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INFLUENCE OF *S*-ADENOSYL-L-METHIONINE ON IRREVERSIBLE BINDING OF ETHYNYLESTRADIOL TO RAT LIVER MICROSOMES, AND ITS IMPLICATION IN BILE SECRETION

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Abstract—A three-day administration of ethynylestradiol (EE) (5 mg/kg per day) is shown to decrease the bile flow in the rat by reducing both the bile salt dependent and independent fractions of the bile. Protection against this effect is observed in rats given *S*-adenosyl-L-methionine (SAMe) in addition to EE. The protection is accompanied by a significant decrease of the binding of EE to liver microsomal proteins suggesting that SAMe administration may favour the estrogen elimination as a methylated derivative.

Ethynylestradiol, a widely used oral contraceptive hormone, has been shown to decrease the bile flow and induce modifications of the bile lipid composition in rats [1–5]. A previous work from this laboratory [6] has shown that this effect is reversed by the administration of *S*-adenosyl-L-methionine (SAMe). Among the various mechanisms postulated to explain these findings, the hypothesis was advanced that the observed protection by SAMe might be the result of the methylation of EE metabolites by which the binding of the estrogen to the microsomal proteins is prevented [7, 8]. As a first step in the evaluation of this hypothesis, the binding of EE to microsomal proteins was studied in animals given [^{3}H]EE and [^{3}H]EE + SAMe, respectively.

MATERIALS AND METHODS

Unlabelled SAMe in the stable form of disulfate-di-*p*-toluene sulfonate was obtained from BioResearch Co., Milan, Italy; [6,7-^{3}H] 17α-ethynylestradiol ([^{3}H]EE), sp. act. 55 Ci/m-mol, was obtained from New England Nuclear, Boston, MA., U.S.A. Unlabelled 17α-ethynylestradiol was purchased from Sigma Chemical Co., St. Louis, MO. PE-50 polyethylene tubing was obtained from Clay Adams, Inc., Parsippany, NJ, U.S.A. Soluene 350 and Instagel were purchased from Packard Instrument Co., Inc.; PPO and dimethyl POPOP from Merck, Darmstadt. Female Sprague–Dawley rats weighing 220 ± 20 g were used for the experiments. Treatment of animals was as follows.

The first group (controls) received 2 % gum Arabic solution (10 ml/kg) by gastric intubation on days 1, 2 and 3. The second group (EE treated) received orally 10 ml/kg of an EE solution in 2 % gum Arabic (5 mg/kg of EE) on days 1, 2 and 3; to the dose assumed on the 3rd day, [^{3}H]EE (5 μCi; 0.03 mg) was added. The third group (EE + SAMe-treated) was treated with EE and [^{3}H]EE but received, in addition, three intramuscular daily doses of SAMe (25 mg/kg) on days 1, 2, 3, and 4; the first daily dose of SAMe was given 30 min before EE treatment and the other two 3 and 6 hr after the first one. All the animals were cannulated on the 5th day, 48 hr after administration of [^{3}H]EE; the animals of the third group were injected with an additional dose of SAMe (25 mg/kg) 1 hr before bile duct cannulation.

The animals, fasted for 12 hr, were anaesthetized by intraperitoneal injection of 1 ml/kg of a 50 % urethan solution before bile duct cannulation.

The common bile duct was surgically exposed by a midline abdominal incision, and cannulated with PE-50 polyethylene tubing. The bile collected during the first and second hour from the cannulation was measured by weight to obtain the bile flow rate. During bile collection, the body temperature was maintained at 36.5–37° using a warming lamp, and saline was instilled intraperitoneally to compensate for the fluid loss.

Analysis of bile composition. Total bile salts were enzymatically determined in the whole bile by the method of Talalay [9]. Phospholipid concentration was measured by the method of Svanborg and Vikrot [10] and cholesterol concentration by the method of Roschlau *et al.* [11].

Irreversible binding of [^{3}H]*EE to microsomal proteins.* Liver microsomes were prepared as described by Remmer *et al.* [12]. Aliquots of the microsomal suspension were used for protein determination according to Lowry *et al.* [13].

Free steroids and steroids bound to "low affinity sites" of microsomal proteins were eliminated as described by Kappus *et al.* [7]; for this purpose, aliquots (4 ml each) of the microsomal suspension were incubated at room temperature for 30 min with 4 ml of 1 % charcoal (Norit A®) suspension in Tris–HCl buffer containing 0.01 % dextran.

