Recycling of docosahexaenoic acid in rat retinas during n-3 fatty acid deficiency

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Abstract

About 50% of the fatty acids in retinal rod outer segments is docosahexaenoic acid [22:6(n-3)], a member of the linolenic acid [18:3(n-3)] family of essential fatty acids. Dietary deprivation of n-3 fatty acids leads to only modest changes in 22:6(n-3) levels in the retina. We investigated the mechanism(s) by which the retina conserves 22:6(n-3) during n-3 fatty acid deficiency. Weanling rats were fed diets containing 10% (wt/wt) hydrogenated coconut oil (no n-3 or n-6 fatty acids), linseed oil (high n-3, low n-6), or safflower oil (high n-6, <0.1% n-3) for 15 weeks. The turnover of phospholipid molecular species and the turnover and recycling of 22:6(n-3) in phospholipids of the rod outer segment membranes were examined after the intravitreal injection of [2-3H]glycerol and [4,5-3H]22:6(n-3), respectively. Animals were killed on selected days, and rod outer segment membranes, liver, and plasma were taken for lipid analyses. The half-lives (days) of individual phospholipid molecular species and total phospholipid 22:6(n-3) were calculated from the slopes of the regression lines of log specific activity versus time. There were no differences in the turnover rates of phospholipid molecular species among the three dietary groups, as determined by the disappearance of labeled glycerol. Thus, 22:6(n-3) is not conserved through a reduction in phospholipid turnover in rod outer segments. However, the half-life of [4,5-3H]22:6(n-3) in the linseed oil group (19 days) was significantly less than in the coconut oil (54 days) and safflower oil (not measurable) groups. Analysis of plasma, liver, and the contralateral (non-injected) eye showed that the specific activity of 22:6(n-3) was less than 1% of the 22:6(n-3) in the injected eye. These results suggest that, during n-3 deficiency, the retina conserves 22:6(n-3) by recycling this fatty acid within the eye.——Stinson, A. M., R. D. Wiegand, and R. E. Anderson.


Supplementary key words: polyunsaturated fatty acids • rod outer segments • essential fatty acids • phospholipids • lipids • membrane lipids

The outer segment of the photoreceptor cell is a highly structured organelle responsible for absorbing light and initiating visual excitation. The rod outer segment consists of stacks of hundreds of membranous discs, which are surrounded by a plasma membrane joined to the inner segment through a connecting cilium. The inner segment contains the nucleus, endoplasmic reticulum, mitochondria, and Golgi apparatus. The presence of these organelles indicates that the inner segment is the metabolic machinery of the photoreceptor cell.

Rod outer segment membranes have a rather simple lipid composition (1). The major phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS), which account for more than 90% of the total phospholipids. The minor components are phosphatidylinositol, phosphatidic acid, and sphingomyelin, which make up the rest of the phospholipids. The most striking feature of rod outer segment membranes is the presence of large amounts of docosahexaenoic acid [22:6(n-3)], which generally comprises about half of the fatty acids of PE and PS (1). In addition, PC, PE, and PS of rod outer segments from a variety of animal species contain large amounts of dipolyunsaturated molecular species, especially 22:6(n-3)–22:6(n-3) (1-6).

Docosahexaenoic acid and its shorter chain precursors are essential fatty acids. Animals cannot synthesize these fatty acids, so they must be obtained from the diet. Since mammalian rod outer segment discs are completely renewed every 10 days (7), one would expect that prolonged dietary deprivation of these essential fatty acids would lead to the depletion of 22:6(n-3) from these membranes. However, rats (8-10) raised on n-3- and n-6-deficient diets and monkeys raised on n-3-deficient diets (11-13) showed only slight decreases in the 22:6(n-3) content of their rod outer segments or retinas. These observations indicate that the retina has the ability to conserve 22:6(n-3) during fatty acid deficiency.

We have previously tested and rejected several hypotheses for the conservation of 22:6(n-3) by the retina (14): a) the retina is capable of de novo synthesis of...
22:6(n-3) (15); b) the retina can reduce the requirements for 22:6(n-3) by either shortening the lengths of the rod outer segments or reducing the number of photoreceptor cells (14); and c) there is a reduction in the renewal rate of rod outer segment discs in the absence of a dietary source of 22:6(n-3) or its precursors (14).

