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EFFECT OF ETHINYLESTRADIOL AND EPOMEDIOL ON BILE FLOW AND BILIARY LIPID COMPOSITION IN RAT

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Abstract—Epomediol (1,3,3-trimethyl-2-oxabicyclo(2.2.2.)octan-6,7-endo,endo-diol) (EPO) is a terpenoid compound shown to reverse 17α-ethinylestradiol (EE)-induced cholestasis in rat. The effect is related to the restoration of normal liver plasma membrane fluidity values. To further characterize the effect of EPO, bile flow and biliary lipid composition were measured in rats treated either with EE or EE associated with EPO. EE significantly reduced the bile flow; this reduction was prevented by concomitant treatment with EPO with an increase in the bile salt secretion rate. EPO alone showed a choleretic effect. The biliary secretion rate of cholesterol was also significantly reduced by EE while being comparable to controls in EE–EPO-treated animals. Phospholipid (PL) biliary excretion was significantly ($P < 0.002$) increased by EE either alone or combined with EPO. After EE treatment, the biliary PL composition showed a reduction in phosphatidylcholine (PC) concentration with a parallel increase in lyso-phosphatidylcholine (LPC) when compared to control animals (PC:LPC ratio 5.0 ± 2.5 vs 26.8 ± 9.9, mean ± SD, $P < 0.005$). EPO administration to EE-treated rats restored the biliary PC:LPC ratio to control values (27.6 ± 10.6). EPO alone did not show any appreciable effect as compared to both control and EE–EPO treated animals. As increased concentrations of LPC have been reported to induce an alteration in the function of membrane lipids and membrane-associated proteins, such as regulatory enzymes for bile acid, cholesterol and phospholipid metabolism, these results suggest that the protective effect of EPO in EE-induced cholestasis may be related to the reversal of the alterations in membrane lipid composition and function induced by EE.

Administration of estrogens may interfere with bile formation in animals and man [1]. The synthetic estrogen 17α-ethinylestradiol (EE‡) causes bile secretory failure characterized by a decrease in the bile salt independent flow [2–4] and by a reduced capacity to excrete organic anions such as bile salts and sulfobromophthalein. The mechanism of the cholestatic effect of EE has been reported to reside primarily in an increased rigidity of sinusoidal liver plasma membrane [5] related to an alteration in its lipid and protein composition [6].

EPO is a synthetic terpenoid compound reported to have choleretic effects in humans and animals [7, 8]. EPO has also been shown to prevent the impairment of sulfobromophthalein transport and bile flow induced by EE, possibly by reversing the reduction in liver plasma membrane fluidity induced by the synthetic estrogen [9]. In spite of these studies, the mechanisms of action of EE and the effect(s) induced by EPO on the EE-induced cholestasis remain to be fully elucidated.

In the present study we have investigated the effect of EE administration on bile flow and biliary secretion rate of bile components such as bile salts, cholesterol and phospholipids. The influence of EPO on the EE-induced changes has also been investigated.

MATERIALS AND METHODS

Animals and treatments. Male Wistar rats weighing 250–300 g were maintained on a standard laboratory diet (GLP, Altromin Rieper, Bolzano, Italy). EE was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Animals were given EE subcutaneously (5 mg/mL in propylene glycol) in a single dose of 5 mg/kg daily for 5 days. EPO was obtained as pure powder (purity higher than 98%) from Camillo Corvi SpA (Piacenza, Italy). Animals received EPO (100 mg/mL in 0.85% saline) i.p. 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. Control animals were treated with corresponding volumes of the appropriate solvent vehicle(s). None of the treatments resulted in significant variations in body weight compared to controls.

Bile flow measurement. After 5 days of treatment, each rat was anesthetized with ketamine (50 mg/kg, i.p.). Body temperature was continuously monitored with a deep rectal probe and maintained at 37.0 ± 0.5° with an infrared lamp. The common bile duct was cannulated with a PE-50 catheter (Clay Adams, Division of Becton, Dickinson and Co., Parsipanny, NJ, U.S.A.). After 15 min to stabilize

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‡ Abbreviations: EPO, epomediol, 1,3,3-trimethyl-2-oxabicyclo(2.2.2.)octan-6,7-endo,endo-diol; EE, 17α-ethinylestradiol; PL, phospholipid; PC, phosphatidylcholine; LPC, lyso-phosphatidylcholine.

