Effects of docosahexaenoic acid on annular lipid fluidity of the rat bile canalicular plasma membrane

Michio Hashimoto,[1,*] M. Shahdat Hossain,[*] Toshio Shimada,[†] Hiroshi Yamasaki,[*] Yoshimi Fujii,[*] and Osamu Shido[*]

Department of Physiology* and Fourth Department of Internal Medicine,† Shimane Medical University, Izumo, Shimane 693-8501, Japan

Abstract **The role of docosahexaenoic acid (DHA) in the fluidity of the annular lipid regions and their associated membrane-bound proteins is still not as well understood as that in the global (bulk) lipid regions. We therefore studied the effects of dietary DHA on the relationship between annular and global lipid fluidity and membrane-bound enzymes such as 5′-nucleotidase and Mg^{2+}-ATPase in the rat bile canalicular membrane. Dietary DHA caused significant increases in 5′-nucleotidase and Mg^{2+}-ATPase activity and in global and annular lipid fluidity, a higher increase in fluidity in the annular lipids than the global lipids, and a decrease in the cholesterol-to-phospholipid molar ratio in the canalicular membrane. Plasma total cholesterol and LDL cholesterol decreased, and fecal cholesterol increased in the DHA-fed rats. No changes were observed in oxidative markers, but glutathione peroxidase increased in the liver with DHA feeding. Annular lipid fluidity, but not global lipid fluidity, correlated remarkably well with DHA, synchronously with the activities of 5′-nucleotidase and Mg^{2+}-ATPase. The data indicate that the DHA-induced increase in annular lipid fluidity is responsible for the increases observed in the enzyme activity. We therefore concluded that the increased activity of membrane-bound enzymes and transporters induced by DHA and the concomitant increase in annular lipid fluidity comprise one of the mechanisms involved in DHA-induced clearance of plasma cholesterol.—** Hashimoto, M., M. S. Hossain , T. Shimada, H. Yamasaki, Y. Fujii, and O. Shido. **Effects of docosahexaenoic acid on annular lipid fluidity of the rat bile canalicular plasma membrane. *J. Lipid Res.* 2001.** 42: **1160–1168.**

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The layer of lipids immediately surrounding the integral membrane proteins, referred to as annular or boundary lipids, plays a mechanically crucial role in maintaining the functional status of the membrane proteins, but experimental evidence of this role in natural membrane proteins is scarce (1, 2). On the one hand, the fluidity probe, which probes equally into all regions of the bilayer membrane (3) regardless of the presence of microdomains, provides the average or global fluidity for the different domains in the membrane. On the other hand, owing to the domain-like nature of the plasma membrane, the fluidity of various membrane domains may vary without a significant change in global fluidity. Thus, although there has been increasing interest in evaluating the role of lipid fluidity in the structure-function relationship of membrane-bound enzymes (4), transporters (5), and receptors (6), measurements of the fluidity of the annular regions, which are essential in determining their effects on the membrane-bound proteins, cannot be obtained directly from measurements of global fluidity. Accordingly, reports on the nature of membrane fluidity with respect to membrane-related functions have been inconsistent and conflicting. For example, decreased liver plasma membrane fluidity has been shown to be associated with both reduced (7, 8) and increased bile flow (9), and no effect on membrane fluidity was observed in yet another study (10). Indeed, membrane fluidity is known to be an important parameter in the regulation of membrane function through its effect on the orientation of integral membrane proteins, its influence on membrane permeability, and its modulation of transmembrane transport processes (11).

DHA is involved in the regulation of various biological functions, and its dietary administration has beneficial effects on the central nervous system, the cardiovascular system, and hepatic lipid metabolism in both human subjects and experimental animals (12–14). The exact mechanism of these beneficial effects is not clearly understood, although this fatty acid is thought to play a significant structural role in the plasma membrane. In this context, we (15) and others (16) have shown that DHA increases the plasma membrane fluidity in aortic endothelial cells and brain synaptosomes. Not much is known, however, about its fluidizing effects on the annular lipid regions. Investigating the effects of DHA on the immediate vicinity of the

Abbreviations: BCM, bile canalicular membrane; DHA, docosahexaenoic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; LPO, lipid peroxidation; ROS, reactive oxygen species; SM, sinusoidal membrane; TMA-DPH, 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene; USI, unsaturation index.

[1] To whom correspondence should be addressed.
e-mail: michio1@shimane-med.ac.jp

membrane proteins may thus help in unveiling the effects of annular fluidity on membrane-bound proteins. DHA has been shown to accumulate in the regenerating liver after partial hepatectomy (17), a postoperative condition associated with alterations in membrane fluidity (18). Dietary fatty acids can influence the physical state and the function of membrane-bound enzymes such as 5′-nucleotidase (19) and Na^+,K^+-ATPase (20). In this study, we selected two plasma membrane marker enzymes, 5′-nucleotidase and Mg^{2+}-ATPase, which are abundantly present in the bile canalicular membranes (BCM) of the liver, and studied the effects of DHA on the relationship between the annular lipid environment and these two enzymes.

