Effect of Altered Dietary n-6-to-n-3 Fatty Acid Ratio on Erythrocyte Lipid Composition and Membrane-Bound Enzymes

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(Received March 27, 2002)

Summary Three experimental diets with varied n-6-to-n-3 fatty acid ratios (120, 40 and 8) were prepared by a suitable blending of safflower oil containing 72.5% linoleic (18:2 n-6) acid and non-detectable levels of α-linolenic (18:3 n-3) acid, and soybean oil having 56.1% linoleic (18:2 n-6) acid and 7.9% α-linolenic (18:3 n-3) acid. These diets were fed to weanling female Wistar/NIN (inbred) rats for 16 wk to assess the impact of altered dietary n-6-to-n-3 fatty acid ratio on erythrocyte membrane (EMS) cholesterol, phospholipids, fatty acid composition and activities of membrane-bound enzymes such as Na+ ,K+ -ATPase, Ca2+ , Mg2+ -ATPase and acetylcholinesterase. Activities of total and ouabain-sensitive-ATPases were significantly higher in the erythrocyte membranes of rats fed diets with a n-6-to-n-3 fatty acid ratio of 40 compared to other groups, whereas the erythrocyte membrane-bound acetylcholinesterase was significantly different among the three groups. The highest and lowest activities for this enzyme were observed in the dietary groups with n-6-to-n-3 fatty acid ratios of 8 and 40 respectively. However, the EMS of rats fed diets with a n-6-to-n-3 fatty acid ratio of 40 alone had significantly higher Ca2+ , Mg2+ -ATPase compared to those of other two groups. Significant increases were observed in absolute amounts of cholesterol, phospholipids and molar ratio of cholesterol to phospholipids in the EMS of rats fed a diet with a very high 18:2 n-6-to-18:3 n-3 fatty acid ratio (120) as compared to those from the dietary group with 18:2 n-6-to-18:3 n-3 fatty acid ratio (40), which had the lowest levels of cholesterol, phospholipids and cholesterol-to-phospholipid molar ratio. On the other hand, the EMS from rats fed a diet with a very low n-6-to-n-3 fatty acid ratio (8) had significantly lower cholesterol and higher proportions of stearic (18:0), oleic (18:1 n-9), eicosapentaenoic (20:5 n-3), and docosahexaenoic acids, and a higher ratio of docosahexaenoic (22:6 n-3) acid-to-α-linoleic (18:3 n-3) acid compared to the EMS from a very high n-6-to-n-3 fatty acid ratio of 120. Although these changes in EM fatty acid profiles were expected of the respective dietary regimens, the observed changes in the activities of membrane-bound enzymes could have resulted from their interaction with membrane cholesterol, phospholipids and fatty acyl chains.

Key Words linoleic acid, α-linolenic acid, n-6-to-n-3 fatty acid ratio, erythrocyte membrane-bound enzymes, fatty acid composition

Cholesterol and phospholipids are the two important lipid components of membranes that regulate various cellular and metabolic processes by altering the activities of membrane-bound enzymes, receptors and also proteins involved in signal transduction mechanisms (1, 2). The polyunsaturated fatty acyl chains of membrane phospholipids not only serve as reservoirs for several bioactive compounds like eicosanoids but also contribute significantly to the physicochemical properties of the membrane lipid micro-environment (3-6).

Besides providing energy and essential fatty acids, namely linoleic (18:2 n-6) and α-linolenic (18:3 n-3) acids, dietary fat modifies membrane lipid components and fatty acid composition, which in turn impact the functioning of membrane-bound enzymes, transport systems, binding of ligands to receptors and finally signal transduction mechanisms and expression of genes encoding lipogenic enzymes (3-6). ATPases are plasma membrane-bound enzymes of many living cells and play a prominent role in the regulation of trans-membrane gradient of ions (4).

