Ethinylestradiol Administration Selectively Alters Liver Sinusoidal Membrane Lipid Fluidity and Protein Composition†

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ABSTRACT: Administration of high-dose ethinylestradiol to rats decreases bile flow, Na,K-ATPase specific activity, and liver plasma membrane fluidity. By use of highly purified sinusoidal and bile canalicular membrane fractions, the effect of ethinylestradiol administration on the protein and lipid composition and fluidity of plasma membrane fractions was examined. In sinusoidal fractions, ethinylestradiol (EE) administration decreased Na,K-ATPase activity (32%) and increased activities of alkaline phosphatase (254%), Mg^{2+}-ATPase (155%), and a 160-kDa polypeptide (10-fold). Steady-state and dynamic fluorescence polarization was used to study membrane lipid structure. Steady-state polarization of diphenylhexatriene (DPH) was significantly higher in canalicular compared to sinusoidal membrane fractions. Ethinylestradiol (5 mg/kg per day for 5 days) selectively increased sinusoidal polarization values. Similar changes were demonstrated with the probes 2- and 12-anthroyloxystearate. Time-resolved fluorescence polarization measurements indicated that EE administration for 5 days did not change DPH lifetime but increased the order component (r_∞) and decreased the rotation rate (R). However, 1 and 3 days after EE administration and with low doses (10–100 μg/kg per day for 5 days) the Na,K-ATPase, bile flow, and order component were altered, but the rotation rate was unchanged. Vesicles prepared from total sinusoidal membrane lipids of EE-treated rats, as well as phospholipid vesicles, demonstrated increased DPH polarization, as did intact plasma membrane fractions. Liver plasma membrane fractions showed no change in free cholesterol or cholesterol/phospholipid molar ratio, while esterified cholesterol content was increased with high-dose but not low-dose ethinylestradiol. High-dose ethinylestradiol treatment produced minor changes in phospholipid polar head groups and fatty acids; however, neither the sphingomyelin/phosphatidylcholine ratio nor the percent of saturated fatty acids was altered. In summary, the sinusoidal liver plasma membrane order component was selectively increased with low- and high-dose ethinylestradiol treatment. This structural change is due to changes in polar rather than neutral lipid composition. These results indicate estrogens may selectively alter plasma membrane lipid and protein domains, and these changes may be integral to the pathogenesis of intrahepatic cholestasis.

Administration of 17α-ethinylestradiol (EE),[1] an estrogenic steroid commonly used in birth control pills, is associated with intrahepatic cholestasis in man and animals (Plaa & Priestly, 1977; Schreiber & Simon, 1983; Metreau & Dhumeaux, 1972). In rats, high doses of EE reduce bile flow and the liver's maximum capacity to excrete organic anions such as bile salts, bilirubin, and bromosulfophthalein (Kaplowitz et al., 1986; Simon & Arias, 1973; Gumucio & Vadivieso, 1971). Previous studies, using both electron spin resonance and fluorescence polarization techniques, have reported that liver plasma membrane fluidity is reduced following EE administration (Davis et al., 1979; Keefe et al., 1979; Storch & Schachter, 1984). The lipid components responsible for this change have been variously attributed to increases in cholesterol and/or cholesterol esters (Davis et al., 1979; Keefe et al., 1979; Simon et al., 1980). Examination of this model has focused attention on the liver surface membrane and, in particular, the canalicular domain as the primary site of abnormalities in the pathogenesis of bile secretory failure (Simon & Arias, 1973). However, other observations suggest the sinusoidal domain may also be abnormal. These observations include decreases in Na,K-ATPase activity (Davis et al., 1979; Reichen & Paumgartner, 1977; Keefe et al., 1979), increased content of low-density lipoprotein receptors (Kovanen, et al., 1979; Ma et al., 1986), and decreased taurocholate uptake using isolated hepatocytes from EE-treated rats (Berr et al., 1984).

The hepatocyte is a polarized epithelial cell with at least two major domains: the sinusoidal/lateral (SM) and the bile canalicular (BCM) (Evans, 1980). The sinusoidal domain is specialized for exchange processes with the blood and contains peptide receptors, cation pumps such as Na,K-ATPase, and the Na–H exchanger (Blitzer & Boyer, 1978; Arias & Forgac, 1984). The bile canalicular or apical domain is specialized for excretion of bile components (Inoue et al., 1983). In addition to domain-specific localization of integral membrane proteins, lipid components and fluidity are also highly polarized (Meier et al., 1984; Schachter, 1984).

Because previous studies have used liver plasma membrane fractions containing various amounts of both sinusoidal and canalicular domains, the significance of the changes in the lipid

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[1] Abbreviations: SM, sinusoidal membrane; BCM, bile canalicular membrane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EE, 17α-ethinylestradiol; DPH, 1,6-diphenyl-1,3,5-hexatriene; 2AS, DL-2-(9-anthroyloxy)stearic acid; 12AS, DL-12-(9-anthroyloxy)stearic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate-buffered saline; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; SPH, sphingomyelin; NS, not statistically different; LDL, low-density lipoprotein.

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components and structure caused by EE and the degree to which these changes contribute to the induced cholestasis could not be determined. This study was, therefore, designed to investigate what changes in plasma membrane fluidity and lipid and protein composition occur in highly purified fractions of sinusoidal and canalicular membrane fractions following administration of ethinylestradiol. The questions addressed are the following: (1) Does EE administration alter protein and lipid properties of the SM, BCM, or both surface membrane domains? (2) What lipid abnormality is responsible for decreased liver surface membrane fluidity?

