ELSEVIER

Estradiol replacement in ovariectomized rats increases the hepatic concentration and biliary secretion of α-tocopherol and polyunsaturated fatty acids

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Previously, we showed that estradiol replacement in ovariectomized rats produced prominent increases in serum and liver α-tocopherol (αTP). The present study was conducted to examine whether the estrogen-induced increase in the liver concentrations of αTP affects its biliary secretion and the fatty acid compositions of hepatic and biliary lipids. Ten ovariectomized rats were assigned to two groups: five rats were implanted subcutaneously with time-release estradiol pellets (OXE; 25 μg/day/rat) and five with placebo (OXP). Twice daily rats were pair-fed a modified AIN-93G diet containing soybean oil. At 5 weeks, bile was collected via a bile cannula hourly for 8 hours during duodenal infusion of a lipid emulsion (565 μmol triolein and 396 μmol Na-taurocholate/24 mL phosphate buffered saline, pH 6.45) at 3.0 mL/hr. During the 8-hour period, no difference was noted in the hourly rate of bile flow (0.95 mL/hr in OXE rats vs. 0.99 mL/hr in OXP rats). The biliary output of αTP for 8 hours was higher in OXE rats (51.6 ± 3.6 nmol) than OXP rats (31.7 ± 2.9 nmol). Likewise, the liver concentration of αTP was higher in OXE rats (81.9 ± 3.5 nmol/g liver) than in OXP rats (53.3 ± 7.4 nmol/g liver). The biliary secretion of phospholipids (PL) for 8 hours was significantly ($P < 0.05$) higher in OXE rats (55.1 ± 4.9 μmol) than in OXP rats (42.3 ± 4.7 μmol). Among the PL fatty acids, the outputs of 20:4 and 22:6n-3 were increased most markedly by estradiol replacement. The total outputs of 22:6n-3 for 8 hours in OXE and OXP rats were 2.95 ± 0.20 μmol and 1.37 ± 0.23 μmol, respectively. In the liver, the concentrations of PL 22:5n-3 and 22:6n-3 were elevated significantly in OXE rats. The present results suggest that estradiol may protect hepatic PL and membranes against oxidative damage by improving the liver status of αTP. (J. Nutr. Biochem. 10:110–117, 1999)

Keywords: estrogen; biliary lipid; vitamin E; fatty acids

Introduction

An early study reported that concentrations of α-tocopherol (αTP) in the liver and other tissues were significantly higher in female rats than in male rats.[1] This gender difference in the body status of αTP, which was corroborated by subsequent studies,[2–4] has been attributed to the opposing effects of estrogen and testosterone on αTP metabolism. In general, ovariectomy decreases and estradiol replacement elevates the tissue concentrations of αTP in ovariectomized rats.[4] In male rats, castration increases the tissue levels of αTP, whereas testosterone injection of castrated males prevents increases in tissue αTP. In contrast, the serum levels of αTP have been shown to be higher in male rats than in female rats.[1] Such gender differences in the tissue or serum levels of αTP are eliminated completely in castrated female and male rats,[4] suggesting that the steroid hormones may regulate the tissue uptake and metabolism of αTP. Despite their pronounced effects on tissue αTP concentrations, little is known about the mechanism whereby those steroid hormones interact with αTP or influence its distribution in vivo. Furthermore, it is unclear whether the sex hormone-induced alterations in tissue αTP status are mediated by

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changes in the distribution of plasma αTP among major lipoproteins and hence the tissue uptake of the vitamin via specific lipoprotein receptors.

In a recent study,[5] we observed that estradiol replacement in ovariectomized rats produces a marked increase in the serum level of αTP and a significant increase in the liver concentration of αTP. The elevation in serum αTP was due primarily to a selective increase in the amount of αTP carried by high density lipoproteins (HDL). The increase in HDL αTP was associated with the increases in HDL cholesterol and phospholipids (PL), suggesting that estradiol may inhibit the oxidation of HDL lipids by increasing αTP carried by HDL. This effect of estradiol is consistent with the observations that estradiol replacement may reduce the incidence of coronary heart disease (CHD) and that the risk of CHD rises substantially in postmenopausal and ovariectomized women.[6–8]

The studies cited above[1–5] strongly suggest that estradiol may improve the overall nutritional or body status of vitamin E, thereby strengthening the antioxidant defense system. Based on our recent study, we have put forth the hypothesis that estradiol may interact with αTP in lipoproteins and cell membranes in vivo. Evidence from various in vitro experiments suggests that estradiol itself may act as an antioxidant to protect αTP against oxidation.[9] Estradiol also has been shown to inhibit the oxidation of lipoproteins and membrane lipids under in vitro conditions.[10–14] However, little information is available concerning whether estradiol or the estradiol-induced increase in αTP status protects membrane lipids against peroxidation in vivo.

The present study was conducted to determine whether estradiol replacement in ovariectomized rats alters the liver concentration of both αTP and the types of fatty acids incorporated into liver lipids. In addition, we examined whether changes in liver αTP and fatty acids affect the biliary secretion of αTP and the fatty-acid makeup of biliary PL.