Table 1. Effect of administration of EE, EPO and EE–EPO on the plasma level of bile salts and cholesterol, and the hepatic cholesterol level

	Controls (8)	EE (8)	EPO (9)	EE–EPO (5)
Plasma cholesterol (mmol/L)	1.4 ± 0.1	0.5 ± 0.3*	1.6 ± 0.2*	0.6 ± 0.3*
Plasma bile salts (μmol/L)	9.2 ± 3.9	23.5 ± 7.2*	8.4 ± 2.6	31.5 ± 18.4*
Hepatic cholesterol (μmol/g)	4.8 ± 0.5	6.2 ± 0.6†	4.4 ± 0.3	6.2 ± 0.1†

Data expressed as means ± SD. In parentheses, the number of animals in each group.
* $P < 0.01$ vs controls.
† $P < 0.001$ vs controls.

flow, bile was collected in pre-weighed tubes every 10 min for 60 min. Samples were pooled and bile flow was expressed as mean ± SD over the experimental period. At the end of the experiment, 1 mL blood was collected by cardiac puncture in a tube containing 25 μL 2.7 mM EDTA Na_2, and the liver was excised, weighed and stored at −20°. Bile and plasma samples were stored at −20° until analysis (within 4 weeks). Preliminary experiments showed that the lipid content of both bile and liver (see below) was stable for up to 2 months under these conditions.

Bile and plasma analysis. Total plasma and biliary bile salts were measured by the kit Sterognost-3α Flu (Nyegaard & Co, Norway) according to the technique of Mashige *et al.* [10]. Total cholesterol in plasma and bile was measured by the enzymatic method [11]. Lipids were extracted from liver or bile as described by Folch *et al.* [12]. In the case of bile, 0.1 M NH_3 was added to the upper phase to remove bile salts [13]. Lipid extracts were concentrated under N_2 and processed immediately. Total phospholipids in bile were measured as the amount of P_i after mineralization as described by Lanzetta *et al.* [14]. To determine the cholesterol hepatic content, the technique described by De Hoff *et al.* [15] was used. Phospholipids extracted from bile were analysed by TLC on 20 cm × 20 cm Silica gel G-60 plates (Stratocrom, C. Erba, Milano, Italy). Separation was obtained by two solvent systems in succession: (a) acetone:*n*-hexane (1:3) (v/v) for 20 cm followed after drying by (b) a mixture of chloroform:methanol:acetic acid:water (50:25:8:2) (v/v) for an additional 15 cm. The position of lipid bands was visualized under iodine vapors and identified by comparison with a mixture of appropriate standards. The bands were scraped off the plates and extracted twice with chloroform:methanol (1:1, v/v). The extracts were pooled and dried under N_2, the residue dissolved in a small volume of the same solvent mixture, and the amount of P_i estimated in aliquots after mineralization according to Lanzetta *et al.* [14].

Statistics. All results were expressed as mean ± SD. A two-tailed Student's *t*-test was used to compare differences between components examined.

RESULTS

Plasma and liver

The effect of the administration of EE, EPO and a combination of the two drugs on the plasma cholesterol level and bile salt concentration is reported in Table 1. The plasma cholesterol level decreased significantly after EE treatment and concomitant administration of EPO did not prevent this decline. By contrast, EPO produced a slight increase in the plasma cholesterol level. The reduction in plasma cholesterol induced by EE was associated with a significant increase ($P < 0.001$) in the hepatic cholesterol content. When EPO was given together with EE, the hepatic cholesterol content was similar to that observed in EE-treated animals. Conversely, EPO alone did not modify the liver cholesterol content as compared to control animals.

The cholestatic effect induced by EE was associated with a significant increase in plasma bile salt levels (Table 1). This increase was not prevented by administration of EPO. As in the case of hepatic cholesterol content, EPO alone did not affect plasma bile salt concentration.