The results of these investigations demonstrate that SM but not BCM from EE-treated rats has a lower lipid fluidity than membranes prepared from pair-fed controls. The difference in fluidity appears to be due to alterations in the phospholipid fraction and not neutral lipids. Furthermore, the decrease in Na,K-ATPase activity is related to increased membrane lipid order and unrelated to increases in esterified cholesterol.

Materials and Methods

Animals. Male Sprague-Dawley rats (Harlon, Indianapolis, IN) weighing 180–220 g were used in all experiments. The control group, which received corn oil injections, was given the same quantity of food consumed by the litter-matched animals, which were injected daily with EE dissolved in corn oil. Except where indicated in the tables, a dosage of 5 mg/kg per day for 5 days was given. Treated animals were given free access to standard rat chow (Purina) and water. All animals were housed under controlled conditions of temperature, humidity, and light (12-h cycle). After each treatment period, animals were fasted 12 h and sacrificed under ether anesthesia at 7:00 a.m.

Chemicals. 17α-Ethinylestradiol and other organic chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). All inorganic chemicals or solvents were purchased from Fisher Scientific Co. (Fair Lawn, NJ) and were of the highest grade available.

Preparation and Isolation of Liver Membranes. Liver sinusoidal and canalicular membranes were prepared simultaneously from the same liver homogenates by using a modification of a method previously developed in our laboratory to isolate renal plasma membranes (Molitoris & Simon, 1985). Briefly, thin liver slices were added to chilled buffer (300 mM mannitol, 5 mM EGTA, 18 mM Tris-HCl, 0.1 mM PMSF, at pH 7.4). The slices were homogenized by using a polytron (Brinkman Co.) in 15 mL of buffer for 45 s. The polytron power output was closely regulated at 4800 rpm with a powerstat (Kimematica GmbH, Switzerland, with ST-10 tip). The solution was centrifuged at 48000*g* for 30 min. The resulting pellet was resuspended in buffer, and 15 mM Mg^{2+} precipitation was followed by centrifugation for 15 min at 2445*g*. The pellet was saved for sinusoidal membrane isolation while the supernatant was centrifuged at 48000*g* for 30 min to obtain the canalicular fraction.

Sinusoidal membranes were isolated from the initial Mg^{2+} precipitation pellet on a discontinuous sucrose gradient using 41% (5 mL) and 37.5% (12 mL) as overlay layers in cellulose–acetate tubes centrifuged at 88000*g* for 3 h in a Beckman Model L8-70 ultracentrifuge and SW 28 rotor. The float layer was carefully harvested (no more than 2-mL volume) from the top of the discontinuous gradient. Both fractions were washed and stored in 0.5 mL of 1 mM $NaHCO_3$ at −2 °C.

Enzyme Assays. Na,K-ATPase was measured after freeze–thawing by using an enzyme-coupled kinetic assay with pyruvate kinase and lactate dehydrogenase (Schoner et al., 1967). Other enzyme assays were measured by using standard assays: alkaline phosphatase by (*p*-nitrophenyl phosphate (Bessey et al., 1946); leucine aminopeptidase (Goldberg & Ratenburg, 1958); cytochrome *c* reductase (Baron & Tephly, 1969); succinic dehydrogenase (Seubert, 1965); *N*-acetylglucosaminidase (Scalera et al., 1980); and UDP-galactosyltransferase (Ahnen et al., 1982). Enzyme activities were measured within 24 h after sample preparation. Assays employed a Beckman Model 25 spectrophotometer equipped with a kinetic unit. Protein was estimated by using crystalline bovine serum albumin (Sigma) as standard (Lowry et al., 1951).

Chemical Analysis. Total lipids were extracted from membrane fractions by the method of Bligh and Dyer (1959). Cholesterol, cholesterol esters, and fatty acids were quantitated by chromatographic methods after derivatization as previously described (Polokoff et al., 1983). Gas–liquid chromatogram peak areas were analyzed by using an automatic integrator after identification of individual peaks by coretention with standard compounds. Total phospholipids (Ames & Dubin, 1960) and individual species after separation by two-dimensional thin-layer chromatography (Esko & Raetz, 1980) were determined as previously described (Molitoris & Simon, 1985).

Serum bile acid measurements on blood obtained from the aorta at time of sacrifice were performed by gas chromatography–mass spectrophotometry as described (Everson, 1987). Serum cholesterol was measured on an autoanalyzer (Allain et al., 1974). Fractionation of total lipid extracts into neutral and polar lipids was performed by silica acid column separation as previously described (Harris et al., 1984; Polokoff et al., 1985).

Gel Electrophoresis. Sinusoidal and canalicular membrane fractions were electrophoresed on 5% SDS–polyacrylamide slab gels (130 × 200 mm). Samples (200 μg) were solubilized with 5% SDS, as previously described (MacKinnon et al., 1977). Gels were stained with Comassie Brilliant blue, and peak height density was quantitated by laser scanning densitometry.

Fluorescence Polarization Studies. Fluorescence polarization and lifetime measurements were done on a 4800 phase-correlation nanosecond polarization spectrofluorometer (SLM Industries, Urbana, IL) with fixed emission and excitation polarization filters. The fluorescence intensity was measured perpendicular ($I_\perp$) and parallel ($I_\parallel$) to the polarization phase of the exciting light to eliminate incidental scattered light by using an excitation wavelength of 360 nm and KV389 emission filters (Schoh, Inc., Dwyer, PA). Nonsignificance of membrane vesicle light scattering was verified by insertion of sample without probe. All samples were run at 35 °C with slits at 4, 4, and 8. The probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes, Junction City, OR), was dissolved in tetrahydrofuran to a final concentration of 0.6 μg/mL. Probes were added to intact membranes in a total volume of 1.2 mL (containing 72 μg of protein) and frequently vortexed for 12–15 min at 35 °C. Fluorescence lifetimes and dynamic depolarization measurements were determined with the same instrument at a frequency of 30 mHz with slits from lamp to sample of 16, 0.5, and 0.5 nm by using DPH in hexadecane (9.62 ns) as a lifetime reference solution. Both phase and modulation lifetimes were measured, and values reported are the mean ± SE. Additional fluorophores were measured by using a HH-1 T format polarization spectrofluorometer (BHL Associates, Burlingame CA) (Polokoff et al., 1985); DL-2-(9-anthroyloxy)stearic acid (2AS) and DL-12-(9-anthroyloxy)stearic acid (12AS) (Molecular Probes, Inc.). The maximal limiting anisotropy taken

Table I: Effect of Ethinylestradiol Administration on Liver and Body Weight and Serum Lipids[a]

	wt gain (g)	LW/BW (%)	serum cholesterol (mg/dL)	serum bile acid (μmol/dL)
control	+10 ± 2	3.9 ± 0.1	85 ± 5	6.7 ± 4
EE[b]	+5 ± 2[c]	4.5 ± 0.1[c]	0.2 ± 0.1[c]	15.5 ± 3[c]

[a] n = 6 in each group. [b] 5 mg/kg for 5 days. [c] P value <0.005.

for DPH was 0.365 (Shinitzky & Barenholz, 1974) and was 0.285 for the anthroyloxy probes (Schachter & Shinitzky, 1977).

Fluorescence polarization measurements were also carried out on multilamellar vesicles prepared from total lipid and polar lipid fractions. The lipid extracts were dried under a stream of nitrogen, fluorescent probes added, and vesicles formed in PBS by vortexing and sonication, as described previously (Polokoff et al., 1985). The amount of total lipid extract or polar lipid fraction used was calculated to be equivalent to the amount contained in 72 μg of initial membrane protein.

Analysis. The steady-state polarization (P) is defined by

$$P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp}) \quad (1)$$

in which $I_{\parallel}$ and $I_{\perp}$ are the components of the fluorescent intensity parallel and perpendicular to the exciting polarization direction, respectively. Anisotropy (r) was determined from

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp}) \quad (2)$$

Measured values for lifetimes (τ) and the tangent of the phase difference of the sinusoidally modulated light (tan Δ) were used to calculate the dynamic (R) component of membrane lipid fluidity from the equation (Lakowicz, 1983)

$$(m \tan \Delta)(2R\tau)^2 + (C \tan \Delta - A)(2R\tau) + (D \tan \Delta - B) = 0 \quad (3)$$

where

$$A = 3B = \omega\tau(r_0 - r)$$

$$C = \tfrac{1}{3}(2r - 4r^2 + 2)$$

$$D = \tfrac{1}{9}(m + m_0\omega^2\tau^2)$$

$$m = (1 + 2r)(1 - r)$$

$$m_0 = (1 + 2r_0)(1 - r_0)$$

$$r_0 = 0.392$$

ω = frequency k (at 30 mHz = 0.1885)

This value for R was used in the equation (Lakowicz, 1983)

$$r = r_\infty + (r_0 - r)/6R\tau \quad (4)$$

to calculate r_∞, the hindered component of anisotropy.

All results are expressed as mean ± SE. A two-tailed Student's t-test was used to compare differences between components examined. Values were considered significantly different if the P value was <0.05.

Results

In Vivo Measures. Rats were pair fed for 5 days, since EE treated rats eat less than untreated controls (Weinstein et al., 1986). Although rats treated with EE gained weight, it was significantly less than pair-fed controls (Table I). As previously reported without dietary control, the liver weight to body weight ratio for EE-treated rats was increased compared to that of control animals, serum cholesterol was markedly decreased, and serum taurocholate levels were significantly increased (Simon et al., 1980; Davis & Roheim, 1978; Chiorantini et al., 1979). Thus, changes in serum lipids and hepatic function are independent of food intake but rather indicate an effect of EE treatment.

Isolation of Liver Plasma Membrane Fractions. The relative enzyme enrichments and percent recoveries for marker enzymes in sinusoidal and canalicular membrane fractions are shown in Table II. In control SM fractions, Na,K-ATPase is enriched 24-fold, while the canalicular marker enzymes, leucine aminopeptidase, Mg^{2+}-ATPase, and alkaline phosphatase, are increased 8–14-fold. In contrast, BCM fractions are enriched 24-fold in leucine aminopeptidase, 40-fold in Mg^{2+}-ATPase, and 34-fold in alkaline phosphatase but deenriched in activities of Na,K-ATPase. Cytochrome c reductase (microsomal enzyme marker), N-acetylglucosaminidase (lysosomal enzyme), and succinic dehydrogenase (inner mitochondral membrane enzyme) were deenriched or minimally enriched in both plasma membrane subfractions. UDP-galactosyltransferase activity was not detected in either fraction, from either control or treated animals (data not shown). These marker enzymes show similar enrichment of activities in sinusoidal membrane fractions after EE treatment. In the bile canalicular membrane fraction relative enrichment and percent recovery of alkaline phosphatase activity were significantly reduced. The explanation for this selective loss is unclear, but it is not due to loss of BCM recovery or intracellular contamination since leucine aminopeptidase and Mg^{2+}-ATPase recovery was unchanged. Rats treated for 1 and 3 days demonstrated similar values (data not shown). These results suggest that ethinylestradiol treatment did not alter the recovery and purity of plasma membrane fractions.

Enzyme specific activities for homogenates and SM and BCM fractions are shown in Table III. Na,K-ATPase specific activity was significantly reduced in both homogenate (28%) and in SM (32%) by EE treatment. In contrast, Mg^{2+}-ATPase and alkaline phosphatase specific activities were significantly increased with homogenates (17.5% and 60%, respectively). Surprisingly, these increased enzyme activities were found in

Table II: Enzyme Enrichment and Percent Recovery in Sinusoidal and Canalicular Membrane Fractions from Control and Ethinylestradiol-Treated Rats

	control[a]		ethinylestradiol[c]		control[a]		ethinylestradiol[c]	
	RE[b]	recovery (%)	RE[b]	recovery (%)	RE[b]	recovery (%)	RE[b]	recovery (%)
Na,K-ATPase	24 ± 1	28 ± 3	24 ± 2	26 ± 4	1 ± 1	0.3 ± 0.2	1 ± 1	0.8 ± 1
leucine aminopeptidase	8 ± 1	11 ± 1.2	9 ± 1	10 ± 2	24 ± 2	8 ± 1	25 ± 1	12 ± 2
Mg^{2+}-ATPase	8 ± 2	12 ± 3	11 ± 3	13 ± 2	40 ± 6	13.2	28 ± 4[+]	12 ± 2
alkaline phosphatase	14 ± 2	19 ± 5	14 ± 3	17 ± 6	34 ± 5	12 ± 2	10 ± 3*	4.5 ± 0.9[d]
cytochrome c reductase	2 ± 0.4	1.4 ± 1.4	1 ± 0.4	0.4 ± 0.5	1 ± 0.3	1.2 ± 0.7	1 ± 0.2	0.7 ± 0.2
succinic dehydrogenase	2 ± 1	2.8 ± 3.0	0.6 ± 0.4	1.1 ± 0.9	0.7 ± 0.2	<0.1	0.6 ± 0.3	<0.1
N-acetylglucosamidase	2.0 ± 1	0.3 ± 0.3	0.9 ± 1.0	0.4 ± 0.3	0.6 ± 0.1	<0.1	0.5 ± 0.5	<0.1

[a] Control: n = 6. Values represent means ± SE. [b] RE: relatve enrichment (membrane fraction/homogenate enzyme specific activities). [c] 5 mg/kg for 5 days. n = 3–12. Values represent mean ± SE. [d] P <0.005.

Table III: Enzymatic Specific Activities[a] in Sinusoidal and Canalicular Fractions from Control and Ethinylestradiol-Treated Rats

enzyme	homogenate	sinusoidal	canalicular
Na,K-ATPase			
control[b]	2.05 ± 0.1	48.5 ± 1.1	3.7 ± 2.7
EE[c]	1.47 ± 0.1[d]	33.2 ± 2.4[e]	2.7 ± 2.7 (NS)[f]
Mg^{2+}-ATPase			
control[b]	3.3 ± 0.3	29 ± 2.4	132 ± 5.7
EE[c]	4.0 ± 0.2[e]	45 ± 2.9[e]	110 ± 6.8[d]
alkaline phosphatase			
control[b]	0.9 ± 0.6	13 ± 0.6	31 ± 2.4
EE[c]	2.3 ± 0.2[e]	33 ± 3.4[e]	22 ± 3.4 (NS)[f]
leucine aminopeptidase			
control[b]	10.1 ± 0.4	84.6 ± 10.2	243 ± 18.5
EE[c]	9.8 ± 3.5 (NS)[f]	82.9 ± 6.5 (NS)[f]	249 ± 10.8 (NS)[f]

[a] μmol h^{-1} (mg of protein)$^{-1}$. Values represent mean ± SE. [b] Control: $n = 6$. [c] EE: ethinylestradiol 5 mg/kg × 5 days, $n = 12$. [d] $P < 0.025$. [e] $P < 0.005$. [f] NS: differences not statistically different.

the SM fraction, while BCM was either significantly decreased (Mg^{2+}-ATPase) or unchanged (alkaline phosphatase) by EE. Leucine aminopeptidase activity was unaltered in all fractions by EE treatment. SM changes were not due to BCM contamination since enrichment of leucine aminopeptidase, a specific BCM enzyme (Roman & Hubbard, 1984), was not changed by the treatment protocol.

Gel Electrophoresis. SM and BCM membrane fractions were analyzed by SDS–PAGE, and EE was found to selectively alter the polypeptide pattern of sinusoidal proteins (Figure 1). Quantitation by densitometry of the Coomasie Blue stained bands indicated no significant alterations except for a consistent 10-fold increase, compared to control, in a polypeptide band with an apparent size of 160 kDa. No significant change in the BCM (Figure 1) polypeptide band pattern was apparent.

Lipid Membrane Fluidity of Liver Fractions. The effect of ethinylestradiol administration on membrane fluidity observed by using steady-state DPH fluorescence polarization with sinusoidal and canalicular membrane fractions is shown in Table IV. Membrane fractions from control animals each demonstrate unique physical characteristics. As others have shown (Whetton, et al., 1983; Lowe & Coleman, 1982; Storch et al., 1983), the BCM was the least fluid. After EE administration, however, only the SM fraction was altered. This membrane domain became more rigid while the BCM (Table IV) and microsomal fractions (data not shown) were unchanged.

