) or total phospholipids (control/cholestatic = 1.19±0.19/1.22±0.19 μmol/mg protein). Furthermore, only the relative content of PS increased slightly (+18%), while the other polar membrane phospholipids (i.e., SPH, PC, PI, PE) and the derived PE/PC and SPH/PC ratios remained unaffected. These data confirm that pharmacological doses of EE mainly affect the lipid composition of the sinusoidal plasma membrane of rat hepatocytes (7), whereas the canalicular membrane lipid composition is only minimally altered.

Fig. 1 illustrates the effects of EE treatment on Na⁺- and ATP-dependent taurocholate uptake in bLPM and cLPM vesicles, respectively. In bLPM vesicles Na⁺-dependent taurocholate uptake was decreased by approximately 40%, thus confirming previous observations in intact hepatocytes (34). However, in "cholestatic" cLPM vesicles an even more dramatic decrease of over 60% was found for the recently described (27, 35–37) ATP-dependent taurocholate uptake portion (Fig. 1 B). No increase in ATP-dependent taurocholate uptake was observed in bLPM vesicles isolated in parallel from the same livers, indicating no redistribution of this bile salt transport system along the surface of hepatocytes as has been shown to occur for electrogenic taurocholate transport in obstructive cholestasis (38). Furthermore, the reduced canalicular ATP-dependent taurocholate uptake activity could be entirely ac-

Table II. Enzyme Enrichment and Percent Recovery in Basolateral (bLPM) and Canalicular (cLPM) Plasma Membrane Subfractions from Normal and Cholestatic Rat Liver*

	Homogenates (specific activity) [§]		Relative enrichment [‡] and (% Recovery)			
	Control	Cholestasis	bLPM		cLPM	
			Control	Cholestasis	Control	Cholestasis
Na ⁺ /K ⁺ -ATPase	1.2±0.3	0.9±0.2	30±4 (3%)	41±9 (3%)	3±2 (0.2%)	4±2 (0.2%)
Mg ⁺⁺ -ATPase	4.3±0.3	5.5±0.4	5±1 (0.6%)	7±1 (0.5%)	48±9 (3%)	25±7 (1.4%)
Alkaline phosphatase	0.2±0.1	1.6±0.3	12±3 (1%)	18±2 (1%)	101±9 (7%)	52±8 (3%)
Leucineaminopeptidase	1.0±0.1	1.0±0.1	3±1 (0.4%)	5±1 (0.4%)	30±3 (2%)	19±5 (1%)
Succinate-cyt.c-reductase	4.2±0.2	4.4±0.4	1.5±0.4	0.9±0.2	0.2±0.1	0.1±0.1
NADPH-cyt.c-reductase	3.3±0.5	2.8±0.4	0.4±0.1	0.5±0.1	0.5±0.1	0.7±0.2
Glucosaminidase	2.3±0.2	2.5±0.2	0.5±0.1	0.6±0.1	0.6±0.1	0.9±0.2
Galactosyltransferase	4.4±1.1	4.6±1.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1

* LPM subfractions were isolated after 5-d treatment with either 1,2-propanediol (controls) or ethinylestradiol (cholestatic). Recoveries of membrane protein (mg/g liver) were as follows. bLPM: controls 0.148±0.049; cholestasis 0.094±0.041 (no significant difference); cLPM: controls 0.101±0.029; cholestasis 0.070±0.035 (no significant difference).

[§] All data represent the means±SD of single measurements in 6 separate membrane preparations (n = 6). Specific activities are expressed as μmol product formed · mg⁻¹ protein · h⁻¹ except for galactosyltransferase (nmol · mg⁻¹ · h⁻¹).

[‡] Relative enrichment is defined as the ratio of specific activity in the LPM to specific activity in the homogenate.

^{||} Significantly different from controls (P < 0.05) as determined by the unpaired Student's t test.