Bile

Data obtained by analysing the different biliary components in the four groups of animals are collated in Table 2.

Figure 1 shows the effect of different treatments on *bile flow*. EE treatment resulted in a significant reduction ($P < 0.002$) in bile flow which was reversed to near control values by concomitant treatment with EPO. The value observed in EE–EPO-treated animals was significantly ($P < 0.02$) higher than that found in rats to whom EE was given alone. EPO alone produced a choleretic effect, bile flow increasing significantly ($P < 0.01$) as compared to controls.

As shown in Fig. 2, the *bile salt secretion rate* was reduced by EE ($P < 0.02$) and increased by EPO either in combination with EE or alone ($P < 0.02$), indicating that EPO reduces the cholestatic effect of the synthetic estrogen by increasing the bile salt-dependent fraction of bile flow. The bile salt secretion rate in EE–EPO-treated animals was not significantly different from controls. A linear correlation ($r = 0.91$, $P < 0.01$) was observed between mean bile flow and mean bile salt secretion rate among the four groups of animals.

Figure 3 illustrates the modifications induced by the different drugs on the *biliary cholesterol secretion rate*. EE produced a significant ($P < 0.01$) reduction in the cholesterol biliary output. Combined treatment with EE and EPO increased ($P < 0.02$ vs EE-treated group) the lipid secretion rate though the value was lower than that observed in controls ($P < 0.02$). EPO treatment resulted in no alteration in cholesterol output compared with controls.

Table 2. Effect of administration of EE, EPO and EE–EPO on different biliary parameters

	Controls (8)	EE (8)	EPO (9)	EE–EPO (5)
Bile flow (μL/hr/100 g)	737 ± 50	576 ± 108*	840 ± 98†	694 ± 80‡
Bile salt secretion rate (μmol/hr/100 g)	10.9 ± 2.8	8.3 ± 2.1§	14.8 ± 4.5†	13.4 ± 2.2‖
Biliary cholesterol secretion rate (nmol/hr/100 g)	290 ± 61	166 ± 35†	238 ± 60	218 ± 50‡
Biliary phospholipid secretion rate (nmol P_i/hr/100 g)	1069 ± 308	1635 ± 395*	1228 ± 396	2236 ± 532*‡

Data expressed as means ± SD. In parentheses, the number of rats in each group.
* $P < 0.002$ vs controls.
† $P < 0.01$ vs controls.
‡ $P < 0.02$ vs EE.
§ $P < 0.02$ vs controls.
‖ $P < 0.001$ vs EE.

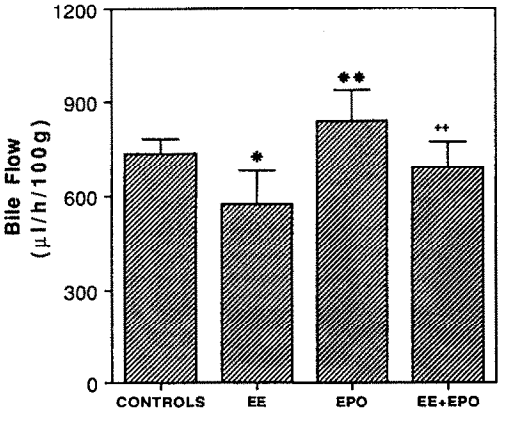

Fig. 1. Bile flow (μL/min/100 g) in control rats (8 rats) and animals treated with EE (8), EPO (9) and EE–EPO (5). Data are expressed as means ± SD. *$P < 0.002$ and **$P < 0.01$ vs controls; ++$P < 0.002$ vs EE-treated rats.