Arachidonic (20:4 n-6) and docosahexaenoic (22:6 n-3) acids are the two long-chain polyunsaturated fatty acids of membrane phospholipids derived from dietary linoleic (18:2 n-6) and α-linolenic (18:3 n-3) acids respectively. Of late, the importance of n-3 fatty acids in the maintenance of health and prevention of chronic degenerative diseases has been recognized (6-10). Based on this, emphasis has been laid on the increased consumption of n-3 fatty acids and decreased intake of n-6 acids, or in other words, to decrease the dietary n-6-to-n-3 fatty acid ratio. It has also been suggested that

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diets should provide an optimal ratio of n-6-to-n-3 fatty acids, which may be between 6–10. This optimal ratio is derived from the fatty acid composition of brain lipids and human milk (8). However, this ratio may vary at different stages of life cycle with respect to different organs and various physiological and pathological conditions (8). There is actually little information available concerning the appropriate amounts and optimal ratio of n-6-to-n-3 fatty acids for the modulatory action on membrane structure and function (8).

In India, like elsewhere in the world, fat consumption is influenced by several factors such as socio-economic status, agronomic conditions, regional preferences, religious beliefs, culinary practices and Government policy in relation to food and oil supply (11). Until the recent past, Indian populations were habitually involved in the usage of a single oil as a cooking medium (11, 12). However, of late, the oil consumption pattern of the Indian population is changing due to emerging scientific information on the beneficial effects of n-3 fatty acids, the possible negative effects of a high n-6-to-n-3 fatty acid ratio and changing Government policy to permit the blending of vegetable oils (11, 13). To this effect, the blending of commonly consumed and largely produced vegetable oils in different proportions has been recommended by our institute to mitigate the possible negative effects of only single vegetable oil consumption (11). In the present study, we have used blends of two vegetable oils, namely safflower oil (a commonly consumed linoleic acid-rich vegetable oil) with soybean oil (largely produced vegetable oil in India), with moderate levels of α-linolenic acid in specific proportions to obtain three n-6-to-n-3 fatty acid ratios. Of the three n-6-to-n-3 fatty acid ratios employed in this study, only 120 is high and the other two ratios (40 and 8) are in the usual range of n-6-to-n-3 fatty acid ratios of Indian (urban and rural) diets (11, 12).

The impact of these blended oils on erythrocyte lipid composition and function in terms of activities of membrane-bound enzymes has been assessed. We have particularly chosen the erythrocytes for this purpose, as they represent the prototype of plasma membranes and are easy to isolate and highly responsive to lipid milieu both under in vitro and in vivo conditions. In addition, the EM lipid and fatty acid compositions reflect not only the habitual dietary fat intake but also the underlying developmental, metabolic changes and potential disease processes.

**MATERIALS AND METHODS**

Substrates and chemicals. The substrates and chemicals used for enzyme assays were obtained from Sigma Chemicals, St. Louis, MO, USA. The solvents used were of analytical grade, and reference standards for gas chromatographic analysis were procured from Nu chek Prep. (Elysian, MN).

Animals and diets. The research proposal concerning this study was submitted to the Institutional Animal Ethical Committee (IAEC) and prior approval was obtained.

Female weanling Wistar/NIN inbred rats from the National Centre for Laboratory Animal Science, NIN, India, were divided into three groups and were reared on a diet containing 20% protein (casein-based) and 10% oil blends (differing in the proportions of linoleic (18:2 n-6) and α-linolenic (18:3 n-3) acids viz. the ratios being 120, 40 and 8, respectively). The compositions of the diets and the proportions in which the safflower and soybean oils were blended are shown in Table 1.

The fatty acid compositions of the oils used in blending and the three oil blends are given in Table 2. The diets were complete with respect to all other essential nutrients and stored at 4°C. Rats were housed individually in a controlled environment with 12-h light and dark cycles and fed ad-libitum for 4 mo. There were no differences in the food intake and gain in body weight of the animals fed the varied n-6-to-n-3 fatty acid ratio containing diets (data not shown). At the end of the ex-
Dietary n-6-to-n-3 Fatty Acid Ratio and Rat Erythrocyte Membrane Enzymes and Lipids

Experimental feeding, blood was collected by capillary tube by ocular vein puncture. Plasma was separated and erythrocytes were washed and processed for plasma membrane isolation as described by Kaplay (14).