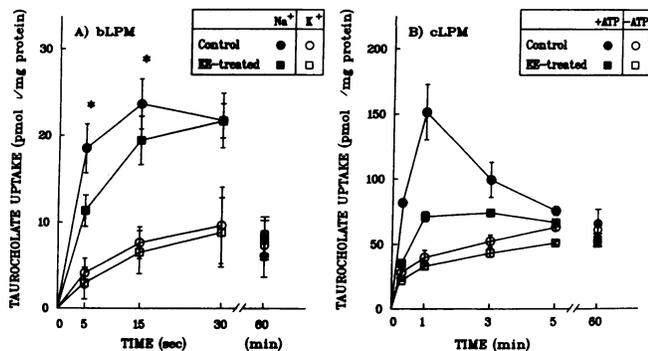


Figure 1. Effects of EE treatment of rats on Na^+ - and ATP-dependent uptake of taurocholate in bLPM and cLPM vesicles, respectively. (A) bLPM vesicles were resuspended (2.5 mg protein/ml) in 0.25 M sucrose, 0.2 mM CaCl_2 , 20 mM HEPES/Tris, pH 7.5 and [^3H]-taurocholate (1.0 μM) uptake determined at 37°C in the presence of outside 100 mM NaCl (●, ■) or KCl (○, □), 50 mM sucrose, 0.2 mM CaCl_2 , 5 mM MgCl_2 , and 20 mM HEPES/Tris, pH 7.5, as described (26). (B) cLPM vesicles were resuspended (2.5 mg protein/ml) in 100 mM KNO_3 , 50 mM sucrose, 10 mM HEPES/Tris, pH 7.5, and [^3H]-taurocholate (2.0 μM) uptake determined at 37°C in the presence (●, ■) and absence (○, □) of 5 mM ATP (Tris salt) in the incubation medium consisting of (final concentrations) 100 mM KNO_3 , 50 mM sucrose, 10 mM MgNO_3 and 10 mM HEPES/Tris, pH 7.5 (27). In all incubations the vesicle protein concentration was adjusted to 50 $\mu\text{g}/100 \mu\text{l}$. All data represent the means \pm SD of triplicate determinations in three separate membrane isolations. *Significant differences between control and “cholestatic” bLPM vesicles ($P < 0.01$).

counted for by a decreased maximal taurocholate transport capacity as demonstrated by a reduction of V_{max} values from 721 ± 50 pmol/mg protein \times min (mean \pm SD) in control cLPM to 264 ± 26 pmol/mg protein \times min in “cholestatic” cLPM (Fig. 2). The corresponding K_m values did not change significantly (control/cholestatic = $3.6 \pm 0.7 \mu\text{M}/5.3 \pm 1.2$

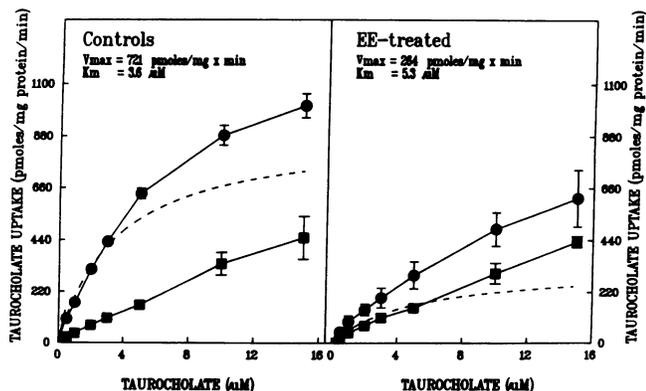


Figure 2. Kinetics of ATP-dependent taurocholate uptake in cLPM vesicles of controls (left) and EE-treated (right) rats. [^3H]-Taurocholate uptake was determined at 20 seconds as described in Fig. 1 B in the presence of increasing extravascular concentrations of unlabeled substrate. ATP-dependent uptake (dashed line) represents the difference between uptake values in the presence (●) and in the absence (■) of 5 mM ATP. The data are the means \pm SD of triplicate determinations in 2 separate membrane preparations. Estimation of the kinetic parameters was performed by a computer-based nonlinear regression analysis.

μM). In addition, the apparent K_m values for ATP were around 0.5 mM in both control (27) and “cholestatic” (data not shown) cLPM vesicles. Finally, estimation of liberated sialic acid in the absence and presence of 0.1% (vol/vol) Triton X-100 (39) revealed that both control and “cholestatic” cLPM vesicles were oriented inside out to 20–30% and right-side out to 70–80%. These figures correspond to our previous observations in normal cLPM vesicles (40) and exclude an altered sidedness of the cLPM vesicles as a possible cause of the observed EE-induced canalicular membrane transport alterations. Thus, EE administration to rats primarily reduced the number of functional ATP-dependent bile salt carriers at the canalicular membrane, while the bile salt affinity of the remaining transport systems was virtually unaffected.