Materials and methods

Animals and diet

Ten female Sprague-Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN USA), which initially weighing 256 ± 9 g (approximately 4 months old), were housed individually in plastic cages with stainless-steel wire bottoms in a windowless room maintained at 22 to 24°C with a daily 12-hour light:dark cycle with the light period from 3:30 AM to 3:30 PM throughout the study. The rats were kept in an animal care facility at Kansas State University, which is approved by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). Upon arrival, the rats were fed a nutritionally adequate diet containing soybean oil and egg white in place of casein (*Table 1*). The diet was formulated according to the AIN-93 recommendations[15] by Dyets Inc. (Bethlehem, PA USA). All rats were given free access to deionized water from a water purification system (Millipore Corporation, Marlboro, MA USA) delivered via a stainless-steel watering system.

Estrogen replacement and meal feeding

At the end of 2 weeks, when rats weighed 271 ± 10 g, they were starved for 13 hours and ovariectomized under halothane anesthesia. After 2 weeks of postoperative recovery, the rats were assigned randomly to two groups: five rats (average weight 280 ± 6 g) were implanted subcutaneously in the posterior neck with 60-day-release 17β-estradiol pellets (25 μg/day; Innovative Research of America, Toledo, OH USA; OXE group) and the other five (average weight 280 ± 5 g) were implanted with placebos containing no estradiol (OXP group).

Table 1 Composition of diet*

Ingredient	g/kg
Egg white	200.0
Corn starch	396.5
Dyetrose (dextrinized corn starch)	132.0
Dextrose	100.0
Cellulose	50.0
Soybean oil†	70.0
Mineral mix	35.0
Vitamin mix	10.0
Biotin (1 mg/g biotin sucrose mix)	4.0
Choline bitartrate	2.5

*Formulated and supplied from Dyets (Bethlehem, PA USA), according to the recommendations of the American Institute of Nutrition.[15]
†Contained 0.02% tert-butylhydroquinone.

Because OXE rats consumed significantly less food than OXP rats, as observed previously,[5] both groups of rats were trained to meal feed and were pair-fed. Both OXE and OXP rats were fed 5 g at 8:30 AM and 8 g at 3:30 PM. The total daily amount of diet fed (13 g) represented 90% of their average food intake before ovariectomy. Each meal was consumed by both groups within 1 hour.

Cannulation of bile duct

After 5 weeks of estradiol implantation, rats were starved for 14 hours and then anesthetized by using a halothane vaporizer with a constant supply of oxygen (2.0% halothane in 1.5 to 2.0 L oxygen/min). Cannulation of the bile duct was performed with a slight modification of the procedure of Knox et al.[16] Briefly, after an abdominal incision was made along the midline, the common bile duct was cannulated with PE10 tubing (Clay Adams, Sparks, MD USA). The cannula was fixed in place with sutures (4-0 silk; Ethicon Inc., Somerville, NJ USA), and the cannula was exteriorized through an incision in the right flank. An intraduodenal infusion catheter was placed by inserting silicone tubing (outer diameter 2.1 mm; Silastic, Dow Corning Medical Products, Midland, MI USA) through the gastric fundus into the upper duodenum, extending it 2 cm down the proximal duodenum, and securing the fundic incision by purse-string sutures (4-0 silk; Ethicon Inc.). The catheter also was exteriorized through the right flank. After the incision was closed, the rat was placed in a restraining cage in a recovery chamber maintained at 30°C and allowed to recover for at least 20 hours. During this period, a maintenance solution (in mmol/L: glucose 277, NaCl 144, and KCl 4) was infused via the infusion catheter at a rate of 3.0 mL/hr with an infusion pump (Harvard Apparatus, Model 935, South Natick, MA USA).

Collection of bile

After the recovery period, the rats were infused at 3.0 mL/hr with a lipid emulsion to facilitate bile flow. The lipid emulsion consisted of 565 μmol triolein and 396 μmol sodium taurocholate in 24 mL phosphate buffered saline (pH 6.45; in mmol/L: Na_2HPO_4 6.75, NaH_2PO_4 16.50, NaCl 115.00, and KCl 5.00). Bile

was collected hourly in preweighed conical centrifuge tubes in ice-filled beakers under subdued light. After bile collection, the rats were euthanized by halothane, and the livers were collected. All samples were stored at −70°C until analysis.

Determination of liver and biliary αTP and PL

Lipids were extracted[17] from bile and finely minced liver samples. The lipid extracts were weighed for total lipid and redissolved in 3 mL of chloroform:methanol (2:1, v/v) containing butylated hydroxytoluene (0.45 mol/L methanol). From the lipid extracts, PL was measured colorimetrically (UV-1201 Spectrophotometer; Shimadzu Scientific Instruments Inc., Columbia, MD USA) by the method of Raheja et al.[18] For αTP analysis, the lipid extracts were filtered through a microfilter membrane (0.45 μm PTFE, Alltech Associates, Inc., Deerfield, IL USA), dried under nitrogen, and dissolved in 150 μL of methanol, as described previously.[19] The concentrations of αTP were determined by using a reverse-phase high performance liquid chomatography (HPLC) column (Alltima C18, 5 μm, 4.6 × 150 mm, Alltech Associates, Inc.) and Beckman System Gold software (Beckman Instruments, Inc., Fullerton, CA USA). Methanol was used as the mobile phase[20] and propelled at 2 mL/min. Detection was monitored at 292 nm (Module 166, Beckman Instruments, Inc.). Under these conditions, αTP was eluted at 4.2 minutes. The standard curve (peak area vs. ng of αTP) was constructed by using αTP standards. Concentrations of αTP from 57 to 340 ng yielded a linear curve ($r = 0.999$).

