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EFFECTS OF ETHINYL ESTRADIOL ON BILE SECRETION AND LIVER MICROSOMAL MIXED FUNCTION OXIDASE SYSTEM IN THE MOUSE*

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Abstract—In the search for an animal model suitable for the study of ethinyl estradiol (EE) induced alterations of bile secretion, we have investigated the effects of three different doses (50–500–5000 μg/kg body wt., orally for 10 days) of EE on bile flow and composition and on liver microsomal mixed function oxidase system in female Albino–Swiss mice. No difference of bile flow was found between control and EE-treated mice. The decrease of bile acid secretion was dose-related and significant in animals treated with 500 and 5000 μg/kg of EE. Cholesterol output was similar in control and EE-treated animals. The molar ratio of bile acid to biliary cholesterol was significantly lower in all groups of EE-treated mice as compared with controls. The specific activities of 3,4-benzpyrene hydroxylase, aniline hydroxylase and NADPH cytochrome-*c*-reductase, as well as the content of cytochromes P-450 and b_5 decreased proportionally, in a dose dependent manner and significantly after 500 and 5000 μg/kg of EE. Our data indicate that mice, following EE, develop a lithogenic bile without obvious cholestasis. Moreover, they demonstrate a decrease of liver microsomal enzyme activities and cytochromes and suggest a relationship between the impairment of liver microsomal mixed function oxidase system and the changes of bile lipid composition.

Estrogens alone or associated with a progestogen have been recognized both on clinical [1] and epidemiological [2,3] grounds to induce alterations of bile secretion. These alterations range from modifications of bile lipid composition, so that bile becomes supersaturated with cholesterol [4,5], to bile flow impairment, i.e. intrahepatic cholestasis [6].

Most of the investigations devised to explain the pathogenesis of bile changes induced by estrogens have been performed in the rat [7,8] and recently also in the hamster [9,10]. Studies in the rat have related the cholestatic effect of estrogens both to the inhibition of the canalicular fraction of bile, which is bile acid independent [11], as also to the interference of these hormones with the hepatic microsomal mixed oxidase system and the turnover of cytochrome P-450 [12–15]. The aims of the investigations in the hamster were to establish the effect of low doses of EE, the common estrogenic component of oral contraceptives, on gallbladder bile lipid composition and on the microsomal rate-limiting enzymes involved in the hepatic synthesis of cholesterol and bile acids [9,10]. In the present paper we describe the effects of three different doses of EE on hepatic bile secretion in the mouse, an animal which seems sensitive to the lithogenic action of this hormone, but, in contrast to the rat, does not develop cholestasis. In the same experiments we have obtained data which demonstrate a decrease in the amount of hepatic cytochrome P-450 and in the liver drug metabolizing activity and suggest a relationship between the reduction of the mixed function oxidase components and the impairment of bile lipid secretion.

MATERIALS AND METHODS

Experimental design. Female Albino–Swiss mice (Charles River, Italy), weighing approximately 21 g, were allocated to four groups of 24 each. They were housed in wire mesh cages in a well ventilated vivarium with 12 hr of light and fed Purina Laboratory Chow with water *ad lib.* EE (17α-ethinyl estradiol, Sigma, St. Louis, MO) was dissolved in 2 per cent gum Arabic and administered orally by gastric drainage in a daily dosage of 50, 500 and 5000 μg/kg body wt., respectively, for 10 days to three groups of animals, while the fourth group was handled similarly, except that no EE was administered with the vehicle, and thus served as control. On the 11th day, 24 hr after the last EE administration, 12 animals of each group, fasted overnight, were anesthetized by intraperitoneal injection of 10 ml/kg body wt. of a 50 per cent urethan solution and then underwent laparotomy. The cystic duct was ligated and the common bile duct was cannulated by a polyethylene catheter (PE 10, Clay Adams, Parsipany, NJ) to obtain hepatic bile. Bile samples were collected over a period of 2 hr and measured by weight to evaluate bile flow rate. During bile collection the body temperature was monitored with a rectal thermometer and maintained at 36–37° using a warming lamp and saline was instilled intraperitoneally to compensate fluid loss. On the same morning the other animals

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of each group of treatment were sacrificed for microsomal enzyme and hemoprotein assays.

