Effects of dietary fish oil supplementation on membrane fluidity and enzyme activity in rat small intestine

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Rats were fed either a fat-free diet supplemented with 10 % menhaden oil or a control diet for four months. Intestinal brush border membranes were isolated; phospholipid fatty acid analysis revealed that the membranes from the fish-oil fed animals had higher levels of palmitoleic ($C_{16:1}$) and eicosapentaenoic ($C_{20:5}$) acids and lesser levels of stearic ($C_{18:0}$) linoleic ($C_{18:2}$) acids compared with controls. The membranes from the fish-oil fed animals had increased levels of alkaline phosphatase activity compared with controls but disaccharidase levels were equivalent in the two groups. Rocket immunoelectrophoresis studies revealed that the increase in alkaline phosphatase activity was due to an increase in the specific activity of the enzyme rather than an increase in the amount of enzyme. Membrane fluidity was assessed by fluorescence anisotropy using diphenylhexatriene and 12-anthroyl stearate as fluorescent probes. The anisotropy of both probes was similar in the two membranes. These studies indicate that fish-oil supplementation alters the fatty acid composition of the intestinal brush border membrane and increases alkaline phosphatase activity without affecting membrane fluidity. Thus the effects of changes in membrane lipid composition on alkaline phosphatase activity appear to result from changes in the local lipid environment of the enzyme rather than from changes in the biophysical characteristics of the membrane.

INTRODUCTION

Dietary manipulation can affect the lipid composition of biological membranes (Spector & Yorek, 1985; Brasitus *et al.*, 1985). Changes in the lipid composition of biological membranes can, in turn, affect the biophysical characteristics of the membrane and the activities of certain membrane-associated enzymes. The intestinal brush border membrane is specialized for digestion and absorption of nutrients; there is considerable evidence that these protein-mediated functions are influenced by the lipid composition of the membrane (Brasitus & Dudeja, 1985b, 1988; Brasitus & Schachter, 1980, 1982). In particular, the activity of one rat intestinal brush border membrane enzyme, alkaline phosphatase, is influenced by changes in lipid composition, namely cholesterol content, membrane phospholipid acyl composition, and phosphatidylcholine levels (Brasitus *et al.*, 1979, 1985; Brasitus & Schachter, 1980, 1982; Seetharam *et al.*, 1985). Arrhenius plots of intestinal brush border membrane alkaline phosphatase reveal changes in the slope near the lipid thermotropic transition which suggests an influence of the lipid environment on enzyme activity (Brasitus *et al.*, 1979; Brasitus & Schachter 1980). Other intestinal brush border membrane proteins including sucrase, lactase and maltase yielded single slopes on Arrhenius plots in the range of 10–40 °C suggesting that their activities were unaffected by phase changes in the membrane lipid. Moreover, changes in phospholipid acyl composition and cholesterol content have no effect on the activities of sucrase, lactase or maltase (Brasitus *et al.*, 1985; Brasitus & Dudeja, 1988).

The effects of alterations in the lipid composition of plasma membranes on enzyme activity may be the result of changes in the fluidity of the membrane or of more localized changes in the lipid environment of the protein. It is not clear whether a change in enzyme activity or other protein-mediated processes during a thermotropic transition are related to changes in the physical state of membrane lipid or the accompanying change in lipid composition in the environment of the protein. Similarly, manipulation of dietary lipids *in vivo* may influence various lipid components of membranes resulting in changes in the lipid environment of specific proteins as well as changes in fluidity (Brasitus *et al.*, 1985). While there is considerable evidence that the functions mediated by some 'intrinsic' proteins of rat brush border membranes are influenced by lipids, the exact mechanism(s) responsible for this phenomenon are relatively undefined.