In contrast to the marked reduction of the ATP-dependent bile salt transport capacity, EE administration had only a minimal or even no effect on DIDS sensitive, electrogenic (intravesicular positive K^+ diffusion potential) taurocholate uptake (41) in cLPM and microsomes, respectively (Fig. 3). However, EE treatment also markedly reduced cLPM-associated ATP-dependent transport of the glutathione conjugate DNP-SG (Fig. 4 A), a cosubstrate of the canalicular multiorganic anion transporter (42), as well as of Na^+ -dependent adenosine uptake (29) (Fig. 4 C). In contrast, and most surprisingly, canalicular $\text{HCO}_3^-/\text{SO}_4^-$ exchange (28) and Na^+ -dependent glycine uptake (30) were markedly stimulated in cLPM vesicles isolated from cholestatic rat livers (Fig. 4 B, D). These latter findings further argue against a higher contamination of “cholestatic” cLPM vesicles with intracellular membranes as a possible cause of the observed reductions in ATP-dependent organic anion transport activities. Thus, the data demonstrate that EE-induced cholestasis is associated with multiple inhibitory and stimulatory membrane transport alterations in rat liver.

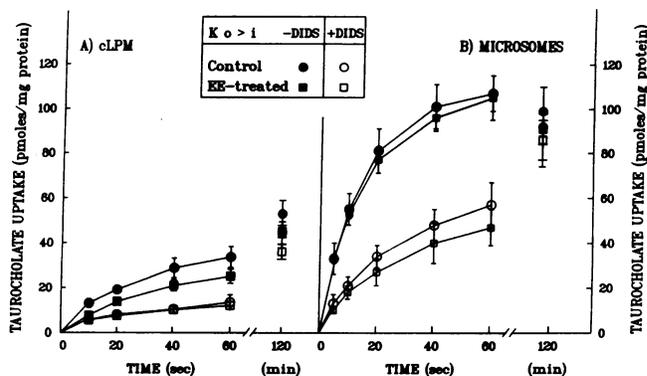


Figure 3. Electrogenic taurocholate uptake in cLPM vesicles (A) and microsomes (B) isolated from livers of control and EE-treated rats. cLPM vesicles and microsomes were resuspended (3.0 mg protein/ml) in 0.25 M sucrose, 0.2 mM Ca gluconate, and 20 mM HEPES/Tris, pH 7.4. All vesicles were preincubated with valinomycin (10 $\mu\text{g}/\text{mg}$ protein) for 5 min at room temperature. Uptake of [^3H]-taurocholate (2 μM) was performed at 25°C by incubating 20 μl vesicle suspension with 80 μl incubation medium consisting of (final concentrations) 100 mM Kgluconate, 50 mM sucrose, 0.2 mM Ca gluconate, 5 mM Mggluconate, and 20 mM HEPES/Tris, pH 7.4, as described (26). Where indicated, 150 μM of the anion transport inhibitor DIDS was also added. The data represent the means \pm SD of triplicate determinations in two separate membrane isolations. cLPM and microsomes were isolated in parallel from the same livers.

