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Rat heart cannot synthesize docosahexaenoic acid from circulating α-linolenic acid because it lacks elongase-2

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Abstract

The extent to which the heart can convert α-linolenic acid (α-LNA, 18:3n-3) to longer chain n-3 PUFAs is not known. Conversion rates can be measured in vivo using radiolabeled α-LNA and a kinetic fatty acid model. [1-^{14}C]α-LNA was infused intravenously for 5 min in unanesthetized rats that had been fed an n-3 PUFA-adequate [4.6% α-LNA, no docosahexaenoic acid (DHA, 22:6n-3)] or n-3 PUFA-deficient diet (0.2% α-LNA, nor DHA) for 15 weeks after weaning. Arterial plasma was sampled, as was the heart after high-energy microwaving. Rates of conversion of α-LNA to longer chain n-3 PUFAs were low, and DHA was not synthesized at all in the heart. Most α-LNA within the heart had been β-oxidized. In deprived compared with adequate rats, DHA concentrations in plasma and heart were both reduced by >90%, whereas heart and plasma levels of docosapentaenoic acid (DPAn-6, 22:5n-6) were elevated. Dietary deprivation did not affect cardiac mRNA levels of elongase-5 or desaturases Δ6 and Δ5, but elongase-2 mRNA could not be detected. In summary, the rat heart does not synthesize DHA from α-LNA, owing to the absence of elongase-2, but must obtain its DHA entirely from plasma. Dietary n-3 PUFA deprivation markedly reduces heart DHA and increases heart DPAn-6, which may make the heart vulnerable to different insults.—Igarashi, M., K. Ma, L. Chang, J. M. Bell, and S. I. Rapoport. Rat heart cannot synthesize docosahexaenoic acid from circulating α-linolenic acid because it lacks elongase-2.

Supplementary key words

diet; heart; deprivation; elongation; synthesis; n-3 polyunsaturated fatty acids

Long-chain n-3 PUFAs, particularly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are reported to be protective against cardiovascular disease (1–8). Thus, a low dietary n-3 PUFA intake and a low plasma DHA concentration are risk factors for cardiac disease (8–10), whereas a high dietary n-3 PUFA content is considered protective (11–13).

In some mammalian tissues, EPA and DHA can be converted from shorter chain α-linolenic acid (α-LNA, 18:3n-3), which is enriched in plant oils, by serial steps of desaturation, elongation, and β-oxidation (14–20). Using our kinetic method and model, we showed in

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unanesthetized rats that rates of conversion of α-LNA to DHA were higher in liver than in brain, and that n-3 dietary deprivation could further increase the liver but not brain rates, in relation to elevated expression of requisite desaturases and elongases (21–23). These enzymes include Δ5 and Δ6 desaturases and elongases-2 and -5. They are expressed in the liver and many other rodent tissues, although elongase-2 has not been identified in rat heart (15–17, 24, 25). Thus, the heart may be incapable of synthesizing DHA from α-LNA, consistent with one study on isolated rat cardiomyocytes (26).

Because of the importance of n-3 PUFAs to cardiovascular function (see above), and because their kinetics have not been thoroughly examined in vivo, we thought it of interest in this study to use our fatty acid method to quantify rates of conversion of circulating unesterified α-LNA to longer chain n-3 PUFAs in the heart of rats fed a diet with an adequate or deficient n-3 PUFA content. Thus, we infused [1-^{14}C]α-LNA intravenously for 5 min in unanesthetized rats, then measured lipid composition and radioactivity in the heart after subjecting it to high-energy microwaving to stop its metabolism. Rats were fed an n-3 PUFA-adequate or -deficient diet for 15 weeks, starting at weaning (21 days). We used our published equations to calculate coefficients and rates of α-LNA conversion to longer chain n-3 PUFAs (21, 22, 27, 28). We also measured cardiac mRNA levels of the desaturases and elongases in the conversion pathways (14, 19, 20).

MATERIALS AND METHODS

Materials

[1-^{14}C]α-LNA in ethanol was purchased from Perkin-Elmer Life Sciences, NEN Life Science Products (Boston, MA). The specific activity was 54 mCi/mmol, and the purity was 98% (determined by HPLC and scintillation counting). Di-heptadecanoate phosphatidylcholine (di-17:0 PC), free heptadecanoic acid (17:0), and TLC standards for cholesterol, triglycerides, and cholesteryl esters were purchased from Sigma-Aldrich (St. Louis, MO). Standards for general fatty acid methyl esters (FAMEs) for GC and HPLC were obtained from NuChek Prep (Elysian, MN). FAMEs for unique n-3 PUFAs (20:4n-3, 22:5n-3, 24:5n-3, 24:6n-3, and 22:5n-6) were purchased from Larodan Fine Chemicals (Malmö, Sweden). 6-p-Toluidine-2-naphthalene sulfonic acid was from Acros Organics (Fair Lawn, NJ). Liquid scintillation cocktail (Ready Safe™) was purchased from Beckman Coulter (Fullerton, CA). Solvents were HPLC-grade and were purchased from Fisher Scientific (Fair Lawn, NJ) or EMD Chemicals (Gibbstown, NJ). Other chemicals and reagents were purchased from Sigma-Aldrich or Fisher Scientific.