Fish oil supplementation (FOS) has previously been shown to result in a marked increase in eicosapentaenoic ($C_{20:5}$) acid and decreases in arachidonic ($C_{20:4}$) and linoleic ($C_{18:2}$) acids (Hock *et al.*, 1987; Garg *et al.*, 1988). To address the issue of whether changes in the enzyme activities of intestinal microvillus membrane proteins are a function of the biophysical parameters of the membrane or of specific changes in membrane lipids, we have studied the effects of dietary supplementation with fish oil on biophysical parameters and enzyme activities in rat intestinal microvillus membranes. In the present study we therefore examined the effects of this dietary manipulation on the lipid composition and fluidity of intestinal brush border membranes, and on the activities of enzymes associated with these membranes.

Abbreviations used: FOS, fish oil supplemented; NP40, Nonidet P40; DPM, 1,6-diphenyl-1,3,5-hexatriene; 12-AS, DL-12-(9-anthroyl)stearic acid.

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MATERIALS AND METHODS

Animals and diet

Male Wistar rats were used throughout the study. Weanling rats were fed either Purina Rat Chow (control) or fat-free test diet with 10% menhaden fish oil (fish oil supplemented; FOS) (Anthony Bimbo, Zapata Haynie, Reedville, VA, U.S.A.) *ad libitum* for 4 months. At the end of this period the animals in each group weighed 500–600 g. The control diet contained 4.5% fat. The fat-free test diet obtained from ICN Nutritional Biochemicals Cleveland, OH, U.S.A. contained 20% casein, 55.4% sucrose, 15–16% Alphacell and 6.0 g/kg of a special fortified mixture that contained all necessary vitamins plus choline chloride. The fatty acid compositions of the control and FOS diets are given in Table 1.

Preparation of brush border membrane

Animals were killed and their small intestines removed. The intestines were divided in half and the segments identified as proximal and distal. Brush border membranes using distal intestinal mucosa were isolated by the method of Kessler *et al.* (1978).

Enzyme assays

Disaccharidases were assayed according to Dahlquist (1964). Alkaline phosphatase was assayed using *p*-nitrophenyl phosphate according to the method of Yedlin *et al.* (1981).

Rocket immunoelectrophoresis

Alkaline phosphatase in brush border membranes was quantified by rocket immunoelectrophoresis as described by Alpers *et al.* (1984). Briefly, the brush border membranes were solubilized overnight with 5% Nonidet P40 (NP40) followed by 70% ethanol precipitation of the solubilized extract at -20°C . The ethanol precipitate was dissolved in 100 μl of 10 mM-Tris/HCl, pH 7.4 containing 0.1% NP40. An aliquot (20 μl) of the solubilized extract was added to a 5 mm well of 1% agarose gel containing 5% polyethylene glycol (PEG) 6000, 0.1% Triton X-100 and a polyclonal antibody raised against purified rat intestinal alkaline phosphatase at 1:2000 dilution. Electrophoresis was carried out at 10 V/cm and 8°C for 5 h. After the electrophoresis, the gels were stained in 5-bromo-4-chloro-1-indolyl phosphate until rockets were clearly visible. The amount of alkaline phosphatase in each rocket was quantified by determining its area (mm^2), which is the product of the measured height and width at half height of the rocket, and comparing it with the area of rocket of a known amount of purified rat intestinal alkaline phosphatase. Samples formed rockets that were within the range of the standard curve.

Lipid analysis

Lipids were extracted from brush border membrane by the method of Bligh & Dyer (1959). Chloroform was evaporated and the fatty acids were methylated with methanolic HCl. Analysis of the fatty acid methyl esters was performed on a Varian 3760 gas chromatograph equipped with a Hewlett Packard 3393A integrator. The

methyl esters were separated on a fused silica capillary column (3.0 m $\times$ 0.25 mm i.d.) with a SP2330 stationary phase (Supelco, Bellefonte, PA, U.S.A.). The initial column temperature was 130°C , which was increased to 190°C at a rate of $5^{\circ}\text{C}/\text{min}$. Aliquots of 1–2 μl of the samples were injected in split mode with a splitter ratio of 1:10 and the fatty acid methyl esters were identified using appropriate standards. Cholesterol was assayed by the method of Courchaine *et al.* (1959) and phosphate was assayed by the method of Raheja *et al.* (1973).