The BCM domain may act as the site of abnormalities in the pathogenesis of bile secretory failure (21, 22). In addition, one possible pathogenic mechanism of liver injury is oxidative damage to membrane-bound proteins and lipids (23), frequently accompanied by alteration in membrane fluidity (24). The membrane-fluidizing effect of DHA, which has been reported to have pro-oxidant properties (25), on the BCM is thus of special significance. Because previous studies have either used liver plasma membrane fractions that contain both BCM and basolateral domains or have centered largely on the relationship between global (bulk) membrane fluidity and Na^+,K^+-ATPase/Mg^{2+}-ATPase (26) and 5′-nucleotidases activity, (19, 27), and have not used purified PUFA such as DHA, or addressed or determined the relevance of oxidative potentials to membrane fluidity, the effects of PUFA on the relationships among membrane-bound enzymes, fluidity, and oxidative potential have not been precisely determined. This study was therefore designed to investigate in detail the effects of chronic administration of DHA on liver BCM annular fluidity and membrane-bound enzymes, and to explore their relationship with the clearance of plasma cholesterol and fecal cholesterol excretion.

MATERIALS AND METHODS

Animals

Rats were provided and killed in accordance with the *Guidelines for Animal Experimentation* of Shimane Medical University (Shimane, Japan), compiled from the *Guidelines for Animal Experimentation* of the Japanese Association for Laboratory Animal Science. Rats were kept in a room that was environmentally controlled at 23 ± 2°C and a relative humidity of 50 ± 10%, with automatic lighting from 08:00 to 20:00. Twenty-four male Wistar rats (300–400 g: Jcl:Wistar) were randomly divided into two groups and fed for 12 weeks: the DHA group was orally fed DHA-95E (300 mg/kg per day; an ethyl ester derivative of all-*cis*-4,7,10,13,16,19-docosahexaenoate with a purity of almost 95%; Harima Chemicals, Tokyo, Japan) gently emulsified in a vehicle of 5% gum arabic solution in ice-cold water before administration, in addition to the standard F1® diet containing no fish products (Funabashi Farm Co., Chiba, Japan); the control group was fed the standard F1® diet and an equal volume of the vehicle. F1® pellet diet intake was 18.0 ± 1.8 g/day per rat for the control rats and 17.9 ± 2.0 g/day per rat for the DHA rats.

Blood preparation and isolation of BCM

After the rats were anesthetized with sodium pentobarbital (65 mg/kg, intraperitoneal), blood was collected from the inferior vena cava into heparinized syringes, put into polyethylene tubes, and centrifuged to collect plasma.

The liver was removed, perfused with chilled 1 mM $NaHCO_3$ solution containing 1 mM PMSF, frozen in liquid N_2, and stored at −80°C. The BCM was isolated as described previously (28). Briefly, 10% homogenate of the liver in chilled buffer (300 mM mannitol, 5 mM EGTA, 18 mM Tris-HCl, 1 mM PMSF, pH 7.4) was prepared with a Polytron homogenizer (PCU 2-110; Kinematica, Littau-Lucerne, Switzerland). After the homogenate was centrifuged at 600 *g* for 10 min at 4°C, the supernatant was further centrifuged at 48,000 *g* for 30 min. The resulting pellet was resuspended in buffer, and precipitation with 15 mM Mg^{2+} was followed by centrifugation for 15 min at 2,445 *g*. The supernatant was again centrifuged at 48,000 *g* for 30 min to obtain the canalicular fraction. The pellet was washed twice with 1.0 mM $NaHCO_3$ containing 1 mM PMSF, suspended in the same washing solution, and stored at −80°C until analysis.

Marker enzyme analysis

The purity of the canalicular subfraction was estimated by determining the activity of marker enzymes: leucine aminopeptidase, alkaline phosphatase for canalicular plasma membranes, succinate cytochrome *c* reductase for mitochondria, NADPH cytochrome *c* reductase for microsomes, glucosaminodase for lysosomes, and galactosyltransferase for Golgi body membranes as described previously (22), and Na^+,K^+-ATPase for basolateral membranes (29).

Activity of 5′-nucleotidase was measured with Sigma (St. Louis, MO) diagnostics, and Na^+,K^+-ATPase and Mg^{2+}-ATPase activity were measured by the method of Samuels and Carey (29) with some modifications. To measure 5′-nucleotidase activity, 50 μg of BCM protein was preincubated with a cocktail of several phosphatase inhibitors to exclude any cross-activity of other ATP/ADP-utilizing enzymes on AMP. This cocktail contained 50 μM βγ-imido-ATP and 1.0 mM NaN_3 for ATPase, 50 μM βγ-methylene-ATP for ADPase, 1.0 mM vanadate for acid phosphatase, and 1.0 mM tetramisole for alkaline phosphatase enzyme.

Total ATPase activity was taken to be micromoles of inorganic phosphate released per milligram of protein per hour in 0.45 ml of the medium, which contained the BCM protein (100–120 μg), 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris-HCl (pH 7.4). After preincubation for 15 min at 37°C, the mixture was incubated for exactly 15 min after the addition of 50 μl of 20 mM ATP. The reaction was terminated by the addition of 0.5 ml of 10% trichloroacetic acid. Mg^{2+}-ATPase activity was measured in the same medium without sodium or potassium, but with the addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated as the difference between the specific activity of total ATPase and of Mg^{2+}-ATPase.