Fatty acid analysis

Total lipid extracts from bile and liver samples were separated by solid phase extraction[21] into cholesteryl esters (CE), triglycerides (TG), free fatty acids (FFA), and PL, using an aminopropyl solid phase column (Bond Elut NH_2; Varian Sample Preparation Products, Harbor City, CA USA) and 17:0 esterified to CE, TG, phosphatidylcholine (PC), and free 17:0 as internal standards. Before samples were loaded, the columns were washed with 0.6 mL of acetone:water (7:1, v/v) and equilibrated by rinsing twice with 2 mL of hexane. Each lipid extract dissolved in 300 μL of hexane:methyl tert–butylether:acetic acid (300:3:0.3, v/v/v) was loaded onto the column, and the following lipid classes were eluted sequentially. The CE fraction was eluted first with 5 mL of hexane and the TG fraction with 5 mL of hexane:chloroform:ethyl acetate mixture (100:5:5, v/v/v). The FFA fraction was eluted with 5 mL of chloroform:methanol:acetic acid (100:2:2, v/v/v). PL was eluted with 5 mL of methanol:chloroform:water (10:5:4, v/v/v) and the chloroform layer containing PL was separated by adding 2 mL water and 1 mL chloroform to this fraction. All fractions from liver lipid extracts and the PL fraction from bile lipids were collected in screw-cap tubes with Teflon lining for fatty acid analysis. The lipid fractions were evaporated under a stream of nitrogen. The lipids were hydrolyzed with 1 mL of 0.5 N methanolic NaOH in boiling water for 15 minutes. After cooling, fatty acids were saponified and methylated simultaneously with 2 mL of 14% BF_3-methanol, as described by Slover and Lanza[22] The fatty acid methyl esters (FAMEs) were redissolved in 150 μL of petroleum ether. Separation and quantitation of FAMEs were performed by capillary gas chromatography on a Hewlett-Packard model 5580A gas chromatograph (Hewlett-Packard, Palo Alto, CA USA) equipped with a Stabilwax-DA capillary column (15 m length × 0.53 mm inner diameter; Resteck Corp., Bellefonte, PA USA), flame ionization detector, and integrator.

Statistics

All statistical analyses were performed using PC SAS (SAS Institute, Cary, NC USA). Student's *t*-test was used to compare two group means.[23] Linear regression analysis was used to determine correlation between variables. Significance was determined at a *P*-value of less than 0.05.

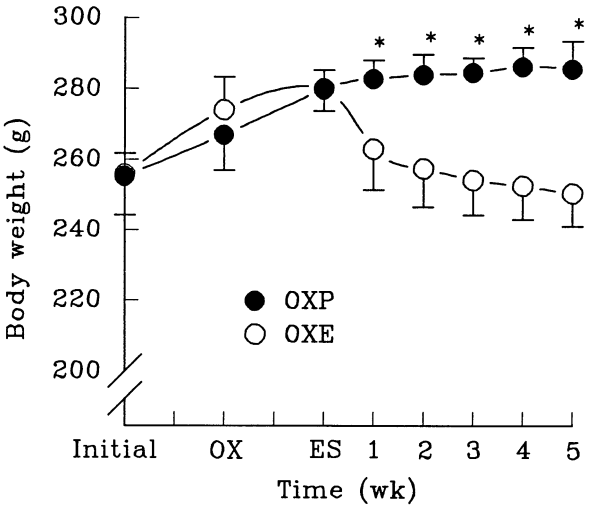

Figure 1 Time-course changes in average body weights following ovariectomy and estradiol implantation. At the start of the experiment, at the time of ovariectomy (OX), and at the time of estradiol implantation (ES), no significant differences were noted between groups in body weight. After estradiol implantation, significant differences were observed at 1 week and thereafter. The average body weight of the OXE group decreased for the first 2 weeks and thereafter remained unchanged, whereas the body weight of the OXP group increased gradually throughout the experiment. OXE–rats implanted with 17β-estradiol pellet. OXP–rats implanted with a placebo pellet. All values are expressed as means ± SD, $N = 5$. Asterisks (*) denote significant differences between groups at $P < 0.05$.

Results

Body weights

Figure 1 shows the effects of ovariectomy and estradiol replacement on body weights. At the start of the experiment and during recovery from ovariectomy, the body weights of rats did not differ between groups. After estradiol implantation, significant differences were observed between groups at 1 week and thereafter, despite the fact that their food intakes were matched by pair feeding. The average body weight of OXE rats decreased from 280 ± 6 g at the time of estradiol implantation to 263 ± 12 g at 1 week and 250 ± 9 g at 5 weeks. In contrast, the body weights of OXP rats continued to increase after placebo implantation and reached 285 g at 5 weeks.

Liver αTP, PL, cholesterol, and fatty acids

Table 2 shows that estradiol replacement significantly increased the concentrations of αTP in the liver. As expressed in nmol per gram of liver, the concentrations of αTP were 81.9 ± 3.5 nmol in OXE rats and 53.3 ± 7.5 nmol in OXP rats. The increase in liver αTP in OXE rats also was evident when it was expressed per milligram of total lipids. The molar ratios of αTP:PL and αTP:cholesterol were significantly higher in OXE rats than in OXP rats. No differences were observed between groups in the concen-

Table 2 The liver concentrations of α-tocopherol (αTP), phospholipid (PL), and cholesterol (CH) at 5 weeks of estradiol treatment*