Enzymatic assays. Na+, K+-ATPase (EC 3.6.1.3) was assayed according to the method of Post and Sen (15). The final volume of reaction mixture was 0.5 mL and had 140 mmol/L NaCl, 14 mmol/L KCl, 3 mmol/L MgCl₂, 3 mmol/L Tris-ATP, 1 mmol/L EGTA and 50 mmol/L Tris-HCl (pH 7.4). Ouabain, when added, was a 1 mmol/L concentration. Ca²⁺,Mg²⁺-ATPase (EC 3.6.1.3) was assayed according to the method of Schatzmann and Rossi (16). The reaction was carried out in a medium containing 100 mmol/L KCl, 2 mmol/L MgCl₂, 3 mmol/L Tris-ATP, 50 mmol/L Tris HCl (pH 7.4). 0.2 mmol/L Tris EDTA, 0.1 mmol/L DTNB (5,5'-dithiobis-2-nitrobenzoic acid), 1 mmol/L acetylthiocholine in a total volume of 1.5 mL. The reaction was stopped by adding 1.5 mL of absolute alcohol, and the colour was read at 412 nm. Membrane protein was estimated according to the method of Lowry et al. (18) using crystalline bovine serum albumin as the standard.

Extraction and analysis of lipids. The method of Folch et al. (19) was employed for the extraction of total lipids using 2:1 chloroform : methanol, to which 25 mg BHT/L was added as an antioxidant. Phospholipid phosphorus was determined according to the method of Bartlett (20) and total cholesterol by the method of Zlatkis et al. (21).

Gas chromatographic analysis. Methyl esters of erythrocyte membrane lipids were prepared by transesterification using methanolic NaOH as described by Kishimoto and Hoshi (22) and quantified using a Perkin Elmer 8500 gas chromatograph equipped with a flame ionisation detector and an electronic integrator. Fatty acid methyl esters were separated on a capillary column (sp-2340 Supelco: 30 m length, 0.25 mm i.d., and 0.2 µm film thickness). Pure nitrogen was used as the carrier gas at a flow rate of 2 mL/min. During the run, the temperature of the column was programmed from 120–140°C with 30°C increments after an isotime of 10 min, then 1 min after attaining 140°C, the temperature was raised to 220°C with a ramp rate of 2.5°C/min and maintained for 1 min at 220°C. From this, the final temperature of 230°C was attained with increments of 0.5°C/min, where it was maintained for 1.0 min. Throughout the run, the injector and detector temperatures were maintained at 230°C. The individual fatty acids were identified from a standard mixture obtained from Nu chek prep (Elysian, MN, USA). Peak areas to concentration were computed by a Perkin Elmer GP-100 integrator.

Statistical analysis. All results were statistically analysed by ANOVA and the level of significance was expressed at p<0.05.

RESULTS

It is evident from the results (Table 3) that with the feeding of a diet having a fatty acid ratio of 120, the higher n-6-to-n-3 acid ratio caused an increase in the highest arachidonic (20:4 n-6) acid content of the EMS. On the other hand, higher levels of docosahexaenoic (22:6 n-3) and eicosapentaenoic (20:5 n-3) acids were observed in the EMS of rats fed diets having n-6-to-n-3 fatty acid ratios of 40 and 8. Besides these expected changes, which are reflections of the dietary fat fed, the erythrocyte membranes of rats fed n-6-to-n-3 fatty acids at a ratio of 8 revealed significantly high proportions of stearic (18:0) and oleic (18:1 n-9) acids, and significantly lower proportions of linoleic (18:2 n-6) and arachidonic (20:4 n-6) acids compared to the other two groups. However, this dietary n-6-to-n-3 fatty acid ratio (8) had no impact on the ratios of either unsaturated to saturated fatty acids or double bond index (data not shown) compared to rats fed diets with n-6-to-n-3 fatty acid ratios of 120 and 40. The total monounsaturated fatty acid (MUFA) content of this group was significantly higher than the other two groups. Although the polyunsaturated fatty acid contents of various groups were not affected by dietary alterations in the n-6-to-n-3 fatty acids ratio, the EMS of rats fed a dietary n-6-to-