Measurements of annular/global fluidity

When excited at its own wavelength of 334 nm, a pyrene molecule in close proximity to another pyrene molecule (monomer, M) forms a monomer-monomer dimer (excimer, E). The ratio of E/M fluorescence intensity can therefore be used as an index of lateral diffusion (lateral mobility of the membrane environment); thus, the higher the ratio, the higher the membrane fluidity. In principle, pyrene diffuses randomly at all nonpolar regions into the bilayer membrane, irrespective of the presence of any microdomain. Accordingly, the resulting E/M ratio gives the average or global fluidity of the membrane. When fluorescence emission is created through energy transfer from the tryptophan of the membrane protein, only the pyrene molecules

localized in the annular lipid are excited and the E/M ratio gives the fluidity for the annular lipids (annular fluidity) (30).

Labeling with pyrene was conducted as described previously (1, 30). Briefly, 100 μl of BCM suspension containing 100 μg of protein was suspended in 2.0 ml of 25 mM Tris-HCl buffer (pH 7.4) containing 137 mM NaCl, 5.4 mM KCl, and 11 mM glucose, incubated in the dark at 37°C for 30 min, transferred to a cuvette, and injected with 2 μl of 10 mM pyrene. After incubation for 5 min, pyrene was excited at a wavelength of 286 nm, and the fluorescence emission spectrum was recorded from 320 to 530 nm. After tracing, pyrene was excited at 334 nm and the intensity profile was again recorded from 320 to 530 nm. The E/M ratio obtained at a wavelength of 286 nm was taken as the annular fluidity, and the ratio at 334 nm was taken as the global (bulk) fluidity, where E is the fluorescence intensity of pyrene excimer at 480 nm and M is the fluorescence intensity of pyrene monomer at 373 nm.

The global fluidity of BCM was also determined by measuring the polarization of the fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH) and its derivative, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), as described previously (15). Fluorescence polarization emitted by these two probes depends on their range and the rate of rotational motion, which is a function of the surrounding medium, which in this case is the plasma membrane; thus, the higher the rotational motion of the probes, the higher the polarization and therefore the lower the fluidity of the plasma membrane, and vice versa. Briefly, 100 μl of a plasma membrane suspension of 100 μg of BCM protein was added to 1.9 ml of 25 mM Tris-HCl buffer (pH 7.4) containing 137 mM NaCl, 5.4 mM KCl, and 11 mM glucose, for a final probe concentration of 1 μM. This dispersion was incubated at 37°C for 60 min for DPH and for 30 min for TMA-DPH. Fluorescence polarization was measured at 37°C with a Hitachi (Tokyo, Japan) 850 spectrofluorometer equipped with rotating polarizers in both the excitation and emission beams, a thermostatic chamber, and a stirrer beneath the cuvette. The effect of light scattering on the intensity values was minimized with a 390-nm cutoff filter and by subtracting the values of unlabeled preparations. Excitation wavelengths of 360 and 365 nm, and emission wavelengths of 430 and 435 nm, were used for DPH and TMA-DPH, respectively. The slit width was 8 nm for excitation and 14 nm for emission for all the probes. Fluorescence polarization was calculated as

$$P = \frac{(I_{VV} - I_{VH} \cdot G)}{(I_{VV} + I_{VH} \cdot G)}$$

where I_{VV} and I_{VH} are the intensities measured parallel and perpendicular to the vertical axis of the excitation beam and G is the correction factor I_{HV}/I_{HH}. Plasma membrane fluidity in this measurement is expressed as the inverse of the polarization (1/P).

Determination of antioxidative-oxidative status

Liver tissue was homogenized at 10% volume in ice-cold buffer. The ice-cold homogenate was centrifuged at 1,000 *g* for 5 min at 4°C and some of the supernatant was used for measuring catalase activity. The remaining supernatant was further centrifuged at 12,500 *g* for 30 min at 4°C. The resultant supernatant containing microsomes and the cytosol was used for glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione *S*-transferase (GST) activity assays.

Catalase activity (EC 1.11.1.6) was determined by measuring the decrease in absorbance of H_2O_2 at 240 nm (31). GPx activity (EC 1.11.1.9) was assayed as described previously (32). GR activity was measured by monitoring the oxidation of NADPH at the expense of the concurrent reduction of oxidized glutathione (GSSG), essentially using the method of Carlberg and Mannervik (33). GST activity was measured with 1-chloro-2,4-dinitrobenzene as described previously (34).

The level of reactive oxygen species (ROS) was determined as described previously (32). Briefly, 50 μl of freshly prepared tissue homogenate was diluted in 4.85 ml of 100 mM potassium phosphate buffer (pH 7.4) and incubated with dichlorofluorescein diacetate in methanol at a final concentration of 5 μM for 15 min at 37°C. The dye-loaded samples were centrifuged at 12,500 *g* for 10 min at 4°C. The pellet was vortexed at 0°C in 5 ml of 100 mM phosphate buffer (pH 7.4) and again incubated for 60 min at 37°C. Fluorescence was measured with a Hitachi 850 spectrofluorometer at wavelengths of 488 nm for excitation and 525 nm for emission.

Lipid peroxidation (LPO) levels were assessed with thiobarbituric acid-reactive substances as described previously (15). LPO levels were expressed as nanomoles of malondialdehyde per milligram of BCM protein.