	OXP	OXE
αTP		
nmol/g liver	53.3 ± 7.5	81.9 ± 3.5[a]
nmol/100 mg lipid	50.9 ± 3.4	69.7 ± 6.5[a]
αTP/PL (nmol/μmol)	1.4 ± 0.2	2.1 ± 0.2[a]
αTP/CH (nmol/μmol)	5.5 ± 0.6	8.0 ± 0.5[a]
PL		
μmol/g liver	39.1 ± 1.0	38.9 ± 1.4
μmol/100 mg lipid	37.7 ± 2.8	33.0 ± 2.1[a]
CH		
μmol/g liver	9.7 ± 0.5	10.2 ± 0.3
μmol/100 mg lipid	9.4 ± 0.6	8.7 ± 0.6
Liver		
Weight, g	6.1 ± 0.2	5.7 ± 0.6
Total lipid, mg/g	104.3 ± 8.0	118.3 ± 8.4[a]

*Mean ± SD, $N = 5$ per group.
[a]Significantly different from OXP ($P < 0.05$).
OXE–rats implanted with an estradiol pellet.
OXP–rats implanted with a placebo pellet.

trations of PL and cholesterol, as expressed in μmol per gram of liver, whereas the total lipid content of the liver was higher in OXE rats than in OXP rats.

Table 3 presents the concentrations of fatty acids present in various liver lipid classes, as expressed in μmol per gram of liver. A significantly greater amount of 16:0 was esterified to cholesterol in OXE rats (3.9 ± 0.8 μmol) than in OXP rats (2.2 ± 0.6 μmol). A slight but significant increase in 18:0 also was noted in OXE rats (0.5 ± 0.1 μmol) compared with OXP rats (0.4 ± 0.1 μmol). No significant differences were noted between groups in the concentrations of 18:1 and 18:2 in the CE fraction. Among the longer-chain polyunsaturated fatty acids (PUFA) in CE, 20:4n-6 was higher in OXE rats (0.46 ± 0.16 μmol) than in OXP rats (0.26 ± 0.09 μmol). Other PUFA including 18:3 and 22:6n-3 were not detectable in the CE fraction. Estradiol treatment produced no significant changes in the concentrations of individual fatty acids incorporated into TG. However, the total amount of fatty acids in the TG fraction was significantly higher in OXE rats (21.8 ± 1.3 μmol) than in OXP rats (14.2 ± 1.4 μmol). In the FFA fraction, the concentrations of all fatty acids except 18:1 and 18:3 were elevated significantly in OXE rats. The concentrations of 16:0 in OXE and OXP rats were 11.3 ± 3.0 μmol/g liver and 6.7 ± 2.3 μmol/g liver, respectively. The concentration of 18:0 also was increased in OXE rats but to a lesser extent. The most striking changes were observed in the PUFA profiles of the FFA fraction. The concentration of 20:4n-6 was significantly higher in OXE rats (4.2 ± 0.8 μmol) than in OXP rats (3.1 ± 0.6 μmol). The concentrations of 22:5n-3 and 22:6n-3 in the OXE group were increased three- and twofold, respectively, relative to those in OXP rats. Likewise, the concentrations of 22:5n-3 and 22:6n-3 esterified to liver PL also were significantly higher in OXE rats. However, the concentrations of 18:2 and 20:4n-6 were slightly but significantly lower in OXE rats than in OXP rats.

Table 3 The liver concentrations of fatty acids in various lipid classes at 5 weeks of estradiol treatment*

Lipid	OXP (μmol/g liver)	OXE (μmol/g liver)
Cholesteryl ester		
16:0	2.15 ± 0.58	3.85 ± 0.78[a]
18:0	0.36 ± 0.06	0.54 ± 0.08[a]
18:1	0.31 ± 0.09	0.57 ± 0.25
18:2	0.37 ± 0.17	0.82 ± 0.49
20:4	0.26 ± 0.09	0.46 ± 0.16[a]
Triglyceride		
16:0	4.97 ± 3.04	8.12 ± 2.94
18:0	0.39 ± 0.14	0.54 ± 0.18
18:1	4.10 ± 2.30	6.83 ± 2.03
18:2	4.20 ± 2.73	6.05 ± 2.51
20:4	0.44 ± 0.25	0.67 ± 0.32
22:6	0.07 ± 0.07	0.12 ± 0.05
Free fatty acid		
16:0	6.73 ± 2.26	11.27 ± 3.01[a]
18:0	2.91 ± 0.45	3.76 ± 0.38[a]
18:1	4.76 ± 2.31	7.80 ± 2.24
18:2	6.90 ± 3.60	13.83 ± 5.46[a]
18:3	0.32 ± 0.23	0.59 ± 0.31
20:4	3.13 ± 0.60	4.23 ± 0.76[a]
22:5	0.21 ± 0.10	0.55 ± 0.24[a]
22:6	0.52 ± 0.16	0.98 ± 0.17[a]
Phospholipid		
16:0	14.50 ± 1.23	12.84 ± 1.83
18:0	11.88 ± 0.58	12.48 ± 0.65
18:1	2.94 ± 0.28	2.55 ± 0.20[a]
18:2	6.99 ± 0.85	4.68 ± 0.81[a]
20:4	12.38 ± 0.93	10.21 ± 1.09[a]
22:5	0.62 ± 0.15	0.89 ± 0.18[a]
22:6	2.50 ± 0.33	3.52 ± 0.26[a]

*Mean ± SD, $N = 5$ per group.
[a]Significantly different from OXP ($P < 0.05$).
OXE–rats implanted with an estradiol pellet. OXP–rats implanted with a placebo pellet.