Analysis of bile composition. Total bile acids were enzymatically determined in bile by the method of Talalay [16]. Phospholipid and cholesterol concentrations have been measured, respectively, by the methods described by Svanborg and Vikrot [17] and by Roschlau *et al.* [18].

Assay of microsomal enzymes and hemoproteins. Liver microsomes were prepared by the procedure of Uemura *et al.* [19]. The microsomal pellets were washed once with 1.15 per cent KCl solution and finally suspended in 0.1 M Tris–HCl buffer (pH 7.5). For 3,4-benzpyrene hydroxylase assay the incubation mixture in a final volume of 1.0 ml consisted of 300 μg of microsomal proteins, 0.25 ml of soluble fraction from control mice (equivalent to that in about 1/8–1/10 of weight of the liver), 5 mM $MgCl_2$, 8 mM glucose-6-phosphate, 0.6 mM NADP, 190 μM 3,4-benzpyrene (added in 20 μl of acetone) and 0.1 M Tris–HCl buffer (pH 7.5) to bring the total volume to 1.0 ml. Reactions were carried out at 37° for 10 min with mechanical shaking in the dark and 3-hydroxybenzpyrene formed was assayed spectrofluorometrically by the method of Nebert and Gelboin [20]. Quinine sulfate was used as the standard and the fluorescence of a known concentration of quinine sulfate was used to calculate the nmoles of 3-hydroxybenzpyrene formed. A quantity of 0.036 nmoles of 3-hydroxybenzpyrene per ml in 1 N NaOH gives a fluorescence intensity which is equal to that given by 0.3 μg of quinine sulfate per ml in 0.1 N H_2SO_4 at an excitation wavelength of 396 nm and an emission wavelength of 522 nm [21].

For the assay of aniline hydroxylase activity, the reaction mixture contained, in a final volume of 1.0 ml, 2.2–2.3 mg of microsomal proteins, 0.25 ml of soluble fraction, 5 mM $MgCl_2$, 8 mM glucose-6-phosphate, 1.2 mM NADP, 8 mM aniline and 0.1 M Tris–HCl (pH 7.5) to bring the total volume to 1.0 ml. Reactions were carried out at 37° for 20 min with mechanical shaking and the *p*-aminophenol formed was assayed by the method of Imai *et al.* [22]. Cytochrome P-450 and b_5 were determined as described by Omura and Sato [23] and Garfinkel [24] using millimolar extinction coefficients of 91 and 163 $mM^{-1}\ cm^{-1}$, respectively.

NADPH cytochrome-*c*-reductase activity was determined spectrophotometrically by the method of Omura and Takesue [25] in the presence of 1.5 mM KCN. The absorption coefficient for the difference in absorbance between reduced and oxidized cytochrome c was 21.1 $nM^{-1}\ cm^{-1}$ at 550 nm [26]. Protein concentration was determined by the method of Lowry *et al.* [27] using crystalline bovine serum albumin as standard.

Statistical analysis. Statistical analysis was obtained by Student's *t*-test. Microsomal enzyme activities and cytochromes were evaluated by the Mann–Whitney *U* test, a non-parametric analysis. All values reported are mean ± 1 standard error.

RESULTS

As shown in Table 1, the low dose of EE (50 μg/kg) did not affect body and liver weight, whereas the intermediate dose (500 μg/kg) reduced significantly the final mean body weight in comparison to control

Table 1. Effect of various EE-treatments on body and liver weight in mice*

Treatment	No. of animals	Body weight (g) Initial	Body weight (g) Final	Liver weight (g)	Liver weight (% of body weight)
Controls	24	21.7 ± 0.3	23.5 ± 0.4†	0.87 ± 0.06	3.74 ± 0.05
EE 50 μg/kg	24	21.6 ± 0.2	22.5 ± 0.3†	0.86 ± 0.03	3.84 ± 0.03
EE 500 μg/kg	24	21.7 ± 0.3	21.9 ± 0.3‡	0.91 ± 0.05	4.17 ± 0.04
EE 5000 μg/kg	24	22.4 ± 0.3	20.4 ± 0.4†‡	1.02 ± 0.03‡	5.00 ± 0.04‡

* Values represent mean ± S.E.M.
† Differs from initial value P<0.01.
‡ Differs from control value P<0.01