Reduced glutathione (GSH) levels were determined by the method of Hissin and Hilf (35). A portion of tissue was homogenized on ice in 3.75 ml of phosphate-EDTA buffer and 1 ml of 25% HPO_3 as the protein precipitant. The homogenate was centrifuged at 100,000 *g* for 30 min at 4°C, and 4.5 ml of phosphate-EDTA buffer, pH 8.0, was added to 0.5 ml of the supernatant. The final assay mixture (2.0 ml) contained 100 μl of diluted tissue supernatant, 1.8 ml of phosphate-EDTA buffer, and 100 μl of *o*-phthalaldehyde (1.0 mg/ml). After thorough mixing and incubation at room temperature for 15 min, GSH levels were determined from the standard curve.

Protein thiols were determined by the procedure of Sedlak and Lindsay (36), separately in two fractions: the purified BCM fraction and the cytosolic fraction (37).

To measure the BCM protein carbonyl content, the BCM was prepared from the 10% homogenate of liver tissue in a buffer containing leupeptin (5 μg/ml), pepstatin A (7 μg/ml), aprotinin (5 μg/ml), and 1% streptomycin. The protein carbonyl content was determined by the method of Nakamura and Goto (38). Briefly, BCM protein (0.5 mg) was precipitated with 10% trichloroacetic acid by incubation for 30 min at 4°C. The precipitate was treated with either 10 mM 2,4-dinitrophenylhydrazine in 2 N HCl or 2 N HCl alone (control) for 1 h at 25°C with a repeated vortex at ~10-min intervals. After the derivatization reaction was completed, the mixture was centrifuged at 11,000 *g* for 30 min, and the precipitates were washed with an excess of chilled ethanol–ethyl acetate 1:1 (v/v) mixture. The pellet was dissolved in 6 M guanidine hydrochloride in 50% formic acid, and its absorbance was determined at 370 nm.

To measure tyrosine and tryptophan content for use as indicators of amino acid oxidation, 50 μg of the canalicular protein was digested with proteinase K as described previously (39) for 2 h at 37°C and then precipitated with 1.6 M perchloric acid (PCA) on ice. The PCA-treated mixtures were centrifuged at 3,000 *g* for 20 min at 4°C, and the supernatants were neutralized with 2 N KOH and kept on ice for 1 h. After centrifugation at 3,000 *g* for 30 min at 4°C, 1 ml of supernatant was added to 1 ml of 0.2 M HEPES-KOH (pH 9.0). Tyrosine fluorescence was measured at 278 and 303 nm for excitation and emission, respectively. Tryptophan fluorescence was determined at 290 and 345 nm.

Lipid analyses

The fatty acid composition and the levels of cholesterol and phospholipid of the BCM were determined as described previously (15). Plasma and fecal total cholesterol and plasma LDL cholesterol were measured with commercial analytical kits (Dai Ichi Pure Chemical Co., Tokyo, Japan).

Protein concentration was estimated by the method of Lowry et al. (40).

Statistical analysis

Results are expressed as means ± SE. Data were subjected to an unpaired Student's *t*-test, and regression analyses for correlation studies were carried out with the computer program StatView™ II (Abacus Concepts, Berkeley, CA). A level of $P < 0.05$ was considered significant.

RESULTS

Relative purity of the BCM

The relative purity of the canalicular membranes isolated from the DHA-fed and the control rats is shown in **Table 1**. No significant differences in either membrane-bound leucine aminopeptidase or alkaline phosphatase activity were found between the liver homogenates of the DHA-fed rats and those of the control rats. Activity of 5′-nucleotidase and Mg^{2+}-ATPase increased significantly in the BCM of the DHA-fed rats. Na^+,K^+-ATPase activity increased in the liver homogenate of the DHA-fed rats. Relative enrichment (the ratio of enzyme activity of the BCM to that of the liver homogenate) of the intracellular marker enzymes succinate cytochrome *c* reductase (mitochondria), NADPH cytochrome *c* reductase (microsomes), glucosaminodase (lysosomes), and galactosyltransferase (Golgi membrane) was 0.30 ± 0.05, 0.80 ± 0.05, 0.5 ± 0.05, and 0.09 ± 0.005 in the BCM of the DHA-fed rats, and 0.25 ± 0.02, 0.75 ± 0.05, 0.60 ± 0.05, and 0.08 ± 0.004 in the BCM of control rats, respectively. No significant differences in relative enrichment of these intracellular marker enzymes in the BCM were found between the two groups. The higher activity of 5′-nucleotidase and Mg^{2+}-ATPase thus was not due to differences in recovery, or to contamination from basolateral membranes or intracellular organelles, which have been documented as similar enrichment factors for Na^+,K^+-ATPase activity (basolateral enzyme marker) and intracellular marker enzyme activity of mitochondria, microsomes, lysosomes, and Golgi bodies. The BCM fraction prepared in the study can thus be considered to be of a purity similar in extent to that in the control and DHA-fed rats.

The present study demonstrated that basal Na^+,K^+-ATPase activity was only 1/25–1/30 of basal Mg^{2+}-ATPase activity (Table 1). This is consistent with the finding that membrane fractions rich in bile canaliculi show only little or no specific Na^+,K^+-ATPase activity, whereas canalicular membrane fractions show the highest Mg^{2+}-ATPase activity (41). The membrane fraction isolated in this study could thus be considered to be the BCM fraction, as described previously (28).

Effects of DHA on membrane fluidity

DHA administration increased both annular and global fluidity (**Fig. 1A** and **B**), and the effect of DHA was furthermore greater on annular fluidity than on global fluidity, as demonstrated by the ratio between the fluidity of the annular and global regions of the BCM (2.44 ± 0.10 for the control rats and 2.92 ± 0.12 for the DHA-fed rats; $P < 0.023$) (Fig. 1C).