Biliary output of αTP

The rates of bile flow did not differ between groups. The average rates of bile flow in OXE and OXP rats were 0.95 ± 0.20 mL/hr and 0.99 ± 0.20 mL/hr, respectively. The total volumes of bile secreted for 8 hours were 7.6 ± 1.6 mL in OXE rats and 7.9 ± 1.6 mL in OXP rats. However, the biliary secretion of αTP was increased markedly in OXE rats. *Figure 2* illustrates the significant differences in αTP outputs that were observed between OXE and OXP rats at every hourly interval. The rate of biliary αTP secretion was significantly greater in OXE rats starting at 1 hour during intraduodenal infusion of a triolein emulsion. In OXE rats, the output of αTP rose sharply to the rate of 5.6 ± 0.6 nmol/hr at 2 hours and plateaued at 7.7 ± 1.3 nmol/hr at 5 hours. In contrast, in OXP rats, the average rate of αTP output was 3.5 ± 0.2 nmol/hr for the first 5 hours. It peaked at 4.7 ± 0.6 nmol/hr at 5 hours and failed to rise further with time. The total biliary outputs of αTP for 8 hours in OXE

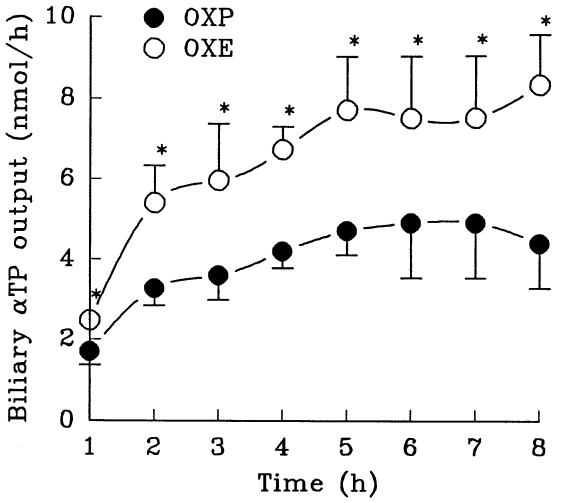

Figure 2 Hourly rates of biliary α-tocopheral (αTP) secretion. The rate of biliary αTP output was significantly greater in OXE rats than in OXP rats starting at 1 hour. In OXE rats, the output of αTP rose sharply to the average rate of 5.6 ± 0.6 nmol at 2 hours, whereas the average rate of αTP output in OXP rats was 3.5 ± 0.2 nmol/hr for the first 5 hours and failed to rise further with time. The total biliary outputs of αTP for 8 hours in OXE and OXP rats were 51.6 ± 3.6 nmol and 31.7 ± 2.9 nmol, respectively. All values are expressed as means ± SD, $N = 5$. OXE–rats implanted with 17β-estradiol pellet. OXP–rats implanted with a placebo pellet. Asterisks (*) denote significant differences between groups at $P < 0.05$.

and OXP rats were 51.6 ± 3.6 nmol and 31.7 ± 2.9 nmol, respectively.

Biliary outputs of PL, cholesterol, and fatty acids

Figure 3 shows the cumulative biliary outputs of PL and cholesterol, both of which were increased significantly by estradiol replacement. The total outputs of PL for 8 hours were 55.1 ± 4.9 μmol in OXE rats and 42.3 ± 4.7 μmol in OXP rats (*Figure 3A*). The average rates of PL secretion in OXE and OXP rats were 6.9 ± 0.6 μmol/hr and 5.3 ± 0.6 μmol/hr, respectively. The biliary secretion of cholesterol also was slightly higher ($P < 0.05$) in OXE rats (3.2 ± 0.2 μmol) than in OXP rats (2.8 ± 0.2 μmol; *Figure 3B*). The average rates of cholesterol output were 0.39 ± 0.03 μmol/hr in OXE rats and 0.34 ± 0.03 μmol/hr in OXP rats.

Table 4 shows the total biliary outputs of PL fatty acids for 8 hours. The outputs of all fatty acids except 18:2 were significantly higher in OXE rats than in OXP rats, reflecting the significant increase in biliary PL secretion in the OXE rats. The outputs of PL 16:0, 18:0, and 18:1 in OXE rats were increased by 19%, 32%, and 34%, respectively, relative to OXP rats. Among PUFA in the PL fraction, the output of 20:4 was increased significantly (32%) in OXE rats, with no significant change in 18:2. Unlike liver PL, biliary PL was devoid of 22:5n-3, regardless of estradiol treatment. Among all PL fatty acids, the cumulative output of 22:6n-3 for 8 hours was increased more markedly (214%) in OXE rats than OXP rats (*Figure 4*). The rate of biliary 22:6n-3 at 1 hour output was significantly greater in OXE (76.4 ± 22.4 nmol/hr) than in OXP rats (38.7 ± 20.0 nmol/hr; *Figure 4*). In OXE rats, it rose sharply to 340.7 ±

