Effects of a High α-Linolenate and High Linoleate Diet on Hemolysis and Lipid Peroxidation of Rat Erythrocytes

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α-Linolenic acid (18:3n-3) is known to autoxidize several fold faster than linoleic acid (18:2n-6). Feeding a high α-linolenate or a high linoleate diet to rats resulted in significant changes in the n-3/n-6 ratios of 20 and 22 carbon highly unsaturated fatty acids in erythrocytes. However, the rates of hemolysis observed in N₂ or O₂-atmosphere were similar between the two dietary groups. No significant amounts of conjugated dienes were detected and no measurable changes in the fatty acid compositions were observed during the incubations, indicating that the hemolysis occurred without involving significant lipid peroxidation. When stimulated with a free radical initiator, [2,2'-azobis-(2-aminodipropionitrile)di(t-amyl)] (AAPH), hemolysis occurred more rapidly, conjugated dienes formed and unsaturated/saturated ratios of phospholipid fatty acids decreased. However, no statistically significant difference was observed in these parameters of the two dietary groups. These results indicate that hemolysis occurs without involving lipid peroxidation but is accelerated by free radicals through lipid peroxidation, and that the difference in antioxidizabilities of α-linolenate and linoleate is not reflected in the rates of hemolysis and autoxidation in rat erythrocytes.

Keywords dietary fatty acid; α-linolenate; linoleate; hemolysis; lipid peroxidation; erythrocyte; α-linolenic acid; linoleic acid

Introduction

Beneficial effects of feeding α-linolenic acid as compared with linoleic acid have been recognized in several animal models of chronic diseases. However, unfavorable side effects have been suspected for long-term feedings of n-3 fatty acids since n-3 fatty acids are generally more easily autoxidizable than the corresponding n-6 fatty acids with the same carbon chains. In fact, the rate of lipid peroxidation of membrane lipids is reported to be faster in the fish oil supplemented group than in the control diet group. According to a free radical theory, free radicals generated in cells attack polysaturated fatty acids (PUFA) to form lipid peroxides, which in turn decompose to produce more free radicals. This kind of chain reaction is presumed to enhance the progress of aging, tumorigenesis and atherosclerosis. Researchers who discuss human nutrition based on this free radical theory often advise people not to take a large amount of n-3 fatty acids. However, the results of our in vivo experiments were against this autoxidation-related lipid peroxide theory; a high α-linolenate diet, as compared with a high linoleate diet, suppressed aging as well as tumorigenesis in rats. Thus, the antioxidizability of fatty acids in diets does not appear to be directly correlated with the ability to produce and proliferate free radicals in vivo.

In this paper, we examined a possible relationship among the antioxidizability of dietary fatty acids, peroxidizability of membrane lipids and red cell stability. Use of a high α-linolenate (perilla seed oil) diet and a high linoleate (safflower seed oil) diet would allow us to examine mainly the effects of α-linolenate and linoleate because these two diets are roughly similar in their amounts of saturated fatty acids, mono unsaturated fatty acids and tocopherols.

Materials and Methods

Diet and Animals Male Sprague-Dawley rats (Shizuoka Laboratory Animals Co., Shizuoka, Japan) at 3 weeks of age were fed experimental diets for 17 or 37 weeks. The diet consisted of conventional laboratory chow (Nihon Clea Co., Tokyo, Japan) treated with hexane to remove endogenous lipids and then supplemented with vegetable oils (10 or 15%) and a vitamin mixture (2%) (Nihon Clea Co.). Either safflower seed oil (rich in linoleate) or perilla seed oil (rich in α-linolenate) (Ohta Oil Co., Okazaki, Japan) was used. Each oil contained 0.1% vitamin E and the diet contained a total of 25 mg vitamin E/100 g. Diets with peroxide values below 50 meq/kg were served. These diets brought about no differences in growth rates and in appearance as compared with a conventional diet. The fatty acid compositions of the diets are given in Table I.

Preparation of Washed Erythrocytes and Hemolysis Assay Rats were anesthetized with nembutal and exanguinated by abdominal aorta in the presence of 3.13% sodium citrate as an anticoagulant (1:5, v/v). Red cells were prepared by centrifugation and washed three times with phosphate-buffered saline. Erythrocytes were suspended in phosphate-buffered saline (pH 7.4) containing 20 mM glucose at a packed cell volume of 2.5%. To keep the suspension sterile during incubations, penicillin (200 unit/ml), streptomycin (1 mg/ml), and fungizone (10 μg/ml, Gibico) were added. Either oxygen (95% O₂ and 5% CO₂) or nitrogen (N₂) was gassed through the incubation mixture for 2 min. Hemolysis was followed at 540 nm by measuring the hemoglobin content of the supernatant after its conversion into cyanmethemoglobin (Wako hemoglobin kit, Wako Co., Ltd.).