In the present study, we have tested four additional conservation hypotheses: a) there is a decrease in the turnover rate of 22:6(n-3)-containing molecular species of rod outer segment phospholipids during n-3 fatty acid deficiency; b) there is a reduction in the turnover rate of 22:6(n-3) esterified in phospholipids of rod outer segment membranes; c) the retina can selectively sequester from the blood the small amount of 22:6(n-3) that is present during n-3 fatty acid deficiency; and d) there is a recycling of 22:6(n-3) (i) within the retina, (ii) between the retina and retinal pigment epithelium, or (iii) among the retina, the retinal pigment epithelium, and the blood. Our results indicate that 22:6(n-3) is conserved by recycling within the eye.

EXPERIMENTAL PROCEDURES

Animals

Weanling Long Evans female and adult (3-4 months) rats were obtained from Charles River (Wilmington, MA) and housed under cyclic lighting conditions (12L:12D) at an illumination of 200-300 lux. Adult rats were fed rat chow, while weanling rats were divided randomly into three groups and fed a semisynthetic diet (AIN-76A Basal Diet containing 0.02% tertiary butylated hydroquinone, an antioxidant, Dyets, Inc., Bethlehem, PA) supplemented with either 10% (wt/wt) hydrogenated coconut oil, 10% (wt/wt) linseed oil, or 10% (wt/wt) safflower oil for 15 weeks. Animals were anesthetized with Nembutal (40 mg/kg body weight IP) and injected intraperitoneally with 80-85% (20). Fatty acid phenacyl esters (FAPE) were prepared using the procedures of Wood and Lee (21) and Hanis et al. (22).

Lipid extraction and separation

Lipids of rod outer segment membranes, plasma, and liver homogenates were extracted according to the procedure of Bligh and Dyer (17). The lipid extract was dried under N2 at room temperature and resolved either into individual phospholipid classes by two-dimensional TLC on silica gel HR (18) or into total phospholipids and neutral lipids by one-dimensional TLC using a KG silica gel plate (10 × 20 cm, 250 µm, Whatman) in a solvent system of hexane-diethyl ether-acetic acid (75:35:1, by volume). The lipid spots were visualized by spraying the chromatoplate with 0.05% 2',7'-dichlorofluorescein in aqueous methanol (75%) and viewing under UV light. Regions on the chromatoplates corresponding to the individual lipid classes were scraped from the plates, butylated hydroxytoluene (BHT, 250 µg) was added, and the lipids were extracted according to the procedure described by Arvidson (19).

Lipid derivatization

The diacylglycerol benzoate derivatives (DGBZ) of rod outer segment PC and PE were prepared as described by Louie, Wiegand, and Anderson (5). Recovery of DGBZ prepared by this method is 80-85% (20). Fatty acid phenacyl esters (FAPE) were prepared using the procedures of Wood and Lee (21) and Hanis et al. (22).
HPLC separation of lipid derivatives

Chromatography of the 1,2-DGBZ molecular species from rod outer segment PC and PE was accomplished by HPLC on a Supelcosil LC-18 column (25 cm x 4.6 mm I.D.). The mixture of 1,2-DGBZ molecular species dissolved in 80-90 µl of acetonitrile-2-propanol 80:20 (by volume) was injected into the column and separated by isocratic elution. The flow rate was 1.0 ml/min and the molecular species were detected by monitoring the absorbance at 230 nm. Identification and quantification of the DGBZ molecular species were accomplished as described by Louie et al. (5).

Separation of FAPE of total phospholipids of rod outer segments was carried out according to the procedures of Wood and Lee (21). The FAPE were eluted with a linear gradient of acetonitrile-water 80:20 to 90:10 (by volume) over 45 min at a flow rate of 2 ml/min. The mobile phase was held at 90:10 for 5 min and then returned to the 80:20 mixture. The eluted FAPE were detected at 242 nm.

Radiolabeled fatty acids were detected using a Radiomatic Flo-One/Beta Series A-250 radioactivity detector (Radiomatic Instruments, Tampa, FL). After emerging from the UV detector, the eluate from the HPLC was mixed with scintillation cocktail (ScintiVerse LC, Fisher Scientific) in a ratio of 1:3 (eluate-scintillation cocktail, by volume) and counted in a 1.5-ml flow cell at 6-sec intervals.