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<tr>
<td>21.1±0.96</td>
<td>0.6±0.14</td>
<td>13.7±0.69</td>
<td>9.9±0.15</td>
<td>9.2±0.42</td>
<td>0.4±0.18</td>
<td>0.8±0.07</td>
<td>31.9±0.35</td>
<td>1.5±0.17</td>
<td>7.4±1.54</td>
<td>2.9±0.27</td>
<td>0.9±0.17</td>
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<td>20.7±0.90</td>
<td>0.5±0.08</td>
<td>14.1±0.89</td>
<td>10.9±0.72</td>
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<td>0.6±0.13</td>
<td>1.2±0.25</td>
<td>28.9±1.64</td>
<td>2.8±0.57</td>
<td>7.0±1.80</td>
<td>0.7±0.24</td>
<td>1.4±0.32</td>
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<td>21.1±0.75</td>
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<td>16.9±0.52</td>
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<td>0.4±0.14</td>
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<td>3.0±0.22</td>
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<td>1.3±0.54</td>
<td>2.8±0.92</td>
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<tr>
<td>31.9±0.35</td>
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<td>15.3±0.43</td>
<td>27.4±1.09</td>
<td>24.0±1.54</td>
<td>24.1±1.96</td>
<td>22.6±1.83</td>
<td>42.3±1.00</td>
<td>56.0±0.60</td>
<td>19.2±3.92</td>
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<td>42.0±0.80</td>
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<td>79.0±0.91</td>
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<td>3.9±0.82</td>
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<td>11.5±0.82</td>
<td>14.8±0.70</td>
<td>44.2±0.84</td>
<td>44.5±1.18</td>
<td>42.4±0.66</td>
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Data are expressed as percent distribution of fatty acid methyl esters. Values are M±SE of six observations. Mean values in the horizontal rows not sharing a common superscript are different at 5% level by ANOVA.

Table 3. Major fatty acids of erythrocyte membrane lipids from rats fed diets with varied n-6-to-n-3 fatty acid ratios.

MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.
Table 4. Lipid composition of erythrocyte membranes of rats fed diets with different ratios of linoleic and α-linolenic acids.

<table>
<thead>
<tr>
<th>n-6-to-n-3 fatty acid ratio</th>
<th>Cholesterol (μmol/mg protein)</th>
<th>Phospholipid phosphorus (μmol/mg protein)</th>
<th>Cholesterol to phospholipid molar ratio</th>
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</thead>
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<tr>
<td>120</td>
<td>0.77±0.053a</td>
<td>0.61±0.07b</td>
<td>1.38±0.18c</td>
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<td>40</td>
<td>0.38±0.08a</td>
<td>0.32±0.06a</td>
<td>1.01±0.11a</td>
</tr>
<tr>
<td>8</td>
<td>0.57±0.07a</td>
<td>0.50±0.06ab</td>
<td>1.17±0.08ab</td>
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Values are M±SE of ≥15 rats. Means within the columns not sharing a common superscript are different at 5% level by ANOVA.

n-3 fatty acid ratio of 120 had the highest total n-6-to-n-3 fatty acid ratio. The ratios of 20:4 n-6-to-18:2 n-6 and 22:6 n-3-to-18:3 n-3, which are indicative of the preponderance of either n-6 or n-3 fatty acid in the diet, suggested that the EMS of rats fed the highest n-6-to-n-3 fatty acid ratio had the highest ratio for the former and the group with the n-6-to-n-3 fatty acid ratio of 8 had the highest value for the latter.

