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REVERSAL OF ETHINYLESTRADIOL-INDUCED CHOLESTASIS BY EPOMEDIOL IN RAT

THE ROLE OF LIVER PLASMA-MEMBRANE FLUIDITY

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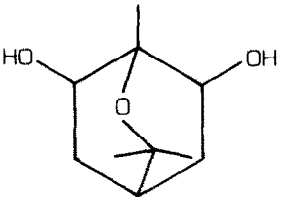
Abstract—Epomediol (EPO) is a synthetic terpenoid compound shown to be active in increasing bile flow and some enzymatic activities of liver plasma membranes in the rat. The possible effect of EPO treatment in the ethinyl-estradiol (EE) induced cholestasis in the rat was investigated by measuring the hepatic transport of sulfobromophthalein (BSP) (plasma clearance and biliary secretion) and bile flow. Liver plasma membrane fluidity was also determined by the steady state fluoresence polarization (P) of diphenylhexatriene (DPH). EE administration (5 mg/kg s.c. for 5 days) was followed by a significant, comparable reduction ($P < 0.001$) in BSP plasma clearance and biliary excretion and in bile flow. Intraperitoneal administration of EPO (100 mg/kg) to EE-treated rats restored both parameters of BSP transport, as well as bile flow, to control values. Liver plasma membrane fluidity was markedly ($P < 0.01$) decreased by EE administration with a concomitant reduction ($P < 0.01$) in Na^+/K^+-ATPase activity. EPO administration significantly increased membrane fluidity to values higher either to cholestatic ($P < 0.05$) or control ($P < 0.05$) animals. On the contrary, EPO did not influence Na^+/K^+-ATPase activity in either EE-treated or control animals. These data indicate that EPO fully reverses the impairments of BSP transport and bile flow induced by EE, possibly by reversing the decrease in liver plasma membrane fluidity induced by the synthetic estrogen. On the contrary, the EE-mediated decrease in Na^+/K^+-ATPase activity was not reversed by EPO.

A number of pharmacological agents including estrogens, anabolic steroids and phenothiazines [1], cause intrahepatic cholestasis in humans and animals by interfering with bile salt-dependent and/or -independent components of bile flow. 17α-ethinyl-estradiol (EE), a sythetic estrogen, produces bile secretory failure characterized by decrease in the bile salt independent flow [2–4] and a reduced capacity to excrete organic anions such as bile acids [5], bilirubin and BSP [6, 7].

The mechanism of the cholestatic effect of EE apparently resides mainly in an increased rigidity of sinusoidal liver plasma membrane as demonstrated by changes in electron spin resonance and fluorescence polarization of the membrane [8, 9]. The increased viscosity was found to be associated to an increase in cholesterol and/or cholesterol esters ratio in liver plasma membrane [8, 9]. Interestingly, administration of the detergent Triton WR 1339 reverses the cholestatic effect of EE restoring the membrane fluidity and lipid composition to control values [4]. Changes in membrane fluidity has been claimed to influence the activity of certain liver plasma membrane enzymes such as Na^+/K^+-ATPase, 5′-nucleotidase and adenylate cyclase as well as the permeability characteristics of the membrane [9, 10]. The activity of Na^+/K^+-ATPase during EE cholestasis has been reported to be either decreased [4, 12–14], or normal [11].



Epomediol

Fig. 1. Molecular structure of epomediol. Molecular weight = 186.

Epomediol (1,3,3-trimethyl-2-oxabicyclo(2.2.2.) octan-6,7-endo,endo-diol) (Fig. 1) is a synthetic terpenoid compound that is reported to have choleretic effects in humans and animals [15, 16]. This action has been demonstrated to be related to an increase in both the bile salt-dependent and -independent fractions of bile flow [16]. *In vitro*, addition of EPO to isolated liver plasma membrane fractions stimulated the activities of Ca^{2+}/Mg^{2+}- and Ca^{2+}-ATPases [17]. This suggests that EPO might reverse EE-cholestasis, which is an animal model for the estrogen-related cholestasis of pregnancy in humans. We have, therefore, tested the effects of EPO on the hepatic transport and biliary excretion of BSP, on bile flow and on liver plasma membrane fluidity in rats with EE-induced cholestasis and in control rats. The results suggest that EPO is effective in EE-

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induced cholestasis, probably by reversing the increased plasma membrane rigidity.