Fluorescence anisotropy

Two fluorophores were utilized in these studies: 1,6-diphenyl-1,3,5-hexatriene (DPH) (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) and DL-12-(9-anthroyl)-stearic acid (12-AS) (Molecular Probes Inc., Junction City, OR, U.S.A.). Steady-state fluorescence polarization studies were performed with an SLM Aminco 4800 spectrofluorometer. The methods used to load the membranes and the quantification of the polarization of fluorescence have been described (Brasitus *et al.*, 1979). It should also be noted that as evaluated by steady-state fluorescence polarization of lipid fluorophores, 'fluidity' has usually been assessed via the fluorescence anisotropy, r , without further resolution of the components that determine r . Recently, however, studies have demonstrated that the rotations of the rod-like fluorophore DPH are hindered in both artificial and biological membranes. Therefore, the fluorescence anisotropy of such a fluorophore is not adequately described by the Perrin Equation but by a modified relationship (Heyn, 1979; Jahnig, 1979): $r = r_{\infty} + (r_0 - r_{\infty}) \cdot [t_c / (t_c + t_f)]$, where r is the fluorescence anisotropy, r_0 is the maximum limiting anisotropy, taken as 0.365 for DPH and 0.285 for 12-AS (Brasitus & Dudeja, 1988), r_{∞} is the limiting hindered anisotropy, t_c is the correlation time and t_f is the mean lifetime of the excited state. The r_{∞} values of DPH in natural and artificial bilayer membranes are high and largely determine r (Brasitus & Dudeja, 1985a, 1988). The r_{∞} values of DPH can, therefore, be used to assess the 'static component of fluidity' (Brasitus & Dudeja, 1985a, 1988). Unlike DPH, anthroxyloxy probes such as 12-AS yield relatively low values of r_{∞} in bilayer membranes (Brasitus *et al.*, 1988a). Their r values mainly reflect t_c , the speed of rotation, and can be used to assess the 'dynamic component of fluidity' of membranes (Brasitus *et al.*, 1988a). The content of each fluorophore in the preparations was estimated fluorometrically as described by Cogan & Schachter (1981). Final molar ratios of probe/lipid ranged from 0.001 to 0.003 and the anisotropy differences noted in these studies could not be ascribed to differences in probe concentrations in the preparations. Corrections for light scattering (suspensions minus probe) and for fluorescence in the ambient medium (quantified by pelleting the preparations after each estimation) were made routinely, and the combined corrections were less than 3% and 5% of the total fluorescence intensity observed for DPH-loaded and 2-AS-loaded membranes respectively. The lifetime, t_f , was estimated by phase and modulation fluorometry at 30 MHz in an SLM 4800 subnanosecond polarization spectrophotometer (SLM-Aminco, Urbana, IL, U.S.A.) as described (Brasitus & Dudeja, 1985a). Differential polarized phase fluorometry was also used to determine the r_{∞} of DPH in membranes from each group as previously described by Lakowicz *et al.* (1979).

RESULTS

The fatty acid compositions of the control and FOS diets were markedly different (Table 1). The major unsaturated fatty acids in the control diet were linoleic ($C_{18:2,n-6}$), oleic ($C_{18:1,n-9}$) linolenic ($C_{18:3,n-3}$) acids. In the FOS diet the major unsaturated fatty acids were eicosapentaenoic ($C_{20:5,n-3}$) palmitoleic ($C_{16:1,n-7}$), oleic ($C_{18:1,n-9}$) and decosahexaenoic ($C_{22:6,n-3}$) acids. Striking differences were noted in the fatty acid compositions of the control and FOS membranes (Table 2). The FOS membranes had higher levels of palmitoleic ($C_{16:1,n-7}$) and eicosapentaenoic ($C_{20:5,n-3}$) acids and lesser amounts of linoleic ($C_{18:2,n-6}$) acid. Membranes from FOS rats had lower levels of saturated fatty acids, a higher percentage of $n-3$, and a lower percentage of $n-6$ fatty