The concentrations of cholesterol and phospholipids showed distinct differences in erythrocyte membranes isolated from the three groups (Table 4). Both cholesterol and phospholipid contents were higher in the EMS of rats with the dietary n-6-to-n-3 fatty acid ratio of 120 when compared to the other two groups with dietary n-6-to-n-3 fatty acid ratios of 40 and 8 respectively. The concentrations of membrane cholesterol, phospholipids and the ratio of cholesterol to phospholipids were significantly decreased in the dietary group with a n-6-to-n-3 fatty acid ratio of 40.

Specific activities of membrane-bound enzymes are shown in Figs. 1, 2 and 3. The activities of Na⁺,K⁺-ATPase and Ca²⁺,Mg²⁺-ATPase were found to be highest in the erythrocyte membranes of rats with dietary n-6-to-n-3 ratio of 40 as compared to those of rats on dietary n-6-to-n-3 fatty acid ratios of 120 and 8. The activity of acetylcholinesterase was considerably higher in groups with n-6-to-n-3 fatty acid ratios of 120 and 8 as compared to that of the dietary n-6-to-n-3 fatty acid ratio of 40.

DISCUSSION

The results of the present study indicate that blended oils with various n-6-to-n-3 fatty acid ratios have profound effects on the lipid composition of the erythrocyte membrane, which in turn, influences the activities of membrane-bound enzymes. An extensive review of related literature indicates that there are several studies including our own regarding the effects of individual vegetable oils with diverse fatty acid compositions and n-6 and n-3 fatty acid contents on erythrocyte membrane fatty acid composition and membrane fluidity (23–25). However, this study is the first of its kind wherein two oils, one with very high levels of linolenic acid (18:2 n-6) and the other with moderate levels of α-
linoleic (18:3 n-3) acid, have been blended to obtain high, moderate and low (120, 40 and 8) ratios of n-6-to-n-3 fatty acids, and related enzymes of significance have been studied.

The results on fatty acid composition suggest that the EMS of the rats that received an oil blend of 40 had intermediary levels of several fatty acids such as stearic (18:0), oleic (18:1 n-9), linoleic (18:2 n-6), arachidonic (20:4 n-6) and docosahexaenoic (22:6 n-3) acids. The EMS of this group further displayed increased activities of Na\(^+\),K\(^+\)-ATPase and Ca\(^{2+}\),Mg\(^{2+}\)-ATPase.

The role of cholesterol in membrane stability and integrity is very well established and cholesterol is a major component of eucaryotic cells. In erythrocyte membrane, an increase in membrane cholesterol level brings about a decrease in the activities of ATPases (26–31). Although the exact mechanism by which higher membrane cholesterol brings about the inhibition of Na\(^+\),K\(^+\)-ATPase is not clear, several studies indicate that cholesterol can cause a substantial modification in the ordering of phospholipids and a decrease in acyl chain volume (1, 30, 31). They are sensitive to alteration in the membrane cholesterol-to-phospholipid molar ratio resulting from increased membrane cholesterol, and thereby inhibit the activities of Na\(^+\),K\(^+\)-ATPase and Ca\(^{2+}\),Mg\(^{2+}\)-ATPase. Na\(^+\),K\(^+\)-ATPase is an important multifunctional protein system located in the plasma membranes of most cells. It is the only ion pump that contains a receptor (4). It is interesting to note that diets with a very high ratio of n-6-to-n-3 fatty acids (120) showed significantly higher levels of cholesterol and phospholipids in their membranes, thereby resulting in a higher cholesterol-to-phospholipid molar ratio as compared to the group with a n-6-to-n-3 ratio of 40; with these membranes displaying decreased activities of Na\(^+\),K\(^+\)-ATPase and Ca\(^{2+}\),Mg\(^{2+}\)-ATPase. The exact mechanism by which EMS cholesterol content increased in the groups of animals that received diets having n-6-to-n-3 fatty acid ratios of 120 and 8 is not clear. However, the feeding of high linoleic acid diets (such as safflower oil) is known to cause cholesterol accumulation in the rat and guinea pig EMS, thereby resulting in a higher cholesterol-to-phospholipid molar ratio as compared to the very rich n-6 fatty acid diets. Regardless of the mechanism, the inhibition of Na\(^+\),K\(^+\)-ATPase through increased levels of membrane cholesterol in the EMS of rats fed very high linoleic acid-containing diets has significant implications for cellular energy metabolism since 20–50% of the total production of cellular energy is used by Na\(^+\),K\(^+\)-ATPase (33). Furthermore, decreased red cell membrane Na\(^+\),K\(^+\)-ATPase has been reported for diabetic rats (34).