DPH-determined global fluidity of the BCM also increased more significantly in the DHA-fed rats than in the control rats (Fig. 1D). The increase in global fluidity in the BCM of DHA-fed rats confirms our previous findings with endothelial cell plasma membranes (15). In contrast with its parent compound DPH, cationic derivative TMA-DPH, a probe frequently used to measure surface membrane fluidity and used in that study (15), did not reveal differences in BCM membrane fluidity between DHA-fed and control rats to the same extent (data not shown).

Effect of DHA on lipid profile of the BCM

Because the lipid constituents primarily reflect the physical state of the plasma membrane bilayer, we also

TABLE 1. Enzyme activity and relative enrichment in liver homogenate and bile canalicular plasma membrane of control and DHA-fed rats

	Control Rats		DHA-Fed Rats	
Enzyme	Homogenate	BCM	Homogenate	BCM
Leucine aminopeptidase	4.02 ± 0.03[a]	131 ± 4.9[b] (32.0 ± 3.0)	3.95 ± 0.30[a]	125 ± 8.50[b] (31.5 ± 5.0)
Alkaline phosphatase	0.30 ± 0.03[a]	14.4 ± 1.30[b] (47 ± 5.0)	0.32 ± 0.02[a]	16.2 ± 1.50[b] (50 ± 5.0)
Na^+, K^+-ATPase	1.75 ± 0.20[a]	4.10 ± 0.50[b] (2.30 ± 0.25)	2.50 ± 0.20[c]	4.90 ± 0.55[b] (1.96 ± 0.20)
Mg^{2+}-ATPase	2.50 ± 0.25[a]	111 ± 11.5[b] (42.0 ± 5.0)	3.00 ± 0.40[a]	149 ± 10.0[c] (49.6 ± 5.0)
5′-Nucleotidase	0.025 ± 0.002[a]	0.5 ± 0.025[b] (19.5 ± 2.0)	0.030 ± 0.004[a]	1.0 ± 0.10[c] (32 ± 3.5)

Results represent means ± SE, n = 7–8 rats per each group. BCM, Bile canalicular plasma membrane. Enzyme activity: $\mu mol \cdot h^{-1} \cdot (mg\ protein)^{-1}$. Values in parentheses indicates the relative enrichment (the ratio of enzyme activity of BCM to that of liver homogenate).

[a,b,c] Values in the same row with different notations are significantly different at $P < 0.05$.

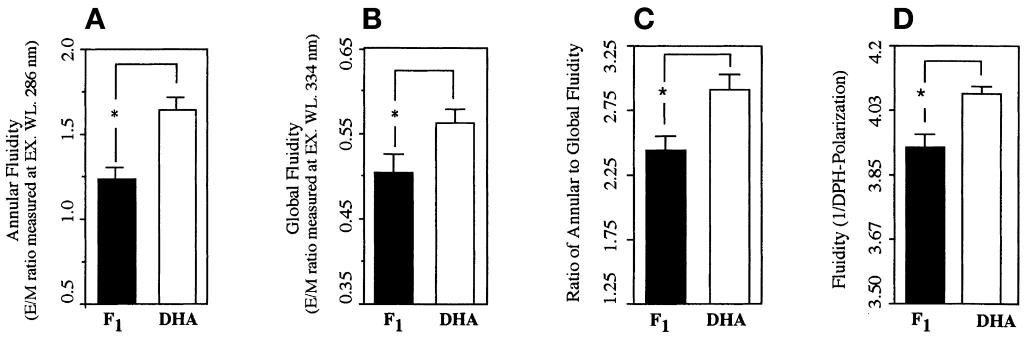

Fig. 1. Effects of DHA administration on membrane fluidity of the rat BCM. A: Annular fluidity. B: Pyrene-determined global fluidity. C: Relative fluidizing effects of dietary DHA on the annular and global regions, represented by annular-to-global fluidity ratios for DHA-fed versus control rats. D: Global fluidity, determined by fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) and expressed as 1/P. Results are shown as means ± SE, for 10–12 rats in each group with duplicate determinations. F_1, Control rats; DHA, DHA-fed rats. * $P < 0.05$, unpaired Student's *t*-test. EX. WL., Excitation wavelength.

measured and compared fatty acid profiles, and cholesterol and phospholipid content, of the BCM for the two groups of rats (**Table 2**). The DHA content of the BCM increased significantly (73%) accompanied by a significant decrease (47%) in arachidonic acid. Eicosapentaenoic acid content increased by 79%. Levels of oleic acid and stearic acid decreased significantly; linoleic acid, linolenic acid, and docosapentaenoic acid levels did not change to any significant extent. The ratio of total unsaturated fatty acids to total saturated fatty acids increased slightly in the BCM of the control group, because of comparatively greater decreases in arachidonic acid levels in the BCM of the DHA-fed rats. The ratio of PUFA to monounsaturated fatty acids increased significantly in the BCM of the DHA-fed rats. The phospholipid content did not change, while the total cholesterol content decreased significantly, accompanied by a significant decrease in the molar ratio of cholesterol to phospholipids in the BCM of the DHA-fed rats. The decrease in BCM cholesterol was accompanied by a significant decrease in plasma total cholesterol and LDL cholesterol, and by an increase in fecal cholesterol.