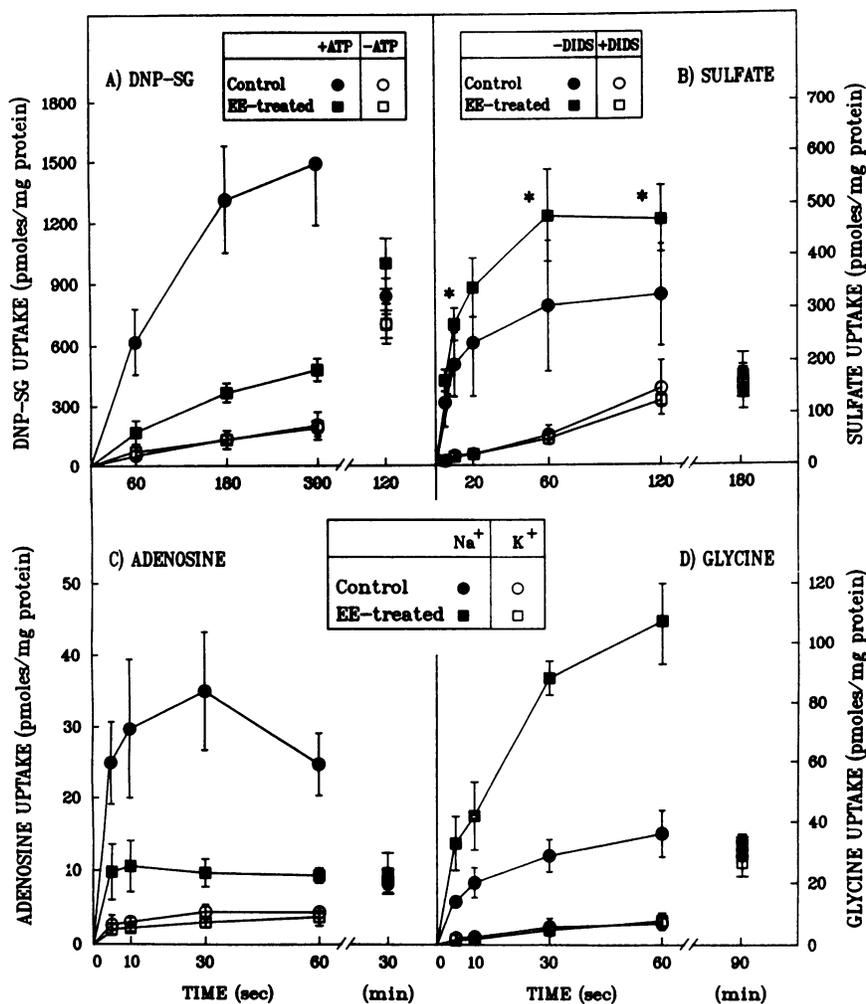


Figure 4. Effects of EE treatment of rats on four different non-bile-salt canalicular transport systems. (A) cLPM vesicles were resuspended (2.5 mg Protein/ml) in 0.25 M sucrose, 20 mM MgCl₂, 10 mM HEPES/Tris, pH 7.4, and preincubated with 100 μM Acivicin (inhibition of γ-glutamyltranspeptidase) at 25°C for 30 min. Uptake of ¹⁴C-labeled DNP-SG was performed by incubating 20 μl vesicle suspension at 37°C in 80 μl incubation medium containing (final concentrations) 100 μM [¹⁴C]DNP-SG, 0.25 M sucrose, 20 mM MgCl₂, 30 mM creatine phosphate, 200 units/ml creatine kinase, 10 mM Tris/HCl, pH 7.4, with (●, ■) or without (○, □) 1 mM ATP (31). (B) cLPM vesicles were resuspended (6.0 mg protein/ml) in 130 mM sucrose, 50 mM KHCO₃, 50 mM Kgluconate, 0.4 mM Cagluconate, 10 mM Mggluconate, 35 mM HEPES/Tris, pH 8.0. The vesicles were pretreated with valinomycin (10 μg/mg protein) and [³⁵S]-sulfate (2 × 10⁷ dpm/ml) uptake determined by incubating 10 μl vesicle suspension at 25°C in 90 μl of incubation medium containing 70 mM sucrose, 100 mM Kgluconate, 0.4 mM Cagluconate, 10 mM Mggluconate, 70 mM HEPES/Tris, pH 8.0 without (●, ■) and with (○, □) 0.5 mM DIDS (28). The outside sulfate concentration was adjusted to 100 μM with neutral K₂SO₄. (*) significantly different from controls (*P* < 0.01). (C, D) The vesicles were resuspended (2.5 mg protein/ml) in 0.25 M sucrose, 0.2 mM CaCl₂, 20 mM HEPES/Tris, pH 7.4. [³H]Adenosine (1 μM) and [³H]-glycine (10 μM) uptakes were determined at 25°C by incubating 20 μl membrane suspensions in 80 μl of incubation medium consisting of (final concentrations) 100 mM NaCl (●, ■) or 100 mM KCl (○, □), 50 mM sucrose,

0.2 mM CaCl₂, 5 mM MgCl₂ and 20 mM HEPES/Tris, pH 7.4 (29, 30). All data represent the means±SD of triplicate determinations in two separate membrane preparations.

Discussion

This study demonstrates that EE administration to male rats causes marked alterations of various transport activities in cLPM vesicles isolated from the cholestatic livers. The documented canalicular transport alterations include a 63% decrease of the ATP-dependent bile salt transport capacity (Fig. 2), a similar decrease of initial ATP-dependent dinitrophenylglutathione and of Na⁺-dependent adenosine uptake rates (Fig. 4) as well as a marked increase of HCO₃⁻/SO₄⁻-exchange and of Na⁺-dependent glycine uptake activities (Fig. 4). These functional transport alterations occurred without significant changes in the sidedness or in the lipid composition of the isolated canalicular membrane vesicles. Furthermore, although in bLPM vesicles similar changes in cholesteryl esters and polar phospholipids were found as previously described (7), the activity of basolateral Na⁺/K⁺-ATPase activity was not significantly altered (Table II), thus supporting the view that an impaired sinusoidal Na⁺/K⁺-ion pumping cannot account for the EE-induced decrease in canalicular bile flow and bile salt secretion (5, 6, 43, 44). Most likely the same is also true for the observed 40% decrease in Na⁺-dependent taurocho-

late uptake into bLPM vesicles (Fig. 1), since for conjugated bile salts the sinusoidal uptake capacity normally exceeds the maximal canalicular secretory capacity about sixfold (45). Thus, our findings do not support the concept that selective alterations in the sinusoidal surface of hepatocytes play a unique role in the pathogenesis of EE-induced intrahepatic cholestasis (7, 10). Rather, our data support, but do not yet prove, the alternative possibility that assigns the primary cause(s) of EE-induced bile secretory failure to the canalicular membrane domain of hepatocytes (2, 9, 46, 47).