The activity of EM-bound Ca\(^{2+}\),Mg\(^{2+}\)-ATPase also showed a significant increase in the group with an intermediary ratio of n-6-to-n-3 fatty acids (40) as compared to the other two groups. Lee et al. (35) extensively studied the behaviour of this enzyme in altered lipid environments and established a lack of apparent association between the degree of unsaturation of the membrane lipids and Ca\(^{2+}\),Mg\(^{2+}\)-ATPase of sarcoplasmic reticulum. However, subsequently Michelangeli et al. (36) demonstrated an interaction of cholesterol with Ca\(^{2+}\),Mg\(^{2+}\)-ATPase that was capable of bringing about the inhibition of activity. In fact, Madden et al. (37) demonstrated a decreased activity of Ca\(^{2+}\),Mg\(^{2+}\)-ATPase with an increased cholesterol content in the sarcoplasmic reticulum. Further, the feeding of cholesterol resulted in increased activity of this enzyme in various tissues (31). However, our results suggest that moderate proportions of both n-6 and n-3 fatty acids of erythrocyte membrane are favourable for the optimal activity of plasma membrane-Ca\(^{2+}\),Mg\(^{2+}\)-ATPase ascompared to the very rich n-6 fatty acid diets. Interestingly, these membranes also had the lowest cholesterol-to-phospholipid molar ratio.

Acetylcholinesterase is yet another membrane-bound enzyme present in erythrocyte membranes and neuronal endings. Although the precise function of acetylcholinesterase in EMS is not clear, it has been implicated in several metabolic processes, maintenance of cell volume, shape, function, and stability of erythrocytes (38). Lipid protein interactions of human erythrocyte membrane acetylcholinesterase have been studied, and it has been concluded that although the enzyme as such is not a lipid-dependent one, it is strongly modulated by its hydrophobic environment. The depressed activity of acetylcholinesterase of EMS has been reported in diabetes, in vitro and in vivo conditions resulting in cholesterol depletion (39, 40). On the other hand, in experimental rats with spontaneous hypertension, along with increased cholesterol and calcium contents of the membranes, there is enhanced activity of acetylcholinesterase of the erythrocyte membrane (41).

In the present study, the elevated cholesterol levels in the EMS of rats fed diets with n-6-to-n-3 fatty acid ratios of 120 and 8 must be responsible for the higher activity of this enzyme. It may be noted that though 1.20 is a very high n-6-to-n-3 fatty acid ratio, the other two ratios reflect the usual range of dietary n-6-to-n-3 fatty acid ratios of normal (high and low) income groups in India. Although, these results cannot be extrapolated to human situations, they may form a basis for the development of oil blends that can be tested for their nutritional and physiological effects in human subjects.

Acknowledgments

We thank Drs. Mohan Ram and Kamala Krishnaswamy, former Directors of National Institute of Nutrition, for their interest in this study. Mrs. P. Lopamudra is thanked for technical assistance. Thanks are also due to Mrs. Padmini Suryaprakash for going through the manuscript, Dr. N. Balakrishna for statistical advice and Mr. S. M. Jeyakumar for figures.

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