Table 1. Fatty acid composition of diets

Fatty acid	Diet ...	Composition (mol %)	
		Control	FOS
$C_{12:0}$		0.1	—
$C_{14:0}$		0.9	13.2
$C_{16:0}$		14.9	25.1
$C_{16:1,n-7}$		1.4	14.9
$C_{18:0}$		3.6	4.4
$C_{18:1,n-9}$		20.8	12.9
$C_{18:2,n-6}$		47.2	2.4
$C_{18:3,n-3}$		5.7	1.2
$C_{20:0}$		0.3	—
$C_{20:1,n-9}$		—	1.7
$C_{20:2,n-9}$		0.4	—
$C_{20:4,n-6}$		0.3	—
$C_{20:5,n-3}$		—	15.6
$C_{22:6,n-3}$		0.8	8.8

Table 2. Fatty acid composition of brush border membranes

Values are means $\pm$ S.E.M. ($n = 4$). * $P < 0.05$; ** $P < 0.01$ compared with control values.

Fatty acid	Composition (mol %)	
	Control	FOS
$C_{14:0}$	1.1 ± 0.1	2.4 ± 0.6
$C_{16:0}$	24.5 ± 0.9	24.5 ± 2.1
$C_{16:1,n-7}$	1.7 ± 0.4	$9.0 \pm 2.7^*$
$C_{18:0}$	19.8 ± 1.6	11.7 ± 4.6
$C_{18:1,n-9}$	23.1 ± 3.1	29.5 ± 10.0
$C_{18:2,n-6}$	16.4 ± 2.5	$5.8 \pm 1.1^{**}$
$C_{20:4,n-6}$	7.4 ± 0.7	5.2 ± 2.8
$C_{20:5,n-3}$	—	$3.7 \pm 1.9^{**}$
$C_{22:6,n-3}$	1.2 ± 0.1	2.9 ± 1.0
Classes:		
Saturated	45.5 ± 1.3	$38.6 \pm 1.7^*$
Monoenoic	24.8 ± 1.5	38.5 ± 5.8
Polyenoic	24.5 ± 0.8	17.7 ± 3.1
$n-3$	0.9 ± 0.3	$6.6 \pm 1.4^*$
$n-6$	23.6 ± 1.0	$11.0 \pm 1.9^{**}$
Ratio $n-3/n-6$	0.04 ± 0.01	$0.60 \pm 0.10^{**}$
Saturation index	0.49 ± 0.02	$0.36 \pm 0.01^{**}$

acids and a higher ratio of $n-3/n-6$. The saturation index for the FOS membranes was lower than for controls. In contrast to the fatty acid composition, there was no significant difference in the cholesterol/phospholipid molar ratio (results not shown).

Measurement of the biophysical parameters of the brush border membranes of the animals fed the two diets was assessed by the rotational mobilities of the two probes, DPH and 12-AS. The anisotropy and limiting hindered anisotropy of DPH in the two groups were similar (Table 3). Fluorescent lifetimes (phase and modulation) of DPH were not different in the control and FOS membranes (results not shown). Likewise, the anisotropy values of 12-AS were similar in the two groups. These data therefore indicate that both the static and dynamic components of fluidity were similar in the two membrane preparations.

The activities of brush border membrane enzymes were assessed in the two membranes. The activity of alkaline phosphatase was 2-fold higher in the membranes from the FOS rats than in the controls (Table 4). Other brush border membrane enzyme activities (lactase, sucrase and maltase) were similar in the two membrane preparations.

The finding that alkaline phosphatase activity was higher in the FOS membranes could be a product of a higher activity per alkaline phosphatase molecule or a product of a greater number of molecules of alkaline phosphatase in the FOS membranes. To address this issue, we assayed the amount of alkaline phosphatase in the membrane by rocket immunoelectrophoresis. This

Table 3. Fluorescence anisotropy of brush border membrane vesicles at 25 °C

Values are means $\pm$ S.E.M. for eight measurements per group

Diet	Anisotropy (r)	Limiting hindered anisotropy (r_∞)
DPH probe:		
Control	0.233 ± 0.008	0.182 ± 0.005
FOS	0.221 ± 0.009	0.167 ± 0.008
12-AS probe:		
Control	0.081 ± 0.002	
FOS	0.078 ± 0.002	

Table 4. Brush border membrane enzyme activities

Values are means $\pm$ S.E.M. ($n = 6$); * $P < 0.05$ compared with control.