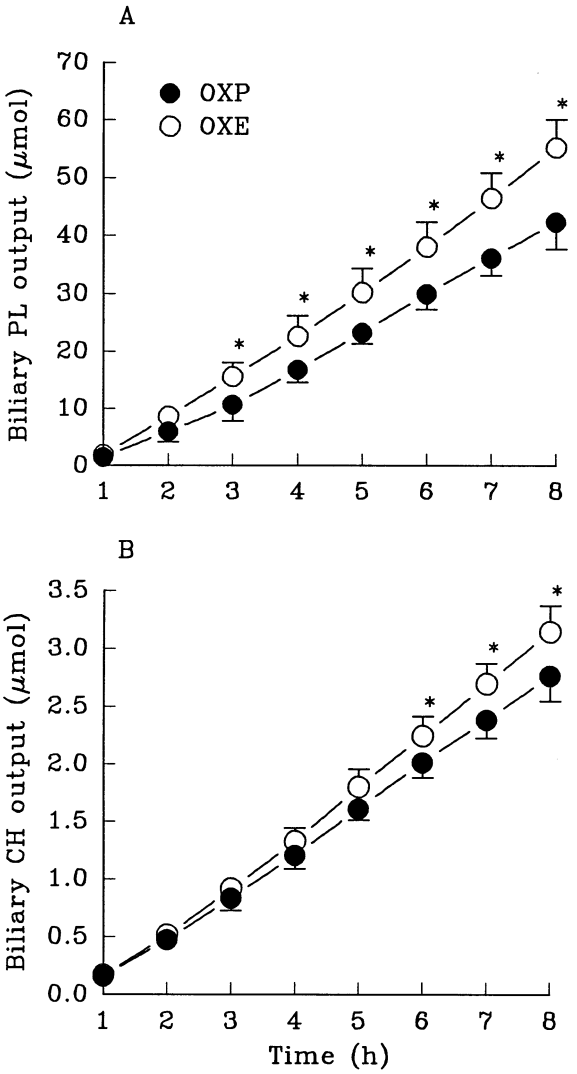

Figure 3 Cumulative biliary outputs of phospholipids (PL) and cholesterol (CH) for 8 hours. *(A)* The total outputs of PL were significantly greater in OXE rats (55.1 ± 4.9 μmol) than in OXP rats (42.3 ± 4.7 μmol). The average rates of PL secretion in OXE and OXP rats were 6.9 ± 0.6 μmol/hr and 5.3 ± 0.6 μmol/hr, respectively. *(B)* The biliary secretion of CH also was slightly but significantly higher in OXE rats (3.2 ± 0.2 μmol) than in OXP rats (2.8 ± 0.2 μmol). The average rates of CH output were 0.39 ± 0.03 μmol/hr in OXE rats and 0.34 ± 0.03 μmol/hr in OXP rats. All values are expressed as means ± SD, $N = 5$. OXE–rats implanted with 17β-estradiol pellet. OXP–rats implanted with a placebo pellet. Asterisks (*) denote significant differences between groups at $P < 0.05$.

55.1 nmol/hr at 2 hours and to 470.7 ± 49.2 nmol/hr at 8 hours. However, in OXP rats the rate of 22:6n-3 output increased to only 141.3 ± 32.2 nmol/hr at 2 hours and peaked at 229.8 ± 61.1 nmol/hr at 8 hours. The total biliary outputs of 22:6n-3 for 8 hours were 3.0 ± 0.2 μmol in OXE rats and 1.4 ± 0.2 μmol in OXP rats. The increased output of biliary PL 22:6n-3 in OXE rats reflected the marked increase in the hepatic concentration of PL 22:6n-3. The hourly rates of biliary 22:6n-3 secretion in both groups were correlated strongly ($r = 0.83$; $P < 0.05$) with those of biliary αTP output (*Figure 5A*). The hourly rates of αTP

Table 4 The cumulative outputs of biliary phospholipid (PL) fatty acids*

PL fatty acids	OXP (μmol/8 h)	OXE (μmol/8 h)
16:0	33.2 ± 3.1	39.5 ± 3.0[a]
18:0	7.3 ± 0.9	9.6 ± 0.6[a]
18:0	5.9 ± 1.1	7.9 ± 0.9[a]
18:2	24.0 ± 3.9	25.6 ± 3.9
20:4	13.4 ± 1.3	17.7 ± 1.9[a]
22:6	1.4 ± 0.2	3.0 ± 0.2[a]

*Mean ± SD, $N = 5$ per group.
[a]Significantly different from OXP ($P < 0.05$).
OXE–rats implanted with an estradiol pellet. OXP–rats implanted with a placebo pellet.

output were correlated significantly ($r = 0.83$; $P < 0.05$) with the rates of PL secretion (*Figure 5B*).

Discussion

This study presents new evidence that estradiol replacement in ovariectomized rats (1) markedly elevates the liver concentrations of αTP and PUFA incorporated into PL and (2) increases the rates and amounts of biliary αTP and PUFA secretion, reflecting the elevated levels of αTP and PUFA in the liver. Regardless of estradiol treatment, biliary PL contained higher concentrations of 16:0 (38 to 39%) and 18:2 (25 to 30%) than other fatty acids, which ranged from 1 to 18%. The pronounced increases in the biliary outputs of