TABLE 2. Effect of DHA on bile canalicular membrane fatty acid profile,[a] cholesterol-phospholipid composition,[a] and plasma and fecal cholesterol concentrations of rats

	Control Rats	DHA-Fed Rats
BCM		
Palmitic acid ($C_{16:0}$)	257 ± 24.0	236 ± 7.50
Stearic acid ($C_{18:0}$)	264 ± 15.4	193 ± 5.50[b]
Oleic acid ($C_{18:1, n-9}$)	87.5 ± 5.90	54.0 ± 2.20[b]
Linoleic acid ($C_{18:2, n-6}$)	173 ± 17.5	159 ± 5.50
Linolenic acid ($C_{18:3, n-3}$)	2.0 ± 0.50	1.40 ± 0.20
Arachidonic acid ($C_{20:4, n-6}$)	266 ± 24.0	140 ± 7.40[b]
Eicosapentaenoic acid ($C_{20:5, n-3}$)	1.90 ± 0.20	3.40 ± 0.25[b]
Docosapentaenoic acid ($C_{25:5, n-3}$)	3.90 ± 0.35	4.80 ± 0.50
Docosahexaenoic acid ($C_{22:6, n-3}$)	62.2 ± 6.20	107.7 ±5.50[b]
TUS/TS	1.25 ± 0.02	1.21 ± 0.04
PUFA/MUFA	6.50 ± 0.20	8.45 ± 0.70[b]
Cholesterol	60.80 ± 4.30	45.60 ± 4.75[b]
Phospholipid	285 ± 4.90	265 ± 16.50
Molar ratio of cholesterol to phospholipid	0.215 ± 0.015	0.170 ± 0.012[b]
Plasma		
Cholesterol (mg/dl)	89.0 ± 5.40	67.0 ± 4.15[b]
LDL cholesterol (mg/dl)	33.5 ± 1.95	24 ± 2.10[b]
Feces		
Cholesterol (mg/g dry feces)	0.50 ± 0.03	0.75 ± 0.02[b]

Results represent means ± SE, n = 8–12 rats per each group. BCM, Bile canalicular plasma membrane; TUS, total unsaturated fatty acid; TS, total saturated fatty acid; MUFA; monounsaturated fatty acid.

[a] Nanomoles per milligram of protein.

[b] $P < 0.05$ versus control rats.

Effect of DHA on the antioxidative-oxidative status of rat liver tissue

Because increased PUFA levels in the membrane cause increased membrane susceptibility to peroxidative attack, which may in turn affect membrane fluidity (15, 42, 43) and membrane-bound enzyme activity (28, 29), the oxidative and antioxidative status of the liver tissue was determined for the two groups of rats and compared (**Table 3**). Markers of antioxidative defense catalase activity remained unaltered; GPx activity, however, increased significantly in the liver. ROS levels in the liver homogenates were not affected. Levels of LPO in the BCM did not change. GSH levels did not change; nor was any alteration in GR or GST activity observed in the liver tissue.

Protein thiol levels in the cytosol of the BCM were not affected by DHA administration, nor were the levels of protein carbonyl, an indicator of oxidative modification of protein damage, in the BCM fraction. Further examination of the effects of dietary DHA administration on BCM protein oxidation, and the accompanying loss of constituent amino acids, such as tyrosine and tryptophan (44), was carried out by fluorometry. The fluorometric studies were considered to be either qualitative or semiquantitative (against 100 μg of BSA protein) assays for tyrosine and tryptophan oxidation in the proteinase K-digested BCM proteins; bityrosine production was also checked. No alteration was detected in the fluorescence intensity of tyrosine or tryptophan (Table 3), the ratio of BCM tyrosine fluorescence intensity versus 100 μg of BSA, or the ratio of BCM tryptophan fluorescence intensity versus 100 μg

TABLE 3. Effect of dietary DHA on antioxidative-oxidative potentials of rat liver

	Control Rats	DHA-Fed Rats
Catalase, catalytic activity (nmol H_2O_2 decomposed/min/mg protein)	120 ± 6.0	125 ± 8.8
Glutathione peroxidase activity (nmol NADPH oxidized/min/mg protein)	175 ± 15.0	237 ± 20.0[a]
Glutathione reductase activity (nmol NADPH oxidized/min/mg protein)	60.0 ± 5.80	6.0 ± 5.50
Glutathione *S*-transferase activity (nmol GSH oxidized/min/mg protein)	250 ± 10.0	265 ± 15.0
Reactive oxygen species (pmol/mg protein)	200 ± 18.5	230 ± 18.5
Lipid peroxides (nmol/mg protein)	4.20 ± 0.30	4.50 ± 0.50
Reduced glutathione (nmol/mg protein)	10.5 ± 1.2	12.0 ± 1.5
Protein thiols		
BCM (nmol/mg protein)	36.5 ± 3.50	40.0 ± 4.20
Cytosol (nmol/mg protein)	75.0 ± 2.50	80.2 ± 4.50
Carbonyl content (nmol/mg protein)	0.40 ± 0.03	0.35 ±0.03
Tyrosine fluorescence intensity (arbitrary units/100 μg BCM protein)	0.85 ± 0.006	0.83 ± 0.005
Tryptophan fluorescence intensity (arbitrary units/100 μg BCM protein)	0.50 ± 0.004	0.52 ± 0.003

Results represent means ± SE, n = 8–10 rats per group. BCM, bile canalicular plasma membrane.
[a] $P < 0.05$ versus control rats.

of BSA [tyrosine: 1.20 ± 0.05 for the DHA-fed rats (n = 10) vs. 1.25 ± 0.08 for the control rats (n = 8); tryptophan: 1.80 ± 0.12 for the DHA-fed rats (n = 10); 1.90 ± 0.10 for the control rats (n = 8)].