Results

Turnover of rod outer segment phospholipid molecular species after intravitreal injection of [2-3H]glycerol

Weanling rats fed coconut oil, linseed oil, or safflower oil diets for 15 weeks were injected intravitreally with [2-3H]glycerol and killed at 1, 2, 3, 4, and 6 days post-injection. Rod outer segment phospholipids were isolated, derivatized, and fractionated into individual molecular species by HPLC. Peaks eluted from HPLC were quantitated and counted for radioactivity. HPLC tracings of PC and PE molecular species from rats fed the three diets are presented in Fig. 1. Since the separation of the DGBZ by reverse-phase HPLC is based on the length of the carbon chain and the degree of unsaturation, the docosahexaenoic species, 22:6(n-3)-22:6(n-3), was the first peak identified after the solvent front. The next major peak was identified as 22:6(n-3)-22:5(n-6); however, GLC analysis showed a multiplicity of coeluting molecular species, with 22:6(n-3)-22:5(n-6) as the major component. Other species found in both PC and PE were 18:1(n-9)-22:6(n-3), 16:0-22:6(n-3), 16:0-20:4(n-6), 16:0-22:5(n-6), 18:0-22:6(n-3), 18:0-20:4(n-6), and 18:0-22:5(n-6). Saturated and monoenoic-saturated species, such as 18:1(n-9)-16:0 and 16:0-16:0, were present only in PC.

HPLC tracings of PC and PE molecular species from the safflower oil and the coconut oil groups showed a decrease in the levels of 22:6(n-3)-22:6(n-3) and an increase in the levels of 22:5(n-6)- and 20:4(n-6)-containing molecular species, such as 22:6(n-3)-22:5(n-6), 18:0-22:5(n-6), 16:0-22:5(n-6), 18:0-20:4(n-6), and 16:0-20:4(n-6). These increased levels of 22:5(n-6)- and 20:4 (n-6)-containing molecular species compensated for and replaced the lower levels of 22:6(n-3)-containing species in the safflower oil and the coconut oil groups. Quantitative analysis of the lipids from rats fed these three diets has been published (14).
The turnover rates of individual molecular species of rod outer segment phospholipids were determined by following the exponential decay of the radioactivity after an intravitreal injection of $[2-^{3}H]$glycerol. Plots of log specific activity versus time of two major molecular species of the rod outer segments, 22:6(n-3)-22:6(n-3) and 18:0-22:6(n-3), are shown in Fig. 2 and Fig. 3, respectively. As demonstrated previously (5, 23, 24), PC is metabolically more active than PE, as indicated by the faster turnover of these two molecular species in PC. The same observation was made for the other 22:6(n-3)-containing molecular species, 16:0-22:6(n-3),

Fig. 2. Linear regression plots of log specific activity of 22:6(n-3)-22:6(n-3) molecular species from the three dietary groups. Animals were injected intravitreally with $[2-^{3}H]$glycerol and killed on days 1, 2, 3, 4, and 6. PC and PE of rod outer segments were separated, derivatized, and fractionated into individual molecular species by HPLC (See Fig. 1). The peak containing 22:6(n-3)-22:6(n-3) was quantitated, collected, and counted for radioactivity. Specific radioactivity is expressed as DPM/nmol fatty acid.

Fig. 3. Linear regression plots of log specific activity of 18:0-22:6(n-3) molecular species from the three dietary groups. Animals were injected intravitreally with $[2-^{3}H]$glycerol and killed on days 1, 2, 3, 4, and 6. PC and PE of rod outer segments were separated, derivatized, and fractionated into individual molecular species by HPLC (See Fig. 1). The peak containing 18:0-22:6(n-3) was quantitated, collected, and counted for radioactivity. Specific radioactivity is expressed as DPM/nmol fatty acid.
18:1-22:6(n-3), and 22:6(n-3)-22:5(n-6) (data not shown). The turnover rates of the same 22:6(n-3)-containing molecular species from the three dietary groups were compared using Analysis of Covariance. The P-values resulting from the pair-wise comparisons (coconut oil vs. linseed oil, coconut oil vs. safflower oil, and linseed oil vs. safflower oil) showed no significant differences between dietary groups at the 0.05 level.

Turnover of docosahexaenoic acid in rod outer segment phospholipids after intravitreal injection of [4,5-3H]22:6(n-3)

Rats raised on the coconut oil, linseed oil, or safflower oil diet for 15 weeks were injected intravitreally with [4,5-3H]22:6(n-3) and killed on days 6, 12, 24, 36, 48, and 60. Fig. 4 shows absorbance (solid line) and radioactivity (dashed line) tracings of FAPE of rod outer segment total phospholipids from rats fed the three diets and killed 60 days after injection. A simple, but rather striking, radio-labeling pattern was observed, with almost all of the label in 22:6(n-3). The label remained in this fatty acid throughout the 60-day period, indicating that no chain shortening or retroconversion occurred in the retina during this time.

The mass of six identified fatty acids eluting from the HPLC (Fig. 4) was determined and normalized to give relative mole%. Fig. 5 shows that the mole% of 22:6(n-3) from the three dietary groups did not change over the 60-day post-injection period. However, differences were observed in the mole% of 22:6(n-3) between the dietary groups. The relative amount of 22:6(n-3) was highest in the linseed oil group (55-60%), lowest in the safflower oil group (30-35%), and intermediate in the coconut oil group (40-45%).