Also shown in Table IV, the SM after EE treatment was significantly less fluid than control membranes, as assessed by steady-state fluorescence polarization using 2AS and 12AS probes. These fluorophores differ from DPH in structure and shape and localize in different domains of the bilayer (Schachter, 1984). Thus, lipid structural and dynamic changes produced by EE involve surface as well as core components

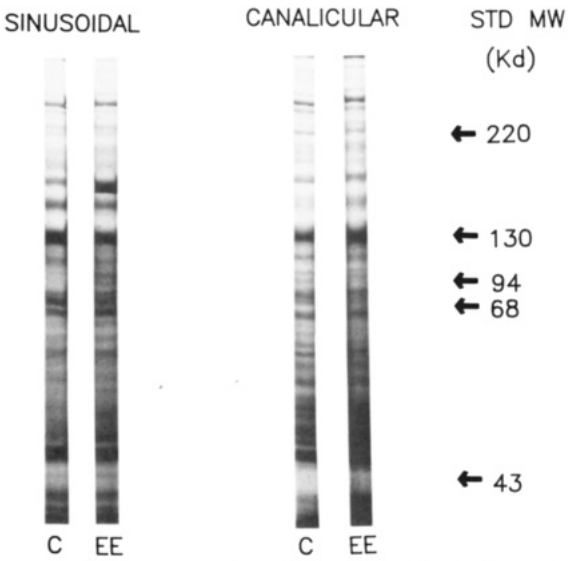

FIGURE 1: SDS–page of sinusoidal and canalicular liver plasma membrane fractions. Ethinylestradiol (5 mg/kg × 5 days) was administered, and SM and BCM fractions were prepared as described under Materials and Methods. Samples were separated in 5% acrylamide containing 0.1% SDS and 6 M urea by slab gel electrophoresis. Molecular markers (MW) were run simultaneously. Gels were stained with Coomasie Brilliant Blue, and the molecular size of bands was estimated from semilogarithmic plots of standards. EE = ethinylestradiol; C = pair-fed control.

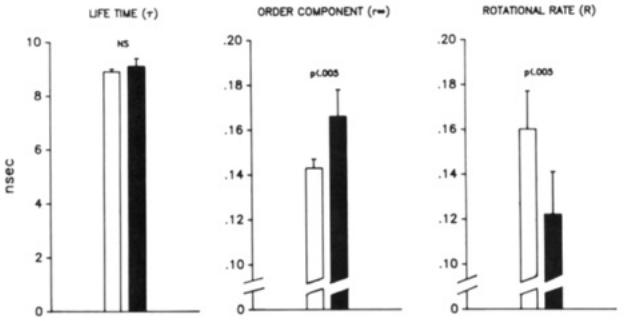

FIGURE 2: Dynamic depolarization measurements of sinusoidal fractions. SM prepared and fluidity components measured as described in text. (Open bars) Control ($n = 6$); (solid bars) EE (5 mg/kg × 5 days) ($n = 10$). Mean ± SE. Rotational rate units, ns^{-1}.

of the SM (Storch & Schachter, 1984). Again, no significant changes in polarization measurements were detected in BCM fractions from EE-treated rats.

Membrane fluidity is generally considered to consist of two major types of motion (Schachter, 1984; Harris & Simon, 1987). The first component is the order parameter r_∞, which is the structural component and relates to the packing of fatty acid acyl chains; the second is referred to as the rotational rate (R) or the dynamic/kinetic component. These components may change independently and thus selectively alter function. Figure 2 demonstrates the effect of EE administration on fluorescence lifetime (τ_f) and the two major components of fluidity in sinusoidal membrane fractions using DPH as the

Table IV: Steady-State Polarization Measurements in Intact Liver Plasma Membrane Fractions[a]

	sinusoidal fraction		canalicular fraction	
probe	control[b]	EE[c]	control[b]	EE[c]
DPH	0.255 ± 0.002	0.267 ± 0.007	0.284 ± 0.010	0.282 ± 0.014 (NS)[d]
2AS	0.188 ± 0.004	0.211 ± 0.005	0.196 ± 0.001	0.195 ± 0.006 (NS)[d]
12AS	0.155 ± 0.004	0.163 ± 0.002	0.163 ± 0.001	0.161 ± 0.008 (NS)[d]

[a] Values represent mean ± SE. [b] Control: $N = 3$. [c] EE = ethinylestradiol 5 mg/kg × 5 days, $n = 4$. [d] NS: differences were not statistically different.

Table V: Change in Sinusoidal Lipid Structure and Hepatic Function: Time Course of Ethinylestradiol Administration[a]

	control	days of ethinylestradiol treatment		
		1	3	5
bile flow [μL min^{-1} (g of liver)$^{-1}$]	2.5 ± 0.3 (3)	1.8 ± 0.1[b] (3)	1.6 ± 0.2[b] (3)	1.5 ± 0.3[b] (3)
Na,K-ATPase sp act.	48.5 ± 1.1 (6)	37.3 ± 1.5 (4)	35.2 ± 3.0 (4)	33.2 ± 2.4 (12)
r_∞	0.143 ± 0.004 (6)	0.154 ± 0.002[c] (4)	0.170 ± 0.005[b] (4)	0.166 ± 0.008[b] (10)
R	0.149 ± 0.005 (6)	0.152 ± 0.005 (4)	0.150 ± 0.005 (4)	0.122 ± 0.019[b] (10)

[a] Values represent mean ± SE. n is given in parentheses. EE administered at 5 mg/kg SQ. [b] $P < 0.005$. [c] $P < 0.05$.