Correlation of BCM fatty acids and membrane fluidity

The relationships between fatty acid content, membrane fluidity, and Mg^{2+}-ATPase/5′-nucleotidase activity were estimated by regression analysis. A highly significant positive correlation was observed between DHA content and BCM annular fluidity, but not between DHA content and global fluidity (**Table 4**). Multiple regression analysis was, furthermore, carried out to determine whether the correlation of DHA with annular fluidity was independent of any confounding effects from other PUFA. Interestingly, the DHA content of the BCM was the only independently significant factor ($R^2 = 0.91$, $P < 0.025$) that contributed to the increase in annular lipid fluidity. No significant correlation between membrane fluidity and molar ratios of either unsaturated to saturated fatty acids or of polyunsaturated to monounsaturated fatty acids was observed. The unsaturation index showed no independent correlation with annular fluidity (data not shown). Significant positive correlations were found between enzyme activity and DHA levels [(Mg^{2+}-ATPase activity) = 0.67 (DHA) + 67.5, $r = 0.70$, $P < 0.05$; (5′-nucleotidase activity) = 0.005 (DHA) + 0.050, $r = 0.50$, $P < 0.05$], and also between annular fluidity and Mg^{2+}-ATPase and 5′-nucleotidase activity [(Mg^{2+}-ATPase activity) = 47.9 (annular fluidity) + 50.9, $r = 0.65$, $P < 0.05$; (5′-nucleotidase activity) = 0.65 (annular fluidity) − 0.19, $r = 0.60$, $P < 0.05$]. These data suggest that increased BCM annular fluidity, together with the concomitant increase in DHA content of the BCM, may be one of the major factors in the activation of these enzymes.

TABLE 4. Correlation coefficients between bile canalicular plasma membrane fluidity and fatty acids

Fluidity	PA	SA	OLA	LLA	LLN	AA	EPA	DPA	DHA	TUS/TS	PUFA/MUFA
Annular pyrene	+0.30 (0.23)	−0.016 (0.95)	−0.25 (0.30)	−0.009 (0.97)	−0.10 (0.67)	−0.30 (0.21)	+0.12 (0.64)	+0.33 (0.25)	+0.75 (0.0007)	− 0.40 (0.07)	+0.21 (0.40)
Global pyrene	−0.23 (0.36)	−0.38 (0.12)	−0.45 (0.06)	+0.48 (0.07)	−0.04 (0.85)	−0.35 (0.15)	+0.44 (0.10)	+0.08 (0.79)	+0.36 (0.15)	+ 0.19 (0.44)	+0.36 (0.14)
Global DPH	+0.30 (0.20)	−0.09 (0.70)	−0.50 (0.04)	+0.25 (0.32)	+0.08 (0.73)	−0.36 (0.15)	+0.36 (0.30)	+0.14 (0.63)	+0.45 (0.07)	−0.44 (0.06)	+0.45 (0.07)

Correlation coefficients were calculated by linear regression analyses. Values in parentheses represent the levels of significance. PA, palmitic acid; SA, stearic acid; OLA, oleic acid; LLA, linoleic acid; LLN, linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; TUS, total unsaturated fatty acid; TS, total saturated fatty acid; MUFA, monounsaturated fatty acid.

DISCUSSION

Our measurements of annular fluidity throw light on the effects of dietary DHA administration on the canalicular membrane bilayer regions where membrane-bound proteins such as 5′-nucleotidase, Mg^{2+}-ATPase, and, obviously, other membrane proteins, transporters, and receptors are localized. The data show that dietary DHA is related to alterations in BCM annular fluidity, and that a DHA-induced increase in the annular fluidity activates 5′-nucletidase and Mg^{2+}-ATPase in the BCM, while at the same time, dietary DHA does not cause an increase in the oxidative potentials of the BCM. Chronic administration of this fatty acid also induced plasma clearance of cholesterol, as evidenced by a significant decrease in plasma total cholesterol and LDL cholesterol and a concomitant increase in fecal cholesterol content.

The effect of dietary DHA on the fluidity of the BCM, in the present investigation, and of synaptosomes, in studies by others (16), contrasts with the unaltered hepatocyte plasma membrane fluidity in rats fed PUFA through black currant oil diets (27). Nevertheless, the effect of dietary DHA on 5′-nucleotidase activity in the BCM is consistent with its increased activity after liver plasma membrane fluidity is enhanced (45). Likewise, a significant decrease in stearic acid, which is reported to have membrane-rigidifying properties, in the BCM of DHA-fed rats (15) is in accordance with the fact that the BCM of the DHA-fed rats is more fluid than that of the control rats. Moreover, an increase in the cholesterol content of the plasma membrane reduces the membrane fluidity of the phospholipid bilayer, and the molar ratio of cholesterol to phospholipid is inversely correlated with membrane fluidity (4, 5). These parameters decrease in the more fluid BCM of DHA-fed rats, as in the more fluid plasma membranes of gallbladder muscle cells (4) and the liver (5). On the other hand, levels of eicosapentaenoic acid, another highly polyunsaturated fatty acid, increased by 80%; this had no effect, however, on either annular or global fluidity. This supports our previous findings that PUFA may differ in their effect on fluidity despite the facts that they are members of the same n−3 family (15, 43) and that enhanced DHA in the BCM caused an increase in membrane fluidity in a highly correlated manner.