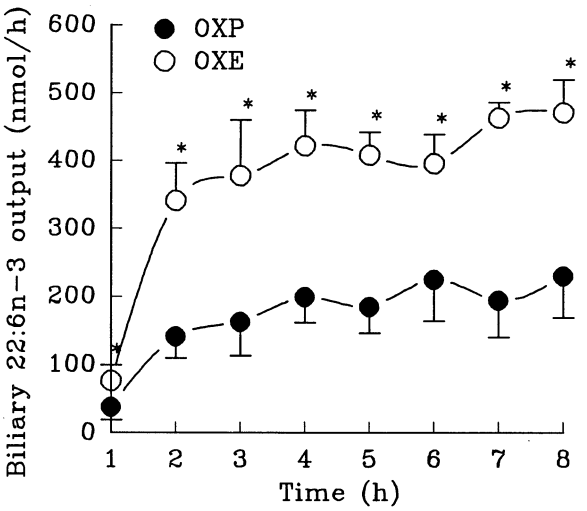

Figure 4 Hourly rates of biliary docosahexaenoic-acid (22:6n-3) secretion. The rate of biliary 22:6n-3 output was significantly greater in OXE rats (76.4 ± 22.4 nmol/hr) than in OXP rats (38.7 ± 20.0 nmol/hr) at 1 hour and thereafter. In OXE rats, it rose sharply to 340.7 ± 55.1 nmol/hr at 2 hours and to 470.7 ± 49.2 nmol/hr at 8 hours. In contrast, the rate of 22:6n-3 output in OXP rats increased to only 141.3 ± 32.2 nmol at 2 hours and reached 229.8 ± 61.1 nmol/hr at 8 hours. The increased output of biliary PL 22:6n-3 in OXE rats reflected a marked increase in its hepatic concentration. All values are expressed as means ± SD, $N = 5$. OXE–rats implanted with 17β-estradiol pellet. OXP–rats implanted with a placebo pellet. Asterisks (*) denote significant differences between groups at $P < 0.05$.

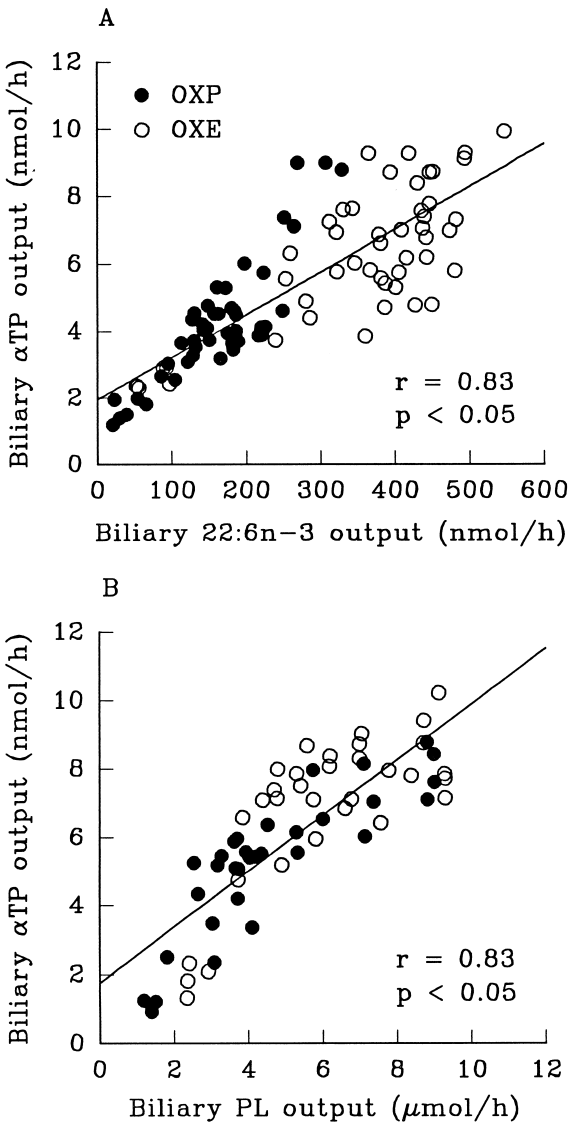

Figure 5 Correlations between the hourly rates of biliary outputs of α-tocopherol (αTP), phospholipids (PL), and PL 22:6n-3. The hourly rates of αTP output were correlated strongly ($r = 0.83$; $P < 0.05$) with the rates of biliary 22:6n-3 output *(A)* and also with the rates of PL secretion ($r = 0.83$; $P < 0.05$) *(B)*.

PL 16:0 and 18:2 in estradiol-treated rats were due primarily to the increase in the total secretion of PL. The relative concentrations (mol%) of these fatty acids in PL were not affected significantly. This observation is in line with the previous finding that the PC containing 16:0 and 18:2 is a predominant molecular species of liver PC and is preferentially secreted into the bile.[24,25] Among the PUFA incorporated into PL, the hepatic concentrations of docosapentaenoic acid (22:5n-3) and docosahexaenoic acid (22:6n-3) were increased significantly in estradiol-treated rats. Likewise, the biliary output of PL 22:6n-3 was elevated most markedly (over 200%) in those rats and output of 20:4n-6 increased moderately (32%). Interestingly, biliary PL was found to be devoid of 22:5n-3. In the liver, 20:4n-6 is produced from 18:2, whereas 22:5n-3 and 22:6n-3 are

synthesized from 18:3n-3 by chain elongation and desaturation. In addition, 22:6n-3 may be retroconverted to shorter chain PUFA.[26] The diet used in the present study contained 7.0% soybean oil and supplied the precursor fatty acids 18:2 and 18:3n-3 at 38.4 mg/g diet and 5.5 mg/g diet, respectively. However, the estradiol-induced increases in those PUFA in the liver and bile were not likely due to a possible stimulation of liver desaturase activities. Previous work[27] showed that estradiol treatment in ovariectomized rats significantly decreased the liver microsomal activity of delta-6 desaturase, a key regulatory enzyme, in the synthesis of 20:4n-6 and 22:6n-3 from 18:2 and 18:3n-3, respectively. Furthermore, the activities of delta-6 and delta-5 desaturases also were inhibited significantly in isolated hepatocytes when preincubated with estradiol.[28]