Table VI: Effect of Ethinylestradiol Dose on Sinusoidal Na,K-ATPase Specific Activity and Components of Membrane Fluidity[a]

dose (μg/kg × 5 days)	Na,K-ATPase sp act.	fluidity parameters	
		r_∞	R
control	48.5 ± 1.1	0.143 ± 0.002	0.149 ± 0.005
10	34.9 ± 5.8	0.173 ± 0.003[b]	0.142 ± 0.004
25	24.5 ± 5[b]	0.167 ± 0.004[b]	0.141 ± 0.004
100	29.8 ± 6	0.180 ± 0.004[b]	0.150 ± 0.002

[a] EE administered SQ daily. Values are mean ± SS for four to six pairs in each group. [b] $P < 0.001$.

probe. Ethinylestradiol administration did not change the mean fluorescence lifetime, indicating that alterations in physical properties do not represent quenching of the fluorescent probe. Sinusoidal membranes from EE-treated rats showed significantly increased order and slower rotational rate (R) of the probe. No significant changes were noted in canalicular membrane fractions for fluorescence lifetime (9.73 ± 0.3 vs 9.65 ± 0.4 ns, r_∞ (0.191 ± 0.006 vs 0.192 ± 0.001), or R (0.205 ± 0.005 vs 0.192 ± 0.008) between control and ethinylestradiol treatment, respectively.

Relationship of Membrane Fluidity to Function. In order to determine whether increased order parameter or decreased rotation rate was the component altering Na,K-ATPase specific activity and bile flow, the time course for changes in membrane physical properties and function was analyzed. Table V shows the changes in bile flow, Na,K-ATPase, r_∞, and R after 1, 3, and 5 days of daily 5 mg/kg EE administration. Bile flow, Na,K-ATPase, and r_∞ significantly altered as early as 1 day after administration of ethinylestradiol and reached a new steady state by at least 3 days of treatment. In contrast, R was not significantly altered at 1 and 3 days but significantly increased at 5 days. The canalicular membrane structural and dynamic parameters remained unchanged at each time period.

These data suggest that alterations in the sinusoidal structural parameter of membrane fluidity may be the major determinant of decreased Na,K-ATPase and bile flow. Whether changes in r_∞ or R are related to function was next examined by determining the effect of different doses of EE for 5 days on SM Na,K-ATPase specific activity and membrane physical parameters (Table VI). Doses of EE as low as 0.01 mg/kg simultaneously decreased Na,K-ATPase specific activity and increased r_∞, while R measurements were unaltered up to 0.1 mg/kg. Similar changes were obtained by using lipid extracts, indicating that the alteration is not due to changes in protein composition (data not shown).

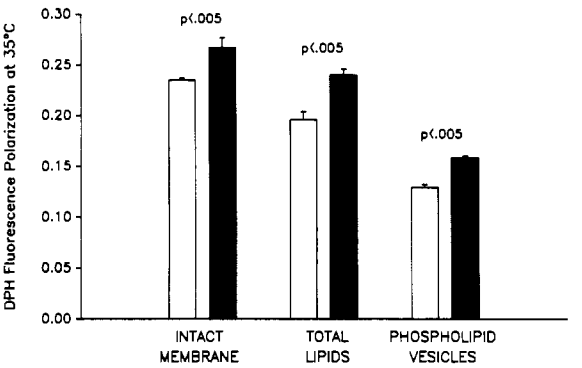

FIGURE 3: Steady-state fluorescence polarization of sinusoidal membrane components. Total lipids and phospholipid vesicles prepared as described under Materials and Methods. (Open bars) Control (n = 6); (solid bars) EE (5 mg/kg × 5 days) (n = 6). Mean ± SE.

Membrane Lipid Compositional Studies. No significant difference in either sinusoidal or canalicular membrane cholesterol, phospholipid, or cholesterol/phospholipid molar ratio was determined after EE treatment (Table VII). However, as previously reported, SM cholesteryl ester content was dramatically increased (Davis et al., 1978; Simon et al., 1980). It was only minimally increased in the canalicular fraction. In the sinusoidal fraction this change was due to an increase in C-18, whose percentage increases from 28 ± 3.0% to 82 ± 4% of esterified cholesterol. However, EE administration of 10 and 25 μg/kg × 5 days did not significantly alter esterified cholesterol membrane content [6.26 ± 2.16 and 7.9 ± 2.3 (NS)] compared to control, yet order parameter and Na,K-ATPase specific activity measurements were changed (Table VI). Thus, increases in cholesteryl esters are unrelated to either structural or functional changes.

The specific components responsible for changes in sinusoidal membrane fluidity were determined by fractionation of membranes into total lipids and phospholipid vesicles. The results of polarization values obtained in control and EE-treated animals are shown in Figure 3. In both groups, removal of proteins reduced polarization values, indicating proteins play a small but significant role in membrane fluidity.

Table VII: Effect of Ethinylestradiol Administration on the Lipid Composition of Liver Plasma Membrane Fractions[a]

fraction	parameters			
	cholesterol (μmol/mg of protein)	phospholipid (μmol/mg of protein)	phospholipid (μmol/mg of protein)	cholesterol phospholipid molar ratio cholesteryl esters (μg/mg of protein)
sinusoidal				
control[b]	0.465 ± 0.020	0.970 ± 0.04	0.48 ± 0.03	4.6 ± 2.0
EE[c]	0.499 ± 0.020	0.913 ± 0.06	0.54 ± 0.04	65.5 ± 18
bile canalicular				
control[b]	0.833 ± 0.030	0.990 ± 0.10	0.86 ± 0.1	0.5 ± 0.15
EE[c]	0.747 ± 0.06	0.897 ± 0.06	0.83 ± 0.07	1.85 ± 0.53

[a] Values represent mean ± SE. [b] Control: n = 12. [c] EE 5 mg/kg × 5 days, n = 6.