MATERIALS AND METHODS

Animals and treatment. Male Wistar rats (five animals per group) weighing 250–300 g, were maintained at standard laboratory chow (GLP, Altromin-Rieper, Bolzano, Italy). 17α-Ethinylestradiol (EE) was obtained from Sigma Chemicals Co. (St Louis, MO). Animals were given EE (5 mg/ml in propylene glycol) subcutaneously in a single dose of 5 mg/kg daily for 5 days. EPO was obtained, as pure powder, from Camillo Corvi Spa (Piacenza, Italy). Animals received EPO (100 mg/ml in 0.85% saline) intraperitoneally in a single dose of 100 mg/kg daily for 5 days. Rats treated with both EE and EPO received separate injections of the two drugs at the same time each day. In a separate group of animals, EE was administered for 3 days before administration for an additional 5 days of EE and EPO according to the previous scheme. Control animals were treated with corresponding volumes of the appropriate solvent vehicle(s).

BSP plasma clearance and biliary excretion. After 5 days of treatment, each rat was anesthetized with pentobarbital, 50 mg/kg body wt intraperitoneally. Body temperature was then continuously monitored with a deep rectal lobe and maintained at 37–37.5° with an infrared lamp. The right jugular vein and carotid artery were cannulated with PE-50 tubing (Clay Adams, Division of Becton, Dickinson and Co., Parsipanny, NJ). The bile duct was cannulated with PE-10 tubing (Clay Adams) and bile collected for 10 min before injection of BSP. Bile was then collected in preweighed tubes every 5 min for 60 min.

BSP (Merck AG, Darmstadt, F.R.G.), 15 μmol/2.0 ml per kg body wt, was injected through the jugular vein and arterial samples were collected into 1.5 ml polyethylene tubes (LP, Milano, Italy) containing 5 U of heparin. Twelve 100 μl samples were collected at 15 sec intervals from 15 and 180 sec after BSP injection.

Arterial samples were spun at 12,000 rpm for 4 min in an Eppendorf centrifuge (model 5412). BSP was then spectrophotometrically determined at 580 nm after addition of 50 μl of plasma or 5 μl of bile to 0.5 ml of NaOH 1 M (BSP extinction coefficient 64/μmol/cm^2 at pH 13). The serial plasma BSP concentrations were plotted on a semilogarithmic scale and the fractional initial disappearance rate of BSP from plasma, k_1 (/min), determined by a least squares fit. The plasma BSP clearance (V_{BSP}) was calculated according to the equation:

$$V_{BSP}\ (\mu mol/min/kg) = k_1(/min) \times dose\ (\mu mol/kg).$$

Liver plasma membrane fluidity. Liver plasma membrane were prepared from separate, comparably-treated, groups of rats according to Ray [18] and stored in liquid nitrogen until use (within 3 weeks). The final pellet was resuspended in 20 mM Tris–HCl pH 7.5 in 150 mM NaCl, and the protein concentration was determined according to Lowry *et al.* [19]. Membrane fluidity of these preparations was determined by fluorescence polarization as described by Shinitzky and coworkers [20] using a spectrofluorometer JASCO FP-770. The probe (1,6-diphenyl-1,3,5-hexatriene, DPH, Eastman Kodak Co., Rochester, NY) was dissolved in tetrahydrofuran to a final concentration of 1 mM (stock solution), kept at −20° and diluted 1:1000 with 20 mM sodium phosphate buffer in saline solution, pH 7.5 immediately before use. Each assay was performed at 37° with 100 μg membrane protein. Samples were excited at 350 nm and the fluorescent emission recorded at 450 nm. The fluorescence intensity was measured perpendicular (I_t) and parallel (I_p) to the polarization phase of the exciting light. The steady-state polarization (P) is calculated according to the ratio:

$$P = (I_p - I_t)/(I_p + I_t).$$

Enzymatic activity assay. Na^+/K^+-ATPase activity in the plasma membrane fractions was measured according to Schoner *et al.* [21]. The degree of purity of liver plasma membrane preparations was assayed by determining the enzymatic activity of 5′-nucleotidase at pH 7.5 [22] and glucose-6-phosphatase [23] at pH 6.5. The release of inorganic phosphate was performed by the method of Widnell *et al.* [24].

Statistics. All results are expressed as mean ± SD. A two-tailed Student's *t*-test was used to compare differences between components examined. Values were considered significantly different when the P value was less than 0.05.

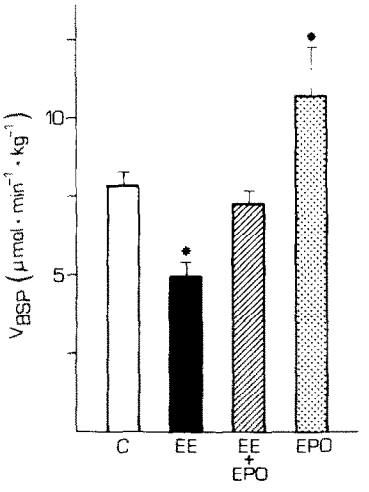

Fig. 2. Plasma disappearance rate (V_{BSP}, μmol BSP/min/kg) in control rats and in animals treated with 17α-ethynilestradiol (EE), epomediol (EPO) and with the combined treatment (EE–EPO). For details see text. Data are expressed as mean ± SD of five animals for each group. (*) $P < 0.005$ vs control and EE–EPO treated rats.