The present data provide the first evidence that estradiol replacement significantly increases the rates and the amounts of biliary PL secretion and that the biliary outputs of PL and PUFA are correlated strongly ($r = 0.83$) with αTP output (*Figure 5*). These findings are consistent with the recent evidence that biliary secretions of PC and αTP across the hepatocyte canalicular membrane in rodents are mediated by a common transporter, mdr2 P-glycoprotein (mdr2 Pgp), which is one of the multidrug resistance (mdr) gene products.[29,30] Direct evidence for the specific role of mdr2 Pgp as PC translocase has been provided by the recent observation that bile from the mdr2 knockout mouse is almost devoid of PC, whereas the secretion of bile acids remains unaffected.[29] Recently, Mustacich et al.,[31] using the mdr2 knockout mice, showed that the biliary secretion of αTP also is dependent on the presence of mdr2 Pgp and that the amounts of PC and αTP secreted into bile are closely related. The simultaneous increases in biliary secretion of αTP and PL high in PUFA in estradiol-treated rats strongly suggest that the hepatic canalicular membrane becomes enriched with αTP and PC high in PUFA by estradiol replacement. The antioxidant effect of estradiol in vivo may be mediated partly by enriching the liver membranes with αTP, thereby protecting membrane lipids (i.e., PL) against peroxidation.

At present, the mechanism whereby estradiol enhances the liver concentrations of αTP is unknown. However, in our recent study,[32] we observed a pronounced increase in the lymphatic absorption of αTP (25 ± 3%) in ovariectomized rats with estradiol replacement compared with those without estradiol replacement (17 ± 4%). The increase in αTP absorption was correlated strongly ($r = 0.71$; $P < 0.0001$) with the lymphatic output of PL. Biliary PL, which is mostly PC, is the major contributor to chylomicron PC, and a sufficient supply of PC via the biliary route is essential for intestinal production of normal chylomicrons, which are secreted into the lymphatics.[33,34] The amount of PC synthesized de novo in the enterocyte is not sufficient for chylomicron synthesis during fat absorption.[35] Evidence also suggests that the PUFA moiety of biliary PC is important for production of appropriate chylomicron PL coat. The lack of PUFA in biliary PL has been shown to result in defective chylomicron formation and impaired fat absorption.[36] Thus, the increased biliary output of PL enriched with PUFA, as observed in estradiol-treated rats, may be a factor contributing to the increased absorption of αTP via chylomicrons. The increased release of αTP into the bile may protect the PUFA-enriched PC against peroxidation in the intestinal lumen, thereby facilitating the enterohepatic recycling of αTP and PC. The pronounced increase in αTP absorption, as produced by estradiol replacement, may explain the significantly elevated concentrations of αTP in various tissues of intact female rats[1–3] and estradiol-treated ovariectomized rats.[4,5]

Another possible mode of estradiol action may reside in its inherent antioxidant properties. The antioxidant activity of estradiol is attributed to its phenolic hydroxyl (-OH) group, which is capable of reducing peroxy radicals. The possibility that estradiol may act as an antioxidant has been suggested by studies involving lipoproteins. Under various in vitro conditions, estradiol and its metabolites have been shown to inhibit the oxidation of low density lipoproteins and HDL.[10–13,37–39] Recent studies also have shown that estradiol partitions effectively into the membrane PL bilayer and interacts with membrane lipids and receptors, eliciting its membrane-mediated actions via nongenomic pathways.[40,41] The hepatocyte membrane has been shown to bind and accumulate estradiol.[42,43] Golden et al.[40] postulated that the cell membrane acts as a sink for steroid hormones including estradiol, which allows their membrane concentrations to be higher than their circulating levels. The antioxidant effect of estradiol on liver microsomal membranes has been demonstrated.[14,44] Under in vitro conditions,[9] estradiol also has been shown to regenerate tocopherols from tocopheroxyl radicals. Such an effect of estradiol, if it occurs on cell membranes in vivo, may prevent the irreversible oxidation of αTP, thus sparing the vitamin. These observations suggest the possibility that estradiol singly, or via interaction with αTP, plays a role in protecting membrane lipids (i.e., PL) against oxidative damage.

Conclusion

The present study demonstrates that estradiol replacement in ovariectomized rats markedly increases the hepatic concentration and biliary secretion of αTP. Estradiol replacement also elevates the liver concentrations of PUFA incorporated into PL. The biliary secretion of αTP and the fatty acid makeup of biliary PL in estradiol-treated rats reflect the elevated levels of αTP and PUFA in the liver. These results suggest that estradiol may protect the hepatic PL and membranes against oxidative damage by improving the liver status of αTP. The simultaneous increases in the biliary outputs of αTP and PL in estradiol-treated rats may serve as a means of protecting the PUFA of biliary PL against peroxidation in the intestinal lumen, thereby enhancing the reabsorption and redistribution of both αTP and PL via the enterohepatic route. Further studies are needed to elucidate the mechanisms underlying the antioxidant action of estradiol in vivo and the biochemical implications of the increases in hepatic PL 22:5n-3 and 22:6n-3 and the decreases in 18:2n-6 and 20:4n-6 in estradiol-treated rats.

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