Table VIII: Effect of Ethinylestradiol on Relative Content of Phospholipid Species[a]

	phospholipid sinusoidal		canalicular	
	control	EE[b]	control	EE[b]
origin	0.6 ± 0.11	0.2 ± 0.4	0.4 ± 0.3	0.2 ± 0.14
LPC	0.8 ± 0.2	0.7 ± 0.1	1.6 ± 0.4	1.2 ± 0.2
SPH	11 ± 0.6	12.9 ± 0.3	22.1 ± 0.8	19.3 ± 0.4[c]
PI	6.4 ± 0.6	5.5 ± 0.2	4.4 ± 0.4	4.7 ± 0.2
PC	44.6 ± 0.6	48 ± 0.7[c]	35.5 ± 1.8	40.9 ± 1.0
PS	7.6 ± 0.3	9.8 ± 0.2[c]	11.2 ± 0.8	11.4 ± 0.1
PE	28.4 ± 0.7	21.4 ± 0.4[d]	23.8 ± 0.9	22.0 ± 0.7
PG	0.2 ± 0.2	0.3 ± 0.4	ND[e]	0.2 ± 0.1
PA	0.2 ± 0.1	0.5 ± 0.2	ND[e]	0.2 ± 0.1
SPH/PC	0.25 ± 0.02	0.27 ± 0.01	0.63 ± 0.02	0.48 ± 0.02
PE/PC	0.63 ± 0.06	0.45 ± 0.05[d]	0.67 ± 0.09	0.54 ± 0.05 (NS)

[a] Values represent mean ± SE for six pairs of membrane fractions in each group. [b] EE was administered at 5 mg/kg × 5 days. [c] $P = <0.005$. [d] $P = <0.0005$. [e] ND: not detected.

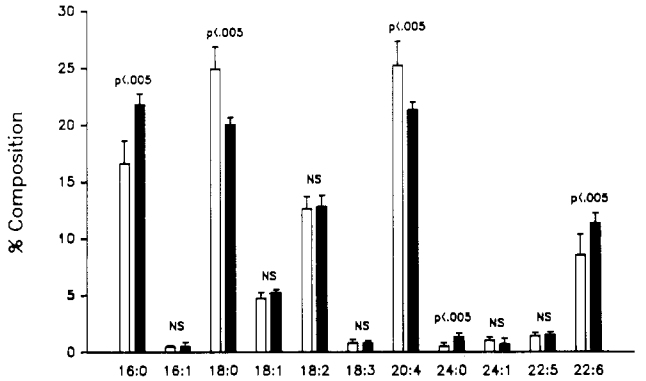

FIGURE 4: Acyl chain composition of total polar lipids from sinusoidal membranes. (Open bars) Control ($n = 3$); (solid bars) EE (5 mg/kg × 5 days) ($n = 5$).

Separation of phospholipids from neutral lipids further reduced polarization values. However, following each extraction the increase in polarization in sinusoidal membranes for EE-treated rats was retained. These results indicate that neither free nor esterified cholesterol was the major determinant of increased order parameter with EE treatment.

Treatment with EE produced minor changes in canalicular and sinusoidal membrane phospholipid polar head groups (Table VIII). In sinusoidal fractions, phosphatidylethanolamine (PE) decreased 24%, while phosphatidylcholine (PC) and phosphatidylserine (PS) increased by 8% and 29%, respectively. Thus, the ratios of PE/PC decreased by 29%, while sphingomyelin/phosphatidylcholine (SPH/PC) was not significantly altered. In the BCM fraction, EE administration significantly increased the sphingomyelin/phosphatidylcholine (SPH/PC) ratio.

Figure 4 shows the fatty acid profile for phospholipids from the sinusoidal membrane fraction after EE administration compared to that of pair-fed controls. Palmitate (16:0), lignocerate (24:0), and docosahexanoic (22:6) fatty acids increased while stearate (18:0) and arachidonate (20:4) decreased. However, no significant change in percent saturation (42 ± 3.5 vs 43 ± 1.3) or double-bond index (1.93 ± 0.17 vs 1.95 ± 0.04) were found.

Discussion

Structural and functional abnormalities have previously been reported in mixed hepatic plasma membrane fractions following administration of EE (Schreiber & Simon, 1983). Recently, the lipid fluidity of microvillus membranes from small intestines (Schwarz et al., 1986) and colon (Dudeja et al., 1987) was reported to be significantly lower than that of control membranes, as assessed by steady-state fluorescence polarization techniques. In agreement with these studies, the present results demonstrate that lipid fluidity and Na,K-ATPase specific activity were altered by EE administration. However, the present studies unexpectedly demonstrated that EE administration selectively decreases sinusoidal membrane fluidity, and this change was unrelated to changes in neutral lipid composition. In addition, we have shown altered sinusoidal protein composition and possible redistribution of canalicular enzymes to the sinusoidal pole.

Ethinylestradiol treatment markedly decreased serum cholesterol concentration compared to that in pair-fed control rats. Decreased serum cholesterol and increased hepatic cholesteryl esters (Davis & Roheim, 1978; Davis et al., 1978) have been related to the induction of hepatic LDL receptors (Kovanen et al., 1979; Ma et al., 1986) and increased clearance of serum cholesterol by the liver (Chao et al., 1979). Consistent with this observation, a polypeptide band of 160 kDa was identified in sinusoidal membrane fractions by SDS–PAGE. Since this band was increased 10-fold by EE and has the molecular size reported for the LDL receptor, it may represent the LDL receptor (Ma et al., 1986). Further studies are in progress to confirm this suggestion.