Enzyme	Diet ...	Enzyme activity (units/mg of protein)	
		Control	FOS
Alkaline phosphatase		0.251 ± 0.050	$0.55 \pm 0.073^*$
Lactase		0.031 ± 0.009	0.024 ± 0.008
Sucrase		0.071 ± 0.015	0.082 ± 0.019
Maltase		0.552 ± 0.108	0.566 ± 0.144

Table 5. Rocket immunoelectrophoresis for alkaline phosphatase

Aliquots of brush border membrane were assayed for alkaline phosphatase activity and total protein to establish the specific activity of alkaline phosphatase. The amount of alkaline phosphatase in the aliquots was assayed by rocket immunoelectrophoresis. Data are presented as means $\pm$ S.E.M. * $P < 0.01$; ** $P < 0.001$ compared with control.

Diet	Alkaline phosphatase		
	(Units/mg of protein)	(Units/mg of alkaline phosphatase)	(μ g/mg of protein)
Control ($n = 11$)	0.24 ± 0.03	0.066 ± 0.005	3.73 ± 0.39
FOS ($n = 6$)	$0.55 \pm 0.07^{**}$	$0.110 \pm 0.016^*$	5.48 ± 1.11

experiment revealed that the units of alkaline phosphatase activity per μ g of alkaline phosphatase were significantly higher in the FOS than the control membranes (Table 5). The alkaline phosphatase content of the membrane (μ g of alkaline phosphatase per mg of protein) was somewhat higher in the FOS membranes than in the controls (5.48 ± 1.11 versus 3.73 ± 0.39) although the difference was not statistically significant ($P = 0.11$). It would therefore appear that the increased alkaline phosphatase activity in the FOS membranes was due primarily to an increase in enzyme activity rather than an increase in the amount of enzyme. However, the alkaline phosphatase content (μ g of alkaline phosphatase per mg of protein) was somewhat higher in the FOS membranes and this may have made some lesser contribution to the increase in alkaline phosphatase activity. The rocket immunoelectrophoresis data also indicate that the difference in alkaline phosphatase specific activities between control and FOS membranes was not an artifact of unequal purification of the brush border membranes from intestinal homogenates. If the difference was due to enhanced purification of the brush border membranes from the FOS animals then the units of alkaline phosphatase activity per μ g of alkaline phosphatase would have been the same in the two groups, and the μ g of alkaline phosphatase per mg of protein would have been significantly higher in the FOS membranes.

DISCUSSION

Earlier studies in rat myocytes (Hock *et al.*, 1987), rat liver (Garg *et al.*, 1988), and monkey neutrophils (Chabot *et al.*, 1987) have demonstrated that dietary supplementation with fish oil results in a marked change in membrane fatty acid composition without affecting the cholesterol/phospholipid molar ratio. As noted in those studies, the predominant $n-3$ fatty acids incorporated in the FOS membranes were $C_{20:5}$ and $C_{22:6}$. Fish-oil fed animals also showed a major decrease in $C_{18:2}$ and a lesser decrease in $C_{20:4}$. These earlier observations demonstrate that fish oil supplementation results in a fairly consistent pattern of changes in fatty acid content in various organs and various species. The effects of fish oil supplementation on fatty acid composition appear to be both the result of direct incorporation of dietary fatty acids into membrane phospholipids and also the result of effects of these dietary fatty acids on fatty acid metabolism (Garg *et al.*, 1988).