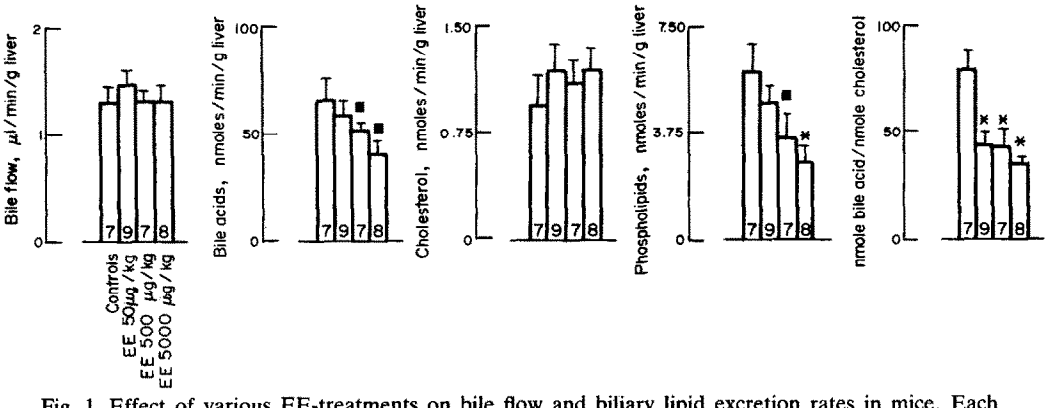

Fig. 1. Effect of various EE-treatments on bile flow and biliary lipid excretion rates in mice. Each column represents the mean ± S.E.M. Numbers in columns refer to the number of experiments. Symbols represent values significantly different from controls; ■ P<0.05; ★ P<0.01.

Table 2. Effect of various EE-treatments on hepatic bile lipid composition in mice*

Treatment	Bile acids (moles %)	Cholesterol (moles %)	Phospholipids (moles %)
Controls (7)	90.8 ± 0.6	1.1 ± 0.1	8.0 ± 0.5
EE 50 μg/kg (9)	90.7 ± 1.0	2.0 ± 0.1†	6.4 ± 0.3
EE 500 μg/kg (7)	90.1 ± 1.2	2.1 ± 0.2†	6.9 ± 1.2
EE 5000 μg/kg (8)	90.1 ± 0.7	2.5 ± 0.2†	6.5 ± 0.5

* Values represent mean ± S.E.M. Numbers in parentheses refer to the number of experiments.
† Differs from control value, $P<0.01$.

value. Furthermore, mice treated with the high dose (5000 μg/kg) lost body weight and showed a significant increase of liver weight in respect to controls.

Bile flow and composition. The effects of the three doses of EE on hepatic bile flow and composition are reported in Fig. 1. No difference in bile flow was found between control and EE-treated mice. The decrease of bile acid secretion was dose-related and significant in animals treated with the intermediate and high doses of EE. Cholesterol output was similar in control and EE-treated animals. Phospholipid secretion was markedly reduced in mice which received the intermediate and high doses of EE. As a result of the decreased biliary bile acid excretion and unchanged cholesterol output, the molar ratio of bile acid to biliary cholesterol was significantly lower in all groups of EE-treated mice in respect to controls. Because of the aforementioned differences in the lipid secretions, there were variations in the relative composition of bile after all the doses of EE. As shown in Table 2, the changes resulted in a slight decrease of the molar percentage of phospholipids and in a significant increase in cholesterol molar percentage.

Hepatic cytochrome P-450 and drug metabolizing activity. As shown in Table 3, EE treatment led to a general decrease of the specific activities of 3,4-benzpyrene hydroxylase, aniline hydroxylase and the flavoprotein component of the microsomal drug-metabolizing system, i.e. NADPH cytochrome-creductase, as well as of the content of cytochrome P-450 and b_5. The reduction of hepatic microsomal enzymes and drug metabolizing capacity was proportional, dose-dependent and significant after the intermediate and high doses of EE.

DISCUSSION

Following EE administration, the bile acid and phospholipid secretions decreased, whereas the cholesterol output remained almost unchanged, so that hepatic bile showed a reduction of bile acid to cholesterol molar ratio. The marked decrease of phospholipid excretion seemed to be related to the significant reduction of bile acid secretion.