RESULTS

Figure 2 shows the BSP plasma clearance (V_{BSP}) in the four groups of animals. EE administration resulted in a significant (mean 40%) decrease in V_{BSP} compared to controls (4.92 ± 0.44 vs 7.74 ± 0.46 μmol/min/kg, $P < 0.005$). Administration of EPO to animals receiving EE restored V_{BSP} to values comparable to control rats (7.31 ± 0.39 vs 7.74 ± 0.46 μmol/min/kg, NS). EPO treatment of

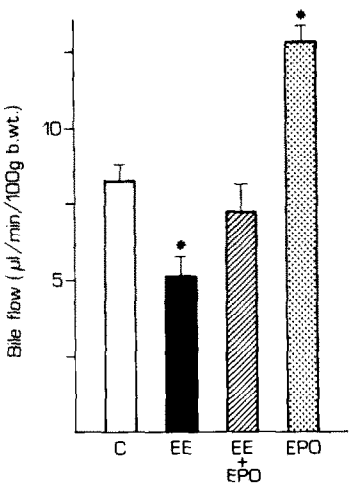

Fig. 3. Bile flow (μl/min/100 g) in the different groups of rats (see Fig. 2 for explanations). (*) $P < 0.005$ vs controls.

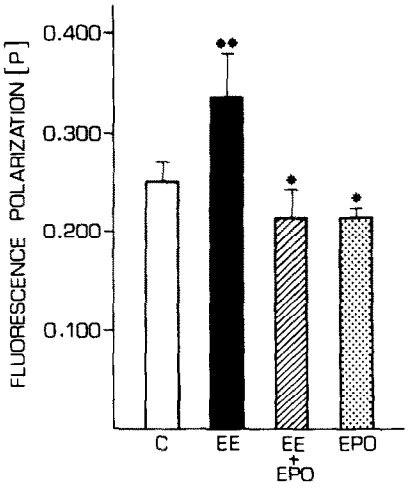

Fig. 4. Liver plasma membrane fluidity (P) as derived for the fluorescence polarization of diphenylhexatriene (DPH). Data are obtained as indicated in Materials and Methods and are expressed as mean ± SD of five different preparations of liver plasma membranes in each group of rats. (**) $P < 0.01$ vs controls; (*) $P < 0.05$ vs controls.

control rats resulted in a significant increase (10.7 ± 1.6 μmol/min/kg, $P < 0.005$) in V_{BSP} when compared either to control or EE–EPO treated rats. In three rats, EE (5 mg/kg) was administered for 3 days before starting EPO and EE administration as reported in Materials and Methods. BSP plasma clearance did not significantly differ from controls (7.47 ± 0.51 μmol/min/kg).

Figure 3 shows the bile flow in the various groups of animals. EE administration led to a significant reduction in bile flow (5.40 ± 0.53 vs 8.23 ± 0.57 μl/min/100 g, $P < 0.005$). Concomitant administration of EPO to EE-treated rats restored bile flow to control values (7.26 ± 0.91 vs 8.23 ± 0.53 μl/min/100 g, NS). EPO administration to control rats resulted in a significant increase in bile flow (12.81 ± 0.53 vs 8.23 ± 0.37 μl/min/100 g, $P < 0.005$). In EE-treated rats, the 60 min biliary recovery of BSP was markedly reduced from control values (68 ± 2 vs 96 ± 3%, $P < 0.005$), but was restored to control values by concomitant EPO administration (93 ± 1 vs 96 ± 3%, NS). In the bile of EPO treated rats 98 ± 2% of injected BSP was recovered. In rats pretreated for 3 days with EE and with EE and EPO for an additional 5 days, both bile flow and biliary recovery of BSP did not show significant difference as compared to controls (8.03 ± 0.61 μl/min/100 g and 94 ± 4%, respectively).

Figure 4 shows the steady-state fluorescence polarization values (P), which are inversely related to membrane fluidity, in liver plasma membrane fractions isolated from the different groups of animals. EE treatment decreased membrane fluidity, as indicated by a significant increase in the P value (0.336 ± 0.049 vs 0.249 ± 0.019, $P < 0.01$). EPO administration to EE treated rats increased membrane fluidity ($P < 0.005$), to P values significantly below those even of control rats (0.213 ± 0.029 vs 0.249 ± 0.019, $P < 0.05$). EPO alone likewise increased fluidity to P values of 0.214 ± 0.010. Thus, EPO treatment induced comparable membrane fluidity, whether given to control rats or those that received EE.