The data presented here demonstrate for the first time that feeding rats on a FOS diet results in significant

changes in the fatty acid composition of the intestinal brush border membrane and in the activity of intestinal alkaline phosphatase. Although fish oil supplementation resulted in a significant change in the fatty acid composition of the brush border membrane, with a decrease in the saturation index, there was no change in the biophysical parameters of the membrane as assessed by fluorescence anisotropy. Although alteration of phospholipid acyl composition is one way of altering the biophysical parameters of the membrane, there is no simple linear relationship between membrane physical properties and the number of fatty acid double bonds (Stubbs & Smith, 1984). The use of a saturation index does not adequately address the complexity of membrane biophysical parameters (Stubbs, 1983; Stubbs & Smith, 1984). Thus, in this study, fish oil supplementation altered the fatty acid composition of the membrane phospholipids without altering the biophysical parameters of the membrane.

In addition to the changes in fatty acid composition, alkaline phosphatase activity was increased in membranes from FOS animals. Rocket immunoelectrophoresis data indicate that this increase in alkaline phosphatase activity was primarily due to an increase in enzyme specific activity, although a lesser increase in the amount of alkaline phosphatase may also have contributed. The mechanism by which fish oil supplementation affects alkaline phosphatase activity is not fully elucidated. Other studies from our laboratory have recently addressed the relationship between alkaline phosphatase activity and lipid composition in the rat enterocyte membrane. Non-specific lipid transfer protein was used to vary the cholesterol/phospholipid molar ratio of rat small intestinal microvillus membrane (Brasitus *et al.*, 1988b). Cholesterol loading or depletion of the membranes was accompanied by a decrease or increase respectively in the lipid fluidity, as assessed by steady state fluorescence polarization techniques. Increasing the cholesterol/phospholipid molar ratio decreased alkaline phosphatase activity, whereas decreasing the ratio increased the activity. Sucrase, maltase and trehalase specific activities were not affected by cholesterol manipulation. However, the addition of benzyl alcohol, a known fluidizer, restored the fluidity of the cholesterol enriched membranes to control levels but did not change the cholesterol/phospholipid molar ratio and failed to alter alkaline phosphatase activity. This study indicates that although alterations in the cholesterol content and the cholesterol/phospholipid molar ratio affect both membrane fluidity and alkaline phosphatase

activity, the effect of cholesterol content on alkaline phosphatase activity is not a product of the change in membrane fluidity.

The effect of phospholipid fatty acid composition on fluidity and enzyme activity has also been previously addressed in a comparison of small intestine microvillus membranes from rats fed diets enriched with unsaturated (corn oil) and saturated (butter fat) triacylglycerols (Brasitus *et al.*, 1985). The corn oil diet increased the overall unsaturation of the acyl chains, enhanced lipid fluidity and increased alkaline phosphatase activity. Enrichment of the diet with corn oil in that study and enrichment with fish oil in the present study each resulted in an increase in alkaline phosphatase activity of approx. 2-fold. However, the effects of the two diets on the lipid composition and the fluidity of the membranes were dissimilar. Both diets decreased the saturation index of the membrane but the specific acyl changes were quite different. Corn oil supplementation increased linoleic ($C_{18:2}$) acid whereas fish oil supplementation increased eicosapentaenoic ($C_{20:5}$) and decosahexaenoic ($C_{22:6}$) acids and decreased linoleic ($C_{18:2}$) acid. In addition, corn oil supplementation increased the fluidity of the membrane whereas fish oil supplementation did not.

Finally, studies of the human erythrocyte sugar transport system have demonstrated that the activity of the transporter is minimally affected by alterations in the physical state of membrane lipids, but rather by relatively minor changes in its immediate lipid microenvironment (Carruthers & Melchior, 1988). Differential sensitivity of intestinal brush border membrane enzymes to treatment of the membrane with filipin has suggested the presence of lipid microdomains in these plasma membranes (Nemere *et al.*, 1983). Taken together, the present study of fish oil supplementation and the earlier studies from our laboratory and others on the effects of cholesterol enhancement and corn oil supplementation on alkaline phosphatase activity suggest that the manipulation of the lipid environment influences alkaline phosphatase activity by the interaction of the enzyme with specific membrane lipids *per se* rather than by changes in the physical state of the lipids of the small intestinal brush border membrane.

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