In order to check the absorption of free steroids by charcoal, aliquots of the microsomal suspensions were added to [^{3}H]EE and worked up as the samples. All the added radioactivity was shown to be absorbed by charcoal.

The samples were centrifuged at 1200 *g* for 1–2 min and 1-ml aliquots of the supernatant (10.08 ± 0.27 mg protein) were digested with 1 ml of soluene 350 and then counted with 15 ml of a 0.5 N HCl/Instagel solution (1:1,v:v; counting efficiency 21.8 per cent as calculated with an internal standard).

Table 1. Effect of SAMe administration on bile flow, bile salts, phospholipids and cholesterol excretion in rats treated with EE

Parameters	Controls	EE	EE + SAMe
Bile flow* (μl min^{-1}g^{-1} liver)	2.18 ± 0.25	0.86 ± 0.08†	1.87 ± 0.24‡
Bile salts (nmoles min^{-1}g^{-1} liver)	78.57 ± 8.96	33.22 ± 4.95†	74.57 ± 6.29‡
Cholesterol (nmoles min^{-1}g^{-1} liver)	1.82 ± 0.13	0.63 ± 0.04†	1.46 ± 0.23‡
Phospholipids (nmoles min^{-1}g^{-1} liver)	16.99 ± 1.94	6.48 ± 0.95†	17.99 ± 2.52‡
nmole bile salts/ nmole cholesterol	51.92 ± 9.11	50.44 ± 5.78	53.16 ± 6.21

* Evaluated on the total of two-hr bile collection. For treatment see Materials and Methods.
Values are expressed as mean ± S.E. for 5 animals.
† $P < 0.001$ vs controls.
‡ $P < 0.005$ vs EE-treated rats.

Statistical analysis. Significance was determined by the Student's t test. Regression lines were computed with the method of least squares described by Snedecor and Cochran [14] and statistically compared.

RESULTS

As shown in Table 1, EE administration induced a significant decrease of the bile flow and rate of elimination of the tested bile components in comparison to controls. The reduced bile salt excretion rate is consistent with a decrease of the bile salt dependent fraction of bile. However, when the molar ratio of bile salts to cholesterol and the per cent molar bile composition were calculated, no difference was observed between EE treated and control rats (Tables 1 and 2). SAMe administration prevented the modifications of bile flow and elimination rate of the bile components (Table 1). In order to estimate the bile salt independent fraction (BSIF), the bile flow for each group was plotted against bile salt excretion. A linear correlation ($P < 0.025$) was observed for all groups of animals (Fig. 1), and no significant differences were noted when the slopes of the three regression lines were compared. BSIF determined as the intercept of the regression lines on the y-axis was 1.02, 0.57 and 0.91 μl^{-1}min^{-1}g^{-1} liver in the controls, EE-treated rats and animals receiving EE + SAMe, respectively.

Liver microsomal binding of [^{3}H]*EE.* The extent of the liver microsomal binding of [^{3}H]EE in rats treated with the estrogen was compared to that in rats receiving the estrogen in combination with SAMe.

As shown in Fig. 2, radioactivity bound to the liver microsomal proteins was significantly decreased ($P < 0.05$) in the EE + SAMe treated animals as compared to the rats given only EE.

Table 2. Effect of SAMe administration on bile composition in rats treated with EE

Treatment	Bile compostion Bile salts moles %	Phospholipids moles %	Cholesterol moles %
Controls	83.52 ± 2.28	15.67 ± 1.85	1.70 ± 0.08
EE	81.11 ± 1.74	17.17 ± 1.69	1.71 ± 0.20
EE + SAMe	83.15 ± 0.59	15.04 ± 0.87	1.68 ± 0.11

Measurements obtained from the first 2-hr bile collection in rats treated with EE and reported as mean ± S.E. for 5 animals.

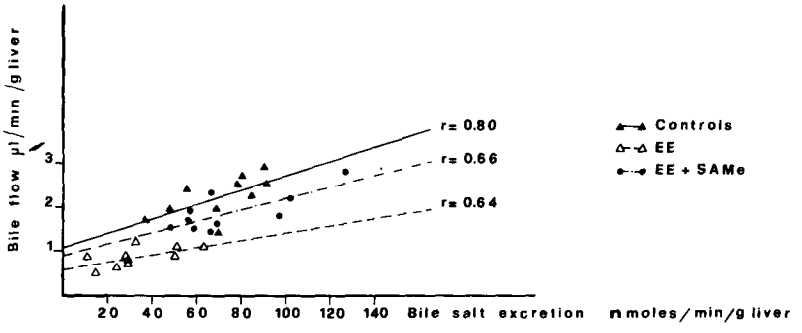

Fig. 1. Relationship between bile flow and bile salt excretion in rats treated with EE or EE + SAMe compared to controls. Each point represents a one-hr bile sample during a 2 hr bile collection period. Regression equation was calculated by the method of least squares. r was significant ($P < 0.025$) for the three groups of animals.