The turnover of docosahexaenoic acid in rod outer segment phospholipids was determined by following the exponential decay of the radioactivity after intravitreal injection of [4,5-3H]22:6(n-3). The rate of turnover is represented by the half-life, which can be calculated from the slope of the linear regression line of log specific activity versus time. Fig. 6 shows the plots of log specific activity of 22:6(n-3) versus time for the three groups. The parameters of these regressions and calculated half-lives are given in Table 1. As determined from the P-value (0.9638) and R² (0.000), the regression line from the safflower oil group showed no dependent relationship between log specific activity and time. The slope of this regression line was not significantly different from zero, indicating that there was no measurable turnover of 22:6(n-3) in this group. However, the regression lines from both the coconut oil and the linseed oil groups were...
significant. The linseed oil group had the fastest turnover of 22:6(n-3), with a half-life of 19 days. The coconut oil group turned over its 22:6(n-3) at a much slower rate, with a half-life of 54 days.

The differences in the turnover rates of 22:6(n-3) from the three dietary groups were determined by comparing the slopes of the regressions using Analysis of Covariance. A P-value of 0.0005 suggested the significant difference between these three dietary groups and prompted follow-up pair-wise comparisons. As shown in Table 1, the linseed oil group was significantly different from the coconut oil and safflower oil groups (linseed oil vs. coconut oil, $P=0.0043$; linseed oil vs. safflower oil, $P=0.0004$). However, there was no significant difference between the coconut oil and safflower oil groups ($P=0.1779$).

**Recycling of docosahexaenoic acid**

The slower turnover rate of 22:6(n-3) in the coconut oil and safflower oil groups indicated that this fatty acid was not lost from the retina during n-3 deficiency. Recycling of 22:6(n-3) between the retina, the retinal pigment epithelium, and the blood was tested by injecting

**TABLE 1.** Linear regression analysis of the turnover of $[4,5-^3H]22:6(n-3)$ from rod outer segment total phospholipids

<table>
<thead>
<tr>
<th>Diet</th>
<th>Slope</th>
<th>SE Slope</th>
<th>$P^*$</th>
<th>$R^2$</th>
<th>$T_{1/2}$</th>
<th>$P'$</th>
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<tbody>
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<td>.0024</td>
<td>.0270</td>
<td>.203</td>
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<tr>
<td>Linseed oil</td>
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<td>.0024</td>
<td>.0000</td>
<td>.666</td>
<td>19.0</td>
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</tr>
<tr>
<td>Safflower oil</td>
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<td>.0032</td>
<td>.9638</td>
<td>.000</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Coconut oil vs. linseed oil</td>
<td>.0043</td>
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<tr>
<td>Coconut oil vs. safflower oil</td>
<td>.1779</td>
<td></td>
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<tr>
<td>Linseed oil vs. safflower oil</td>
<td>.0004</td>
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</tbody>
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$^*$The functional dependence of log specific activity on time was tested using Analysis of Variance. A $P$-value less than 0.05 indicates that the slope is significantly different from zero.

$^1$The turnover rate of [4,5-^3H]22:6(n-3) is presented as the half-life, calculated from the slope of a linear regression ($T_{1/2} = \log 2/\text{slope}$) plotting log specific activity of 22:6(n-3) versus time. No half-life could be calculated for the safflower oil group (ND).

$^2$The difference in the turnover rates of [4,5-^3H]22:6(n-3) among the coconut oil, linseed oil, and safflower oil groups was determined by comparing the slopes using Analysis of Covariance. $P$-values less than 0.05 indicate that the slopes are different.
[4,5-3H]22:6(n-3) intravitreally into the right eye of rats and monitoring the appearance of radioactivity in contralateral (uninjected) eye, plasma, and liver, 6 and 22 days post-injection. At both time points, the specific radioactivity of 22:6(n-3) from rod outer segment phospholipids from the injected eye was several hundred times greater than in the uninjected eye, plasma, or liver (Fig. 7).

DISCUSSION

The retina has the ability to conserve its high level of 22:6(n-3) during n-3 and/or n-6 fatty acid deficiency (8-14). The mechanism of conservation is not known, although several hypotheses have been proposed (14): a) the retina is capable of de novo synthesis of 22:6(n-3); b) the retina can reduce the requirements for 22:6(n-3) by either shortening the lengths of the rod outer segments or reducing the number of photoreceptor cells; c) there is a decrease in the turnover rate of 22:6(n-3) during n-3 fatty acid deficiency; d) there is a decrease in the turnover rate of 22:6(n-3) in phospholipids of rod outer segment membranes; e) the retina can selectively sequester from the blood the small amount of 22:6(n-3) that is present during n-3 fatty acid deficiency; f) there is a recycling of 22:6(n-3) within the retina, between the retina and retinal pigment epithelium, or among the retina, the retinal pigment epithelium, and the blood. Hypotheses a-c were tested in previous studies and rejected (14, 15).