Table 1. Enzymatic activities in liver plasma-membrane fractions in the different groups of animals studied

	Na^+/K^+-ATPase	5′-NT/G6Ph
Controls	8.5 ± 0.2	13.4 ± 2.7
EE	6.6 ± 0.8*	12.6 ± 3.1
EE + EPO	6.8 ± 1.2*	14.2 ± 2.4
EPO	8.02 ± 0.8	12.2 ± 2.2

Each group consists of five animals. Activity is expressed as μmol Pi/mg prot/hr. Data are expressed as mean ± SD.
G6Ph = glucose 6-phosphatase; 5′-NT = 5′-nucleotidase.
* $P < 0.01$ vs controls.

To assess if the effects induced by EPO could be related to a detergent action of the drug, 100 μg of liver plasma membrane preparation was incubated *in vitro* in the presence of (volume) 1 and 10 mM EPO for 10 min at 37°. Membrane fluidity, measured by P values, was not changed by this *in vitro* exposure to EPO.

Enzyme activities of the different preparations of liver plasma membrane are shown in Table 1. EE-treatment was followed by a significant reduction of Na^+/K^+-ATPase; EPO did not influence this activity in either EE-treated or control animals. In addition, the 5′-NT/G6Ph ratio was similar in the four groups of animals indicating a comparable degree of purity in the plasma membrane preparations obtained from each group of rats.

DISCUSSION

17-α-Ethinyl-estradiol, a synthetic estrogen, has been demonstrated to produce cholestasis in man and animals by reducing bile flow and biliary excretion of cholephylic organic anions [4–8]. The cholestatic effect was mainly related to a reduced

fluidity of liver plasma-membrane probably due to the increased cholesterol and/or cholesterol esters in the heptocyte membrane [8, 9]. Davis *et al.* [25] reported that EE significantly increases hepatic cholesterol ester concentrtions by acting on the hepatic microsomal cholesterol acyl-CoA transferase. In addition, in a preliminary report, Arias and Kinne [14] demonstrated that EE primarily affects the sinusoidal domain of the hepatocyte plasma membrane. More recently, Rosario *et al.* [13] showed that EE, also at low dose, alters sinusoidal liver plasma membrane lipid structure, mainly by changing the polarity of the fatty acids in the membrane phospholipids.

Data reported in this paper confirm and expand previous reports. Administration of 5 mg/kg of EE was followed by a marked impairment in either BSP clearance and biliary secretion or in bile flow. A concomitant reduction of liver plasma membrane fluidity and Na^+/K^+-ATPase activity was observed also. Our study showed that all these effects of EE, except for the decrease of Na^+/K^+-ATPase activity, may be reversed completely by concomitant treatment with the terpenoid compound, EPO.

Interestingly, EPO induced also a significant increment of liver plasma membrane fluidity in control animals, and comparably increased fluidity also in the EE-treated rats to levels above those of controls. Since EE treatment has been demonstrated to affect lipid composition of the membrane [4, 8, 13], it is possible that the primary site of action of the drug may be the lipid milieu of the membrane. In any case, its action is not linked to a "detergent" effect of the drug, since membrane microviscosity was unaffected when EPO was added to liver plasma membrane preparations *in vitro*.

The finding that EPO induces an increase in membrane fluidity in control animals may be the basis of the observed increment in plasma membrane enzymatic activities of Ca^{2+}/Mg^{2+}- and Ca^{2+}-ATPase, previously reported [17]. Plasma membrane composition and physicochemical characteristics have been shown to affect the activity of some membrane protein [26]. In line with this concept and previous data [4, 8, 13], EE treatment was associated with a significant reduction of Na^+/K^+-ATPase activity of liver plasma membrane. EPO administration however, either alone or after EE, did not induce any increment in Na^+/K^+-ATPase activity, even though it restored normal or increased membrane fluidity. This is in line with the view that the Na^+/K^+-ATPase activity is not linearly correlated with membrane fluidity and is not decreased in parallel with fluidity in EE-treated rats [27], as assessed from steady-state DPH polarization [13, 28].

From these data we conclude that EPO is able to completely reverse the cholestatic effects of EE, probably by restoring liver plasma-membrane fluidity to a normal or even higher value. This protective effect was obtained at high dose (100 mg/kg) so that one must be cautious in extrapolating these data to humans. The possible effects of lower doses of EPO and/or drug metabolite(s) in preventing EE-induced cholestasis is currently under investigation in our laboratory. This might be of therapeutic importance, since EE cholestasis in rats is an animal model for the estrogen-related cholestasis of pregnancy. EPO which does not produce fetal abnormalities in rat [29], might be worthy of clinical trials in humans with cholestasis of pregnancy if it is effective in lower pharmacological doses in rats.

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