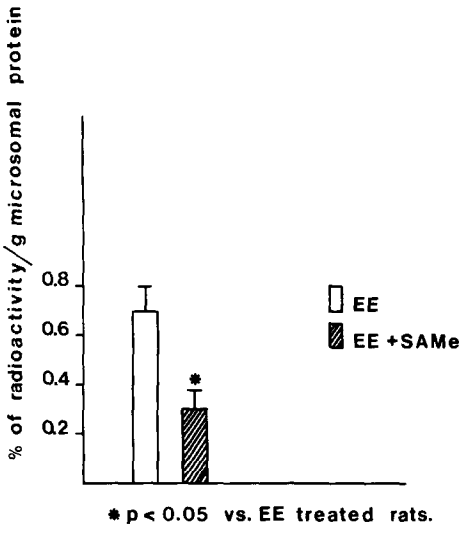

Fig. 2. Microsomal binding of EE *in vivo*. Radioactivity firmly bound to microsomes 48 hr after 6,7-[^{3}H]EE (5 μCi) administration. Values are expressed as mean ± S.E. for 5 animals.

DISCUSSION

From the results reported it appears that EE at the given doses induces a decrease of bile flow by inhibiting both the bile salt dependent and independent fractions of the bile. In contrast, previously reported data [6] obtained with doses of EE (2.5 mg/kg for three days) which were lower than those used here, showed a prevalent anticholeretic action of EE due to the selective inhibition of BSIF without any significant change of bile salt output. This is in agreement with the finding of other authors [2, 4, 15, 16], suggesting the existence of a dose-dependent effect of EE on bile secretion, changing from inhibition of BSIF at lower doses to inhibition of both biliary fractions when higher doses of the estrogen are employed. As shown in Table 1, cholesterol excretion was significantly reduced in EE-treated rats, but since bile salt excretion was reduced to the same extent, no variation of the ratio of bile salt to cholesterol was evident with respect to the controls.

It was previously shown by Davis and Kern [5, 17] that after administration of 5 mg/kg of EE, a decrease of bile salt/cholesterol ratio is obtained when the bile excreted during 14 hr after bile duct cannulation is analyzed.

The shorter time for collection in our experiments may explain the unaltered ratio obtained in agreement with the observation reported by Eriksson [18] and, more recently, by Hardison and Apter [19]. These authors pointed out that the decrease of the ratio of bile acids to cholesterol is detectable in the EE-treated animals only when the time of bile collection is prolonged as in the experiments described by Davis and Kern. In spite of the above mentioned discrepancy between our previous [6] and present results, the protection exerted by SAMe against the effect of the estrogen was observed in both experiments. Moreover, as already reported [6], no effects on the bile flow and composition is observed in the animals treated with SAMe alone. Among the variety of mechanisms by which SAMe may act as a protective agent, its methylating activity was taken into consideration. As a matter of fact, the transmethylation in mammalian cells by exogenous SAMe was already demonstrated by our previous studies [20]. The metabolic fate of the estrogen involves the hydroxylation at position 2 [21] followed by the transfer of the methyl group of SAMe to the 2-hydroxy group [22]. According to Kappus and Bolt [8], this transformation prevents the binding of EE with the hepatic microsomal proteins.

On the other hand, it was suggested by Remmer [23] that the irreversible binding of EE and its reactive intermediary metabolites to the liver microsomal proteins may account for the alteration observed in bile secretion.

Notwithstanding the little evidence of the mechanisms by which the reactive metabolites can induce cholestasis [24], the EE administration was recently reported to impair some liver microsomal activities participating in the bile salt metabolism [25–27]. Therefore, the binding of EE to the liver microsomal proteins was evaluated in two groups of rats given [^{3}H]EE and [^{3}H]EE + SAMe, respectively: the radioactivity found in the group treated with association of [^{3}H]EE and SAMe was significantly lower than in the group receiving [^{3}H]EE alone. SAMe administration might, therefore, protect against the effects exerted by the estrogen, methylating the drug and reducing its binding to the microsomal proteins.

Work is in progress in our laboratories on the EE metabolism, in order to evaluate possible modifications induced by SAMe treatment.

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