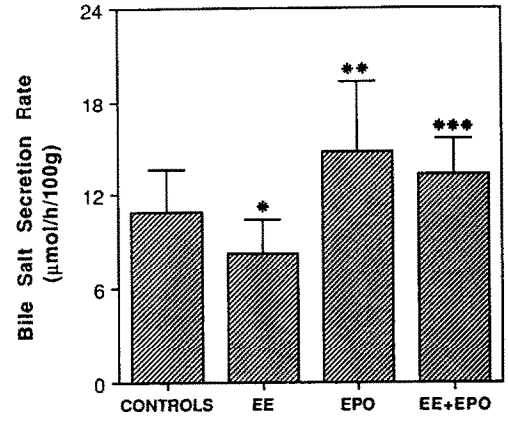

Fig. 2. Biliary bile salt secretion rate (μmol/hr/100 g) in the different groups of rats (for explanations see legend to Fig. 1). *$P < 0.02$ vs controls; **$P < 0.01$ vs controls; ***$P < 0.001$ vs EE-treated rats.

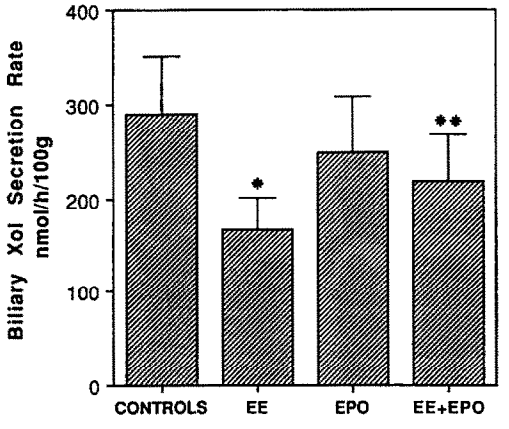

Fig. 3. Biliary cholesterol secretion rate (μmol/hr/100 g) in the different groups of rats (for explanations see legend to Fig. 1). *$P < 0.01$ vs controls; **$P < 0.02$ vs EE-treated rats.

As shown in Fig. 4, EE administration resulted in a significant increase in the *biliary PL secretion rate* ($P < 0.002$) which was increased further ($P < 0.001$) when EE was administered together with EPO. Conversely, no difference was observed in EPO-treated rats as compared to controls. As reported in Table 3, by analysing the composition of different PL classes in bile, EE treatment was associated with a significant ($P < 0.005$) increase in LPC secretion with a concomitant reduction in the PC concentration. When EE was administered with EPO, the percentage of LPC was restored to values comparable to either controls or EPO-treated rats. No alteration was observed in the other biliary phospholipid components.

DISCUSSION

EE, a synthetic estrogen, produces cholestasis via a decrease in bile flow, liver plasma membrane fluidity and the transport of organic anions [2–4, 9, 16]. This model of cholestasis in animals is considered adequate for the study of intrahepatic cholestasis caused in humans by oral contraceptives or during pregnancy [17]. The cholestatic effects of

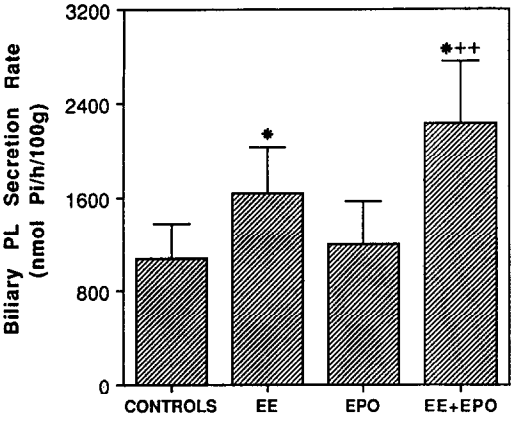

Fig. 4. Biliary phospholipid secretion rate (nmol P_i/hr/100 g) in the different groups of rats (for explanations see legend to Fig. 1). *$P < 0.002$ vs controls; $^{++}P < 0.02$ vs EE-treated rats.

EE have been shown to be reversed by EPO both in rats [9] and humans [18].