The present study showed the BCM annular lipids to be more fluid than the global lipids. This effect allows the canalicular membrane-bound proteins greater motional freedom in the annular region than in the global region. Although the mechanism whereby dietary DHA administration exerts a greater fluidizing effect on the annular lipid regions, as indicated by the increased annular-to-global fluidity ratio, is not clear, the following points can be stressed. Dietary DHA administration may cause the DHA molecules of the BCM to preferentially localize in the annular region, leading to a higher concentration of DHA surrounding the annular proteins. Because DHA increases membrane fluidity, the increased DHA may thus exert a greater fluidizing effect in the annular region. This postulation is consistent with the suggestions that PUFA (46) including DHA (13) preferentially accumulate in the lipid domains that surround the annular proteins, and that the annular lipid regions are relatively poor in cholesterol (2). However, the fluorescence data used to determine annular lipid fluidity may not directly provide evidence that DHA molecules accumulate at higher concentrations in the annular regions, because annular fluidity was also found to be greater than bulk fluidity in the control group. It is therefore also possible to speculate that the amount of DHA in both the annular and global areas may be the same, but the spatial effects of DHA on the respective fluidity of the two areas are different, with a greater effect occurring in the annular lipid region. Whatever the mechanism, DHA exerts a greater effect on annular fluidity than on global fluidity.

The direct cause-and-effect relationship between BCM fluidity, the enzymes studied, and the hepatic transport of bile and lipids is difficult to infer. Cholestasis induced by chlorpromazine is associated with decreases in Mg^{2+}-ATPase activity and membrane fluidity of the rat liver plasma membrane (26). In our study, dietary DHA resulted in increased Mg^{2+}-ATPase activity; DHA may thus improve some forms of liver cholestasis through interaction between DHA-modulated annular fluidity and Mg^{2+}-ATPase in the canalicular membrane. This speculation is based on the evidence that dietary fish oil increases bile flow (47), and supplementation with DHA from egg phospholipid alters the bile metabolism by increasing bile acid (48). Moreover, evidence indicates that liver bile flow is operated instead through canalicular ATP-binding cassette (ABC)-type proteins such as multidrug resistance-associated protein 2 (MRP2)/sister of P-glycoprotein (P-gp) (bile salt export pump, BSEP) (49). The function of P-gp is modulated by increases in fluidity of rat BCM (50). Thus, the DHA-induced increase in fluidity of the annular region that surrounds the membrane-bound Mg^{2+}-ATPase/ABC transporters may improve the function of bile flow. The canalicular ectonucleotidases, including 5′-nucleotidase, function in the liver, the major organ that synthesizes and salvages adenosine and other purine nucleotides (51). The DHA-induced increase in nucleotidase activity may thus improve these liver functions, although the mechanism remains unresolved.

Biliary cholesterol secretion is also controlled by the P-gp/ABC-type protein 1 transporter (52, 53). We have observed that dietary DHA significantly increases fecal cholesterol. It is not surprising, therefore, that DHA-induced decreases in plasma and BCM cholesterol and increases in fecal cholesterol are partially mediated through the fluidizing effect of DHA on the canalicular membrane-bound cholesterol and/or bile flow-related transporter functions. In the present experiments, plasma LDL cholesterol levels decreased in the DHA-fed rats. The hepatic LDL receptor is responsible for the removal of >70% of LDL from rats (54). Hepatic uptake of LDL particles has been demonstrated to be a function of plasma membrane (annular) fluidity, usually increasing with fluidity (6). Biliary secretion of hepatocytes is more favored by specialized membrane domains with increased fluidity (55). De-

creases in plasma total cholesterol and LDL cholesterol in the DHA-fed rats may thus be due to decreased endogenous synthesis, increased hepatic uptake of cholesterol from the blood flow, and the subsequent increase in biliary fecal excretion.

Increased presence of PUFA in the membrane increases the amount of LPO, leading to structural modification and/or cross-linking of proteins, an alteration in membrane fluidity, and finally, functional impairment (43, 56). This means that DHA, a highly purified PUFA with six double bonds, could be a susceptible target for free radicals, and may ultimately cause the structure and function of 5′-nucleotidase and Mg^{2+}-ATPase to deteriorate. In this study, however, the unaltered oxidative potential of free radicals such as LPO, ROS, thiols, and carbonyl content, further confirmed by tyrosine and tryptophan oxidation, suggests that dietary DHA administration (300 mg/kg per day) is not likely to provoke oxidative potential in the BCM proteins.

Our study suggests that DHA causes increased fluidity, with a greater influence on the annular lipids than on the global lipid regions, without exerting any oxidative potential on the liver canalicular plasma membrane, and consequently improves the activity of membrane-bound 5′-nucleotidase and Mg^{2+}-ATPase enzymes and other BCM-related hepatocellular transport functions.

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