Animals

The protocol was approved by the Animal Care and Use Committee of the National Institute of Child Health and Human Development and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80-23). Fischer-344 (CDF) male rat pups (18 days old) and their surrogate mothers were purchased from Charles River Laboratories (Portage, MI) and were housed in an animal facility with regulated temperature and humidity and a 12 h light/12 h dark cycle. The pups were allowed to nurse until 21 days of age. Lactating rats had free access to water and rodent

chow (formulation NIH-31 18-4; Zeigler Bros., Gardner, PA), which contained 4% (wt/wt) crude fat and whose fatty acid composition we previously reported (27, 28). α-LNA, EPA, and DHA contributed 5.1%, 2.0%, and 2.3% of total fatty acids, respectively, whereas linoleic acid (LA, 18:2n-6) and arachidonic acid (AA) contributed 47.9% and 0.02%, respectively. After weaning, the pups were divided randomly into n-3 PUFA-adequate and -deficient diet groups. They had free access to food and water, and their food was replaced every 2 or 3 days, when body weight was recorded.

Ten rats in the n-3 PUFA-adequate group and 7 rats in the n-3 PUFA-deficient group were studied for the radioisotope infusion study, and 10 additional rats were studied from each group for the analysis of cardiac mRNA levels (see below).

n-3 PUFA-adequate and -deficient diets

The n-3 PUFA-adequate and -deficient diets were prepared by Dyets, Inc. (Bethlehem, PA) and were based on the AIN-93G formulation (29). Their exact compositions are presented in our prior publications (22, 30). Both diets contained 10% fat, but only the adequate diet contained flaxseed oil. The adequate diet contained 7.8 μmol/g α-LNA (4.5% total fatty acid), a minimum level for dietary n-3 PUFA adequacy in rodents (31, 32). The deficient diet contained 0.25 μmol/g α-LNA (0.2% total fatty acid). Other n-3 PUFAs were absent from both diets. Both contained 40 μmol/g LA (23–24% total fatty acid).

Surgery

A rat was anesthetized with 1–3% halothane (Shirley Aldred and Co., Ltd., UK). Polyethylene catheters (PE 50, Intramedic™, Clay Adams™; Becton Dickinson, Sparks, MD) filled with heparinized saline (100 IU/ml) were surgically implanted into the right femoral artery and vein, after which the skin was closed with staples and treated with 1% lidocaine (Hospira, Inc., Lake Forest, IL) for pain control. The rat then was loosely wrapped in a fast-setting plaster cast taped to a wooden block, and allowed to recover from anesthesia for 3–4 h. Body temperature was maintained at 36–38°C using a feedback-heating element (Indicating Temperature Controller; Yellow Springs Instruments, Yellow Springs, OH). Animals were provided food the night prior to surgery, but not on the morning of surgery.

Radiotracer infusion

A rat was infused via the femoral vein catheter with 500 μCi/kg [1-^{14}C]α-LNA (22, 27, 28). An aliquot of [1-^{14}C]α-LNA in ethanol was dried under nitrogen, and the residue was dissolved in HEPES buffer (pH 7.4) containing 50 mg/ml fatty acid-free BSA, to a final volume of 1.3 ml. The mixture was sonicated at 40°C for 20 min and mixed by vortexing. A computer-controlled variable speed pump (No. 22; Harvard Apparatus, South Natick, MA) was used to infuse 1.3 ml tracer at a rate of 0.223 ($1+ e^{-1.92t}$) ml/min (t in min), to rapidly establish a steady-state plasma radioactivity (22, 28, 33). Arterial blood (180 μl at each time point) was collected in centrifuge tubes (polyethylene-heparin lithium fluoride-coated; Beckman) at 0, 0.25, 0.5, 0.75, 1.5, 3, 4, and 5 min after starting infusion. At 5 min, the rat was euthanized with an overdose of sodium pentobarbital (100 mg/kg i.v.; Ovation Pharmaceuticals, Inc.), and the head and torso were immediately subjected to high-energy focused beam microwave irradiation (5.5 kW, 4.8 s) (Model S6F; Cober Electronics,

Stamford, CT). The heart was removed and confirmed visually to be entirely browned (cooked); if not, it was discarded. Its weight was recorded, and then it was stored at −80°C until analyzed. Arterial blood samples were centrifuged at 18,000 *g* for 2 min, and plasma was collected and frozen at −80°C.

Separation and analysis of stable heart lipids

Total heart lipid was extracted by the procedure of Folch, Lees, and Sloane Stanley (34). The aqueous extraction phases were washed once with an equal volume of chloroform to remove residual lipid, and aqueous and total lipid radioactivity was counted (see below). Total lipid extracts were separated into neutral lipid subclasses by TLC on silica gel 60 plates (EM Separation Technologies; Gibbstