Effect of Dietary n-3 Fatty Acids on the Composition of Long- and Very-Long-Chain Polyenoic Fatty Acid in Rat Retina

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Summary The effect of dietary supplementation with n-3 fatty acids, primarily docosahexaenoic acid (DHA) with high purity, on the fatty acid composition, especially very-long-chain fatty acids (VLCFA) longer than DHA, with four or six double bonds, in the rod outer segment (ROS) membranes of young Sprague-Dawley rats was investigated. After several weeks of feeding, diets high in n-3 fatty acids increased the DHA level significantly, while there were decreased levels of most n-6 fatty acids, such as arachidonic acid and 22:5n-6. Six kinds of VLCFA were detected by gas chromatography-mass spectrometry (GC-MS). Feeding a high n-3 fatty acid diet significantly increased the content of some n-3 VLCFAs such as 26:4n-3 and 30:4n-3 in ROS membranes, but not all detected n-3 VLCFAs. This study demonstrates that the dietary level of n-3 fatty acids not only affects the level of DHA, but also the levels of VLCFA in ROS membranes.

Key Words docosahexaenoic acid, very-long-chain fatty acids, retina, rod outer segment

In recent years, the discovery of very-long-chain fatty acids (VLCFA) longer than docosahexaenoic acid (DHA), with four or six double bonds, has extended the study of membrane lipid composition. They can only be found in a few specialized tissue lipids such as rod photoreceptors of the retina (1, 2), sperm, testes (3), and brain (4). The rod outer segments (ROS) are highly specialized structures composed of stacks of membranous disks in a plasma membrane and enriched in polyunsaturated fatty acid, primarily DHA, and also VLCFA of n-6 and n-3 (5, 6). Many studies have been done on the relationship between different ratios of dietary n-6/n-3 fatty acids and direct changes of visual functions. Because of the very low content of VLCFA, there have only been a few studies on the dietary effect of the change in VLCFA composition in ROS membranes. Our experiment was designed to investigate if the content of VLCFA in ROS membranes depends on the amount and species of n-3 fatty acids in the diet fed.

Our previous study has shown that the DHA proportion in the retina can be manipulated by direct adding dietary DHA in young rats, but not mature rats (7, 8). In this study, young rats were subjected to a short-term feeding paradigm in which they were fed diets of varying DHA concentrations (ranging from negligible to approximately 9.7% of total energy) with a constant amount of linoleic acid (18:2n-6). This was used to identify the change in VLCFA content in the ROS membrane of the retina. Since the content of VLCFA is very low and no standard can be used as a comparison, it is very difficult to analyze and identify them clearly. Many methods have been used in recent studies with the development of various instruments and methods, such as 13C-labeled fatty acids measurement by gas chromatography-combustion-isotope ratio mass spectrometry (9, 10), [1-14C]C26:4n-6 measured using the Nuclear-Chicago Isocap/300 liquid scintillation counter (11), and gas chromatography-mass spectrometry (GC-MS) (5, 12). In our experiment, GC-MS was used.

Materials and Methods

Animals and diets. The composition of the experimental diet is shown in Table 1. Forty-two 4-wk-old male Sprague-Dawley rats were first fed the basal diet containing 5% (w/w) olive oil for 6 d, and then randomly assigned to seven diet treatments containing 10% (w/w) fatty acid for 30 d. Animals were housed individually under a controlled temperature of 22±1°C, humidity of 50–60%, and a 12-h light/dark cycle. To avoid autoxidation of DHA, the diet was prepared beforehand without adding DHA and stored at –20°C. DHA was stored at –75°C in nitrogen and mixed with the diet every day immediately before feeding. Food and water were available ad libitum. Each diet was made available to the rats in the evening and

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Abbreviations: DHA, docosahexaenoic acid; ROS, rod outer segment; VLCFA, very-long-chain fatty acid.
removed the next morning. There was no significant difference in the amount of diet taken by these rats, nor a difference in body weight. Rats were anesthetized first with ether and Nembutal as follows using intraperitoneal injection. After removing the blood and organs, rat retinas were extruded through a slit made across the entire cornea (13), quickly frozen on dry ice, and stored at −75°C until used.

Fatty acid analysis. Isolations of ROS membranes were performed at 4°C. Two retinas were pooled for separation of the ROS. ROS was isolated by discontinuous sucrose gradient centrifugation as described by Stinson et al. (14). Purity of ROS was confirmed using polyacrylamide gel electrophoresis. Total lipids were extracted from the ROS membrane using the Folch method. Solvent (chloroform: methanol, 2:1 by volume) containing 2.25 mg/L of 2, 6-di-tert-butylphenol was used as an antioxidant. Methyl esters of total lipids were prepared with 0.6 M methanolic-HCl as described by Kates (15). Argentation TLC was used to resolve fatty acid methyl esters on the basis of degree of unsaturation (5).

Fatty acid methyl esters were injected into a DP-35 column (0.25 μm, 30 m × 0.32 mm; J&W Scientific Columns from Agilent Technologies, USA) and analyzed by an electronic impact mass selective detector. The injection temperature was 280°C and the GC-MS transfer line temperature was 300°C. The initial oven temperature was 110°C, which was increased to 200°C at 20°C/min, followed by an increase to 270°C at 3°C/min, and maintained for 30 min. The carrier gas was helium, at a low flow rate of 1.8 mL/min and in a split-less mode. The identification of VLCFA was performed using a Hewlett Packard 5970 mass selective detector with a Hewlett Packard comp 216 data system.

Statistical Analysis. Data were assessed by one-way factorial ANOVA and multiple comparison tests. Significant effects of treatment were defined utilizing Scheffe’s method as a post-hoc test. All data were expressed as mean ± standard deviation (SD).

Results
The GC-MS technique provided definitive identification of the series of fatty acids for polyenoic fatty acids containing diagnostic ions with m/z 79 as the base peak. Tetraenoic acids had a higher ion intensity for m/z 150, 164 and M-71, confirming that these belong to the n-6 series of fatty acids. Pentaenoic and hexaenoic n-3 series acids were enriched in the diagnostic ions m/z 108, 131, M-29, and M-69. In addition to m/z 108 for n-3 and m/z 150 for n-6 series, m/z 164 and M-71 for n-

### Table 1. Fatty acid composition of dietary lipids (for 4-wk rats).

| Group          | 1  | 2  | 3  | 4  | 5  | 6  | 7  |
|----------------|----|----|----|----|----|----|----|
| LA level (en%) | 4.89 | 13.43 | 5.17 | 4.61 | 4.63 | 4.37 | 3.98 |
| ALA level (en%)| 0.11 | 0.09 | 5.47 | 1.80 | 2.77 | 1.54 | 0.04 |
| EPA level (en%)| 1.04 | 5.13 | 3.12 | 2.19 | 2.19 | 0.26 |    |
| DHA level (en%)| 0.95 | 1.71 | 2.99 | 5.63 | 9.69 |    |    |

a: en%, % total energy.
6 and m/z 131, M-69, and M-29 for n-3 series were also reliable diagnostic ions (5). According to the characteristics of the MS spectra above, the structures of six kinds of very-long-chain fatty acids can be inferred from the electron impact mass spectra (i.e. 24:4n-6, 24:6n-3, 26:4n-3, 26:6n-3, 28:6n-3, and 30:4n-3), which were indicated by arrows in the GC spectrum (Fig. 1 (A)). The polyunsaturated fatty acid composition in ROS membranes is shown in Table 2. As expected, significant increases in the DHA levels of the ROS membranes were produced in both the high DHA feeding group (G7) and the n-3 fatty acids-mixed groups (G4, G5, G6) as compared to the high linoleic acid group (G2). Significant differences were also obtained between the diet group with a lower linoleic acid level (G1) and G4, G6, and G7. However, the high α-linolenic acid feeding group (G3) did not show a significant increase in the DHA level. A similar tendency was observed in other n-3 VLCFAs such as 26:4n-3 and 30:4n-3, but not in 24:6n-3, 26:6n-3, or 28:6n-3. Moreover, it is difficult to interpret why the only n-6 VLCFA, 24:4n-6, had an increasing tendency in the n-3 fatty acids-fed groups (G3–G7) as compared to the linoleic acid-fed groups (G1 and G2), although not all increases were significant.

**Discussion**

Many recent studies have indicated that the fatty acid composition of the retinas can be changed by intaking varies ratios of n-6/n-3 fatty acids during the developing age. Generally, feeding a high n-3 fatty acid diet increases n-3 fatty acids while decreasing n-6 fatty acids as compared to feeding a low or n-3 fatty acid-deficient diet (7). In this study, diet did not alter total saturated or monounsaturated fatty acids, while obvious changes were obtained among polyunsaturated fatty acids, such as DHA, ARA, and some very-long-chain fatty acids. The content of DHA in the ROS membranes increased approximately 10% in the G7 group as compared to the G2 group. But only a slight increase in VLCFA was observed.

In the analysis of VLCFAs, there were only a few references. Some of them conflicted with each other (5, 12), in which the flowing sequence of the fatty acids in the GC spectrum was reversed. Moreover, there were few studies on the quantification of these fatty acids, as they have a very low content within only special tissues. From our GC and GC-MS spectra, six kinds of very-long-chain fatty acids with four or six double bonds were detected after the peak of DHA. According to our GC spectra, the sum of the VLCFA contents was approximately 6.5% of the total fatty acids. Although it is difficult to get an obvious changing tendency like DHA by dietary manipulation, some of them showed significant changes (Table 2). Thus, intaking n-3 series fatty acids, especially DHA, could increase the content of some VLCFAs as seen in 26:4n-3 and 30:4n-3. But there was a conflict observed in 24:4n-6. Hargeaves and Clandinin have found strong correlations between the dietary fat content of n-6 and n-3 fatty acids and membrane content of long-chain n-6 and n-3 fatty acids in brain membranes (16, 17). This turnover could result from fatty acid incorporation during phospholipid synthesis or by direct substitution of n-6 for n-3 fatty acid by
phospholipid deacylation-reacylation cycle. Both of these metabolic pathways are active in the retina and might be important for maintaining appropriate membrane components in specialized cells, and be responsible for supplying components for ROS membrane renewal.

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