Our finding that EE increases cholesterol molar percentage of hepatic bile in mice is in accordance with previous reports that estrogens induce lithogenic bile in women [4,5], in the rat [28] and in the hamster [9]. Not only the intermediate and high dose of this hormone, but also the low dose, which is similar to that employed by Bonorris *et al.* [9] in the hamster, was able to induce a significant change of bile lipid composition. Although cholesterol concentration of mouse bile is very low, well below saturation [29], its molar percentage increased significantly in response to EE, as it does in man [4] who has a higher bile cholesterol concentration. Previous studies have indicated that EE reduces bile acid synthesis and secretion in rat [28] and inhibits cholesterol 7α-hydroxylase, the rate-limiting enzyme for the conversion of cholesterol to bile acids, in the hamster [9]. The impairment of microsomal enzymes involved in steroid hydroxylation described by some authors [30,31] is consistent with our data which report a decrease of the hepatic microsomal drug metabolizing activity and of the liver content of cytochromes P-450 and b_5. Moreover, since 7 α-hydroxylase activity is P-450 dependent [32], we can assume that there is a reduced bile acid synthesis in our animals which could be responsible for the reported changes of bile lipid composition. The mechanism by which EE decreases hepatic microsomal enzyme activities and cytochromes is not yet established. According to Davis and Kern [33], EE affects the lipid composition and physical characteristics of liver microsomal membranes; the reduced synthesis of bile acids could be secondary to these changes in membrane lipid composition. These data, however, have not been con-

Table 3. Liver microsomal enzyme-specific activities and cytochromes following EE-treatment in mice*

Treatment	3,4 Benzpyrene hydroxylase (nmoles/mg/min)	Aniline hydroxylase (nmoles/mg/min)	Cytochromes (nmoles/mg) P-450	b_5	NADPH cyt. *c* reductase (nmoles/mg)
Controls (4)	6.095 ± 0.266	1.232 ± 0.200	0.906 ± 0.11	0.51 ± 0.04	135.35 ± 18.22
EE 50 μg/kg (4)	4.643 ± 0.637	1.145 ± 0.216	0.761 ± 0.98	0.486 ± 0.04	96.92 ± 8.85
EE 500 μg/kg (4)	4.433 ± 0.479†	0.984 ± 0.149†	0.705 ± 0.05†	0.456 ± 0.02	93.37 ± 7.85†
EE 5000 μg/kg (4)	3.419 ± 0.120‡	0.969 ± 0.111†	0.384 ± 0.19‡	0.412 ± 0.10†	84.00 ± 7.7‡

* Values are expressed as the mean ± S.E.M. Numbers in parentheses refer to the number of experiments.
† $P<0.05$ vs controls.
‡ $P<0.01$ vs controls.

firmed by Bonorris *et al.* [9] who failed to find any variation in cholesterol content of hepatic microsomal fractions in the hamster.

As suggested by Mackinnon *et al.* [14], the general reduction of the components of the drug metabolizing system following EE administration does not seem explicable in terms of competitive inhibition by substrate. Alternatively, the reduction of the drug metabolism and of the cytochrome P-450 level could be related to the formation of reactive metabolites of EE which are able to bind irreversibly to microsomal proteins, thereby inducing biochemical lesions [34,35]. Whether this mechanism might really play a role in the alterations of bile lipid composition observed in EE treated mice is, however, unclear at this time.

In spite of the decreased bile acid excretion rate, we have not observed bile flow reduction in the mouse, whose bile acid independent canalicular fraction of bile does not seem to be inhibited, but, on the contrary, stimulated by EE. This finding is in contrast with previous data, showing a selective inhibition of canalicular (Na^+K^+)-ATPase by EE in the rat [11,36]. In this respect mice seem to resemble humans who always show a significant increase of cholesterol saturation of bile following EE [4,37], but only occasionally develop cholestasis [1].

In conclusion, EE reduces bile acid secretion, increasing cholesterol saturation of hepatic bile in the mouse. In this animal species, hitherto unstudied, EE does not decrease bile flow. The change of bile lipid composition seems to be consistent with a general impairment of the hepatic microsomal mixed function oxidase system which is involved in steroid hydroxylation and, therefore, in bile acid synthesis. Our data suggest a possible role of cytochrome P-450 and related activities in the pathogenesis of estrogen induced lithogenic bile also in humans. However, much more remains to be learned about bile secretion and gallbladder function in these conditions and the mouse may be a promising, low cost experimental model of investigation.

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