Most importantly, this study indicates that a decreased canalicular ATP-dependent bile salt transport capacity is primarily responsible for the EE-induced impairment of bile salt secretion in intact liver (1–3, 44, 46). Consequently, this recently described canalicular bile salt transporter (27, 35–37) appears to play an important physiological role in the maintenance and regulation of bile salt dependent bile flow in normal liver. Although its exact relationship to the previously characterized potential sensitive bile salt secretory pathway (26, 39, 48) is not yet known, evidence has recently been provided that both electrogenic and ATP-dependent taurocholate transport might be mediated by the same or closely related canalicular transport

protein(s) (36). Since electrogenic taurocholate uptake was higher in microsomes than in cLPM vesicles and not or only minimally affected by EE administration (Fig. 3), this study indicates instead that both transport activities are mediated by different transport systems. Although this assumption as well as the exact cause of the EE-induced decrease in the canalicular ATP-dependent bile salt transport capacity require further investigation, the data strongly indicate that EE administration reduces the number of functionally active ATP-dependent bile salt carriers in the canalicular membrane either by reducing their biosynthesis and insertion into or by increasing their degradation and retrieval from the canalicular membrane (48).

Similar to impaired bile salt secretion, the estrogen-induced hepatic excretory failure for other organic anions such as bilirubin and bromosulfophthalein (BSP) (1–3) can also be explained by a canalicular transport defect as evidenced by the decreased ATP-dependent transport activity for DNP-SG (Fig. 4), which represents a typical cosubstrate (31) of the canalicular multiorganic anion transporter (42). Interestingly, the EE-induced decrease of both ATP-dependent transport activities (for taurocholate and DNP-SG) was associated with a similar decrease in Na⁺-dependent uptake of adenosine into cLPM vesicles (Fig. 4). Since ATP-dependent transport has been shown to be mediated by the inside-out-oriented portion of cLPM vesicles (35, 39), while Na⁺-dependent adenosine uptake relates to the right-side-out-oriented cLPM vesicles (29), the latter transport system is thought to be involved in the reuptake of nucleosides from the canalicular lumen back into the cells. Thus, a close functional interdependence between the intracanalicular availability of nucleotides, their degradation by the canalicular ecto-ATPase (49), and the reuptake of their degradation products (nucleosides) appears to exist in normal liver.

Finally, our data demonstrate that EE treatment of rats not only decreases, but also can stimulate certain canalicular transport activities (Fig. 4). The pathophysiologic significance of the increased HCO₃⁻/SO₄⁻-exchange activity cannot be easily judged since the physiological role of this anion exchange system is not yet known (28, 50). However, the increased Na⁺-dependent glycine uptake activity is reminiscent of the previously reported increase in basolateral alanine uptake in mid-pregnant rats (51). This latter observation was supported in the present study by the finding of a fivefold increase in Na⁺-dependent alanine uptake into bLPM vesicles isolated from EE-treated as compared with control rats (data not shown). Thus, increased reabsorption of amino acids from both portal blood plasma and the canalicular lumen (30, 52) represents a further compensatory mechanism for the estrogen-induced increase in the hepatocellular protein and amino acid turnover and metabolism (51). Since glycine represents a degradation product of reduced glutathione (GSH), the canalicular secretion and intrabiliary hydrolysis of which have been shown to significantly contribute to the generation of canalicular bile salt independent bile flow (53), increased canalicular reabsorption of amino acid constituents of GSH could also significantly contribute to the well-described decrease in the bile salt independent fraction of bile flow in estrogen-induced cholestasis (1–3, 43, 46).

In conclusion, this study provides the first direct evidence that estrogen-induced cholestasis is associated with multiple

canalicular in addition to the previously demonstrated sinusoidal membrane transport alterations in rat liver. Although the exact molecular mechanisms of the observed canalicular membrane transport alterations require further investigations, the findings suggest that most EE-related bile secretory defects might primarily originate from inhibition of excretory and stimulation of reabsorptive mechanisms at the canalicular pole of hepatocytes.

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