It has been well established that phospholipids of rod outer segment membranes are in a dynamic state of rapid turnover (5, 23, 24). Also, different molecular species within a phospholipid class or the same species between different phospholipid classes have different turnover rates (5, 24). In general, when [1H]glycerol is used to label rod outer segment phospholipids, there is rapid turnover of PC species, intermediate turnover of PE species, and slow turnover of PS species. The results of the present study confirm and extend these reports. However, between the three diet groups, no significant differences were found in the turnover rates of 22:6(n-3)-containing molecular species, as measured by the disappearance of radioactive glycerol (Figs. 2 and 3). We therefore reject the hypothesis that the retina conserves 22:6(n-3) by lowering the turnover rates of 22:6(n-3)-containing phospholipid molecular species of rod outer segment membranes during n-3 deficiency.

Hypothesis (e), i.e., a reduction in the turnover rate of 22:6(n-3) in rod outer segment phospholipids, was tested by following the fate of [4,5-3H]22:6(n-3) injected intravitreally. The half-life of 22:6(n-3) in the linseed oil group was 19 days, compared to 54 days in the coconut oil group. No half-life could be calculated for the safflower oil group. The renewal of integral proteins (14) and the turnover of glycerol in glycerolphospholipids (Figs. 2 and 3) of rod outer segments were not affected by n-3 deficiency. However, the differences in half-lives of labeled 22:6(n-3) indicate that the metabolism of this fatty acid is affected by diet. When n-3 fatty acids are readily available in the diet, 22:6(n-3) turns over in the retina either through oxidation or by returning to the circulation. However, in n-3 deficiency, 22:6(n-3) does not leave the retina. Rather, it is recycled, either within the retina via deacylation-reacylation pathways known to exist in rod outer segments (25, 26) or between the retina and the pigment epithelium.

Recent studies suggest that 22:6(n-3) is recycled between the retina and the pigment epithelium. Gordon and Bazan (27) injected radiolabeled 22:6(n-3) intravitreally...
into frogs and monitored its cellular localization by autoradiography. The densities of silver grains over the distal tips of rod outer segments and outer segment phagosomes in the pigment epithelium were similar, leading them to suggest that 22:6(n-3) was present in the shed outer segment tips. Chen et al. (28) measured the molecular species of PC and PE in frog rod outer segments and pigment epithelium before and after light-induced shedding of outer segment tips. There was a significant increase in molecular species containing 22:6(n-3) in the pigment epithelium following the shedding event. If we apply these results to the present study, we would conclude that 22:6(n-3) is conserved in the retina through recycling between the retina and the pigment epithelium.

The results presented in Table 1 also provide information on hypothesis (f), i.e., that the retina can selectively sequester small amounts of 22-carbon PUFA from the blood during n-3 deficiency. If the retina incorporated unlabeled 22:6(n-3) from the blood during the 60 days of the study, the specific activities of 22:6(n-3) in the rod outer segments should decrease with time because of dilution of the label. Indeed, this occurred for the linseed oil group, but not for the safflower oil group. Therefore, we conclude that, although the retina can sequester 22:6(n-3) from the blood, there are limitations. During the early phase of n-3 fatty acid deficiency, the retina responds by sequestering 22:6(n-3) from the blood. However, during prolonged n-3 deficiency, the uptake of 22:6(n-3) from the blood is drastically reduced and the retina maintains its levels of 22:6(n-3) by recycling within the eye.

In summary, we have shown that the retina conserves 22:6(n-3) during n-3 fatty acid deficiency by retaining this fatty acid within the eye. On the basis of other studies (27, 28), we suggest that 22:6(n-3) is recycled between the retina and the pigment epithelium. The pigment epithelium must have a remarkably efficient system to direct the flow of fatty acids back to the retina, given the large amount of material that this tissue phagocytizes each day (10% of the rod outer segments). The mechanism(s) by which this highly selective flow of lipids between the pigment epithelium and the retina is achieved is not known. Proteins that bind retinoids have been identified in the retina (29), and cytosolic fatty acid binding proteins have been characterized in a number of tissues (30). It seems likely that the transfer of 22:6(n-3) and other fatty acids between the retina and the pigment epithelium would be mediated by some type of carrier protein.

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REFERENCES