When the effect of 2,2'-azobis(2-aminodipropionitrile)di(t-amyl) (AAPH), an azo compound, was examined, AAPH was added at a concentration of 74 mM to 20% erythrocytes suspended in phosphate-buffered saline (pH 7.4). The mixture was shaken gently during the incubation at 37°C.

Analysis of Fatty Acid Composition and Measurement of Conjugated Dienes Lipids were extracted from erythrocytes with chloroform: methanol (2:1, v/v), and phospholipids were separated by thin-layer chromatography.

Table I. Fatty Acid Composition of the Diets

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>% of total fatty acids</th>
<th>High linoleate (safflower)-diet</th>
<th>High α-linolenate (perilla)-diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>16:0</td>
<td>7.9</td>
<td>7.1</td>
<td>6.1</td>
</tr>
<tr>
<td>18:0</td>
<td>1.8</td>
<td>1.6</td>
<td>1.4</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>17.3</td>
<td>16.1</td>
<td>17.8</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>17.1</td>
<td>15.6</td>
<td>17.4</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>51.7</td>
<td>49.9</td>
<td>50.9</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.7</td>
<td>9.1</td>
<td>0.7</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>0.3</td>
<td>9.1</td>
<td>0.3</td>
</tr>
<tr>
<td>n-3/n-6</td>
<td>3.0</td>
<td>3.2</td>
<td>3.0</td>
</tr>
</tbody>
</table>

a) The diet contained 10% high linoleate oil (safflower oil) or high α-linolenate oil (perilla oil). The fatty acid compositions (w/w %) were quantitated by gas chromatography. Fatty acids are expressed by the carbon chain: the number of double bond, and the first double bond numbered from the methyl terminus is designated as n-9, n-6 or n-3.
Fatty acids were analyzed as methyl esters by gas chromatography as described previously.18,19 Conjugated dienes were estimated by measuring the absorbance at 233 nm (ε=23000 M⁻¹ cm⁻¹) in cyclohexane solution.21,22

Results

Fatty acid compositions of erythrocyte phospholipids are shown in Fig. 1. Despite a significant difference in the proportions of linoleate and α-linolenate in the diets, the proportion of linoleate varied little and no significant amount of α-linolenate was found even in the high α-linolenate group. The major difference was observed in the 20 and 22 carbon highly unsaturated fatty acids (20:4n−6 vs. 20:5n−3 and 22:4n−6 vs. 22:5n−3).

Hemolysis of erythrocyte suspension was followed for up to 7d. Under the conditions, the extent of hemolysis reached a plateau level (70%) within 24h (Fig. 2). The rate of hemolysis tended to be slightly higher in the high linoleate group than in the high α-linolenate group but the difference was not statistically significant. When incubation atmosphere (O₂-CO₂) was replaced with nitrogen, the rate of hemolysis was affected a little. Quite similar results were obtained by using diets with 15% oils instead of 10% (data not shown).

Fatty acid compositions of the erythrocytes were determined before and after the incubations. Even after 7d of incubation, no significant changes in the fatty acid compositions were observed (data not shown). These results indicate that the highly unsaturated fatty acids (HUFA) are quite resistant to autoxidation when present in the membranes in the presence of antibiotics, which was confirmed by measuring the production of conjugated dienes; very little conjugated diene was detected (below 10 nmol/mg fatty acids).

When a free radical generating agent, AAPH, was added to the incubation mixture at a concentration of as high as 74 mm, hemolysis occurred more rapidly23 (data not shown). Changes in the fatty acid compositions of the erythrocytes during the 3h incubation with AAPH were observed as shown in Fig. 3. The decrease in the proportions of 20:4n−6 and 22:6n−3 with a concomitant increase in the proportion of palmitate (16:0) was clearly seen in the high linoleate group and similar changes were also observed in the high α-linolenate group. In accordance with the decrease in highly unsaturated fatty acids, significant amounts of conjugated dienes were formed (Fig. 4). However, no statistically significant difference was observed between the two dietary groups.

Fig. 1. Fatty Acid Compositions of Rat Erythrocyte Total Lipids

Sprague-Dawley rats were fed a diet supplemented with a high linoleate oil (safflower oil) or a high α-linolenate oil (perilla oil) for 17 weeks. Figures represent means ± S.E. for 3 rats. Statistical significance by Student’s t-test is shown as a) p<0.05, b) p<0.01 or c) p<0.001 for the high linoleate vs. the high α-linolenate groups. □, high-α-linolenate; ○, high-linoleate.

Fig. 2. Effect of Dietary n−3/n−6 Balance on Hemolysis of Rat Erythrocytes

Each point represents the mean for 3 rats, each assayed in duplicate. Rats fed either the high α-linolenate or high linoleate diet for 7 weeks were used.

High α-linolenate: • ○
High linoleate: △ △

Fig. 3. Effect of AAPH on Fatty Acid Compositions of Total Lipids of Erythrocytes

Values are means ± S.E. for 3 or 4 rats. Statistical significance by Student’s t-test is shown as a) p<0.05 or b) p<0.01.

Fig. 4. AAPH-Stimulated Lipid Peroxidation Measured as Diene Conjugate Formation

Sprague-Dawley rats were fed test diets for 37 weeks. AAPH was added at a concentration of 74 mm to 20% erythrocytes suspended in phosphate buffered saline (pH 7.4). Each point represents the mean ± S.E. of determinations for 4 to 5 rats. △, high-linoleate; ○, high-α-linolenate.
Discussion

Cell damages by free radicals and lipid peroxides have been clearly shown *in vitro* in the absence of appropriate amounts of antioxidants. However, vitamins E and C serve as endogenous antioxidants and the causal relationship between the lipid peroxide formation *in vivo* and pathogenesis of tumor, aging and atherosclerosis still remains to be clarified. The present results indicate that HUFA in the membranes are quite resistant to autoxidation in the presence of endogenous antioxidants; no significant diene formations nor losses of HUFA were observed even after 7 d of incubation under sterile conditions.

Although conjugated dienes are formed from the methylene bridges of fatty acids and are more specific indices of lipid peroxidation, their measurement is semiquantitative and less sensitive than TBA-RS. Under similar conditions, Einsele et al. observed volatile hydrocarbon formation possibly as a consequence of lipid peroxidation in human red blood cells, but the amount of hydrocarbons produced was very little; below 4 nmol/mg fatty acid/5d (unpublished observations). Recently, Fraga et al. examined the effects of dietary PUFA on the lability of red blood cells in humans. Although arachidonic acid content was correlated with lipid peroxidation in the stronger oxidative conditions (in the presence of hydroperoxide), variations in PUFA content in the human diet did not affect the rate of oxidative reactions in red blood cell membranes. While preparing this manuscript, Miyagawa reported that lipid peroxide concentrations measured by chemiluminescens were not significantly different in the liver, brain or blood of rats fed diets containing perilla oil, safflower oil or olive oil.

We have previously shown that these high Δ-linolenate and high linoleate diets induce no measurable difference in erythrocyte deformabilities, whole blood viscosities or hematological indices in rats. Therefore, the beneficial effects of increasing Δ-linolenate in diets, as compared with linoleate, which were observed in suppressing the aging process and tumorigenesis *in vivo*, cannot be ascribed to the difference in the peroxidizabilities of n-3 and n-6 fatty acids nor in the physicochemical properties of membranes so far measured. Needless to say, the balance of n-3 and n-6 eicosanoids, which are other kinds of peroxidation products of HUFA, is affected by these diets and is probably deeply involved in these diseases. However, easily oxidizable compounds are generally good antioxidants. Therefore, we point out another possibility that easily autoxidizable fatty acids serve more effectively *in vivo* as free radical scavengers rather than free radical propagators, trapping generated free radicals. The resultant lipid peroxides would be converted to more stable hydroxy fatty acids in the presence of appropriate amounts of endogenous antioxidants (vitamins E, C and glutathione) and peroxidases. In their absence, HUFA may propagate free radicals which damage cellular components, leading to the enhancement of pathogenic disorders. This interpretation is consistent with an observation that a free radical initiator, AAPH, induced lipid peroxidation only after the consumption of endogenous vitamin E in erythrocytes as reported in details by Frei et al. The results by Hammer et al. are also consistent with this interpretation; the rate of ascorbate-induced lipid peroxidation in rat liver microsomes was faster in the group with fish oil supplement than in the group with corn oil supplement with more vitamin E, but was even slower in the fish oil group in the presence of comparable amounts of vitamin E in the diets. Relatively good correlation observed *in vitro* between the degree of unsaturation of membrane lipids and peroxidizability of the membranes appear not to be applicable directly to the phenomena *in vivo*.

References