Previous studies have shown that membrane enzymes, lipids, and fluidity are different for the SM and BCM (Schachter, 1984). Using a new method to isolate these plasma membrane fractions, we have confirmed these reports. Administration of EE apparently did not alter the isolation characteristics of plasma membrane domains since enrichments and percent recovery of domain specific marker enzymes Na,K-ATPase and leucine aminopeptidase were unchanged. In mixed membrane fractions, Na,K-ATPase specific activity was decreased by EE treatment (Davis et al., 1978). Similarly, its activity was reduced in SM fractions. However, increased homogenate Mg^{2+}-ATPase and alkaline phosphatase specific activities were present only on the sinusoidal surface. Failure to detect increased enzyme activities on the BCM may be due in part to reduced recovery of alkaline phosphatase with EE treatment. However, similar redistribution for alkaline phosphatase has been found by using histochemistry in bile duct ligated rats (Komoda et al., 1984). Thus, we speculate that EE administration selectively alters sinusoidal proteins.

Previously it was shown that high-dose EE treatment decreased membrane fluidity by using steady-state fluorescence measurements with DPH (Keefe et al., 1979). We confirmed this observation and now show that decreased fluidity is selective to the sinusoidal surface. In addition, other probes of fluidity including 2AS and 12AS all demonstrated decreased fluidity specifically localized to the sinusoidal surface.

Steady-state fluorescence polarization measurements in biological membranes are composed of two components: a fast

decaying (kinetic) component related to the rotational rate of the probe and an order parameter, the slowly decaying component that is determined by the angular contraints of probe motion within the lipid bilayer (Harris & Simon, 1987). Therefore, we examined by time-resolved fluorescence polarization spectrometry whether decreased lipid fluidity after treatment with EE reflected alterations in both the dynamic and static components. As expected, the order parameter was significantly increased. In addition, the dynamic or kinetic component (R) was decreased by EE administration. Dose–response and time course studies of changes in r_∞ and R were measured in order to determine which change in these components of lipid fluidity were important for the alteration in hepatic function. These studies indicate that decreased Na,K-ATPase specific activity is directly related to changes in polarization and r_∞ but unrelated to R. Furthermore, decreased bile flow (cholestatis) was correlated with increased SM order parameter and decreased Na,K-ATPase specific activity, suggesting a cause–effect relationship.

To determine which component of the sinusoidal membrane was responsible for decreased fluidity, total lipid extracts and phospholipid vesicles were prepared for polarization measurements and lipid composition. Proportional increases in polarization remained after removal of proteins and neutral lipids by extraction and silicic acid chromatogrphy. Therefore, neither free nor esterified cholesterol was responsible for increased polariztion. We (Davis et al., 1978; Simon et al., 1980) have previously suggested that increased plasma membrane viscosity following EE treatment is due to incorporation of cholesteryl esters. Although cholesteryl ester content was increased, especially in the sinusoidal fraction after EE treatment, this increase did not appear to influence measurements of polarization or Na,K-ATPase specific activity. Following low-dose EE administration (<0.1 mg/kg) cholesteryl esters did not change, but Na,K-ATPase specific activity decreased and polarization values increased.

The specific lipid abnormality accounting for the markedly increased viscosity of phospholipid fractions from EE-treated animals is unclear. In addition to cholesterol, changes in sphingomyelin content and fatty acid composition are the major determinants of membrane fluidity. In the present study, although minor changes in phospholipid species and fatty acid composition were measured, the spingomyelin/phosphatidylcholine ratio, the double-bond index, and the percent fatty acid saturation were not altered. Indeed, the PE/PC ratio was significantly decreased by 40%. Although this change is in the predicted direction for a more fluid membrane, alterations less than 50% are generally not detected by measurements of lipid fluidity (Schroeder et al., 1976).

As discussed by Stubbs (1983), it is difficult to relate specific alterations of fatty acid composition to the changes in membrane lipid structure, and thus specific fatty acid alterations may at least in part account for the altered fluidity. In addition, it has been reported (Storch & Schachter, 1984) that a dietary regimen which induces acyl chain desaturases and membrane fluidity also reverses EE-induced cholestasis. Thus, modulation of membrane lipid fatty acid composition may be an important mechanism to regulate plasma membrane fluidity.

These studies demonstrate that in contrast to the enterocyte and colonocyte EE treatment modifies hepatic basolateral membranes (SM) and not the brush border (BCM) (Schwarz et al., 1986; Dudeja et al., 1987). In addition, lipid changes accounting for similar alterations in fluidity differ since changes in the cholesterol to phospholipid ratio do not play a role in hepatocyte altered fluidity as they apparently do in distal small intestinal and colonocyte microvillus membranes.

The functional importance of these changes in order parameter to Na,K-ATPase, bile flow, and cholestasis is suggested by associated changes with both dose–response and time course. The observation that doses as low as 0.01 mg/kg altered SM fluidity and Na,K-ATPase suggests that changes may account for cholestasis in humans as well. However, the link between decreased Na,K-ATPase and decreased bile flow is circumstantial, for a number of other processes localized to the sinusoidal surface may be altered as well. For instance, Na–H exchange is reported to be decreased (Arias, 1985). Whatever the mechanism, these studies direct attention to the primary role of alterations in the sinusoidal surface in the pathogenesis of EE-induced intrahepatic cholestasis (Berr et al., 1984; Arias, 1985). Whether other forms of cholestasis may be due to similar abnormalities is unknown.

In summary, the present studies indicate that EE selectively decreased SM fluidity. This change may account for decreased Na,K-ATPase specific activity, decreased bile flow, and increased serum bile acids. Although major alterations in fluidity were measured in the phospholipid fraction and fatty acid composition is altered, the exact biochemical abnormality cannot be ascertained.

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Registry No. EE, 57-63-6; ATPase, 9000-83-3; 16:0, 57-10-3; 24:0, 557-59-5; 22:6, 32839-18-2; 18:0, 57-11-4; 20:4, 506-32-1; cholesterol, 57-88-5; taurocholic acid, 81-24-3.

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