EE administration stimulates low density lipoprotein receptor activity with an increase in either the binding of lipoproteins by liver plasma membrane [19] or hepatic catabolism of low density lipoprotein [20]; both cause the marked hypo-cholesterolemia observed after EE administration. In addition, EE has been reported to stimulate the activity of the hepatic microsomal enzyme cholesterol acyl-CoA-transferase, resulting in an increase in the hepatic concentration of cholesterol esters [21]. The activity of cholesterol 7α-hydroxylase is also reduced by EE causing a reduction in bile acid synthesis [22]. The increase in the hepatic content of cholesterol esters has been regarded as being the basis of the higher microviscosity of liver plasma membranes, particularly at the basolateral domain [6], found after EE administration. This increase is accompanied by a reduction in the activity of Na^+,K^+ ATPase [4, 5, 8], a key enzyme in the Na^+-dependent uptake of bile salts [23]. EPO administration to EE-treated rats was associated with a normalization in both the bile salt secretion rate and bile flow while the plasma level of bile salts remained above control values. These findings indicate that EPO reverses the inhibition of bile salt synthesis induced by EE probably by restoring normal values of microsomal plasma membrane fluidity. Such a conclusion is also supported by the linear correlation found between bile flow and bile salt secretion rate. The increase in bile salt plasma levels above control values in EE–EPO-treated rats may have been due to Na^+,K^+ ATPase activity which was shown to be still below the control value in EE–EPO-treated rats [9]. The reflux of bile salts from the biliary duct system to plasma induced by EE, as reported previously [24], may be excluded in view of the observation that short-term EE treatment, as in this case, has no effect on biliary permeability [25].

A significant increase in the excretion rate of biliary phospholipids was also observed after EE administration. The increase is associated with the alteration in PL species since a significant decrease in PC, with a concomitant increase in LPC, was observed. The increase in the biliary LPC content induced by EE may be related to a reduction in LPC acylation, a process controlled by the activity of the acyl-CoA-LPC-acyl transferase. This enzyme is embedded into microsomal lipid membrane and its activity is regulated by the plasma membrane lipid environment [26]. Thus, the altered lipid composition and microviscosity induced by EE may be the cause of the increased biliary LPC content.

The ratio of the biliary secretion of PC:LPC was reduced by EE and restored to the control value when EE was given in association with EPO. This finding indicates that the drug is able to reverse the EE-induced alteration in PL biliary composition. This observation may be the basis of the effect of EPO on the degree of membrane fluidity, since it has been reported that this terpenoid compound, when given in association with EE, restores the liver plasma membrane fluidity to control values [9].

LPC has been shown to have an important disruptive effect on several cellular functions as well as membrane protein distribution [27]. Accordingly, the increased biliary excretion of LPC, paralleled by a concomitant reduction in PC, in EE-treated rats probably reflects the membrane lipid alterations induced by the synthetic estrogen. Since the biliary excretion of phosphatidylcholine species reflects the PL composition of plasma membrane [28], the normalization of the PC:LPC ratio in bile found upon concomitant treatment with EE and EPO may account for the reversal in the EE cholestatic effect [9].

From these data we conclude that the effects observed after EPO treatment on EE-induced

Table 3. Effect of administration of EE, EPO and EE–EPO on biliary phospholipid composition expressed as the percentage of total phospholipids

	Controls (4)	EE (4)	EPO (4)	EE–EPO (4)
LPC	4.35 ± 2.35	17.65 ± 5.19*	4.48 ± 1.60	3.71 ± 1.48
PC	88.90 ± 2.78	74.70 ± 4.41*	88.45 ± 2.81	90.63 ± 1.56
PC:LPC	26.8 ± 10.0	5.0 ± 2.5*	22.3 ± 9.5	27.6 ± 10.6
Others†	6.75 ± 2.33	5.00 ± 2.74	7.07 ± 2.20	5.66 ± 1.98

Data expressed as means ± SD. In parentheses, the number of animals in each group.
* $P < 0.005$ vs other groups.
† Others, phosphatidylethanolamine, lysophosphatidylethanolamine, sphingomileline, phosphatidylserine, phosphatidylinositol.

cholestasis in rats are most probably accounted for by the restoration of the microsomal plasma membrane microviscosity and/or lipid environment to control values. This effect is followed by a normal activity of the several enzymes operating at this level, in particular those regulating bile acid synthesis and PC and cholesterol metabolism.

Further studies currently being carried out in our laboratory, particularly the analysis of the lipid and protein membrane composition after EE administration, will provide further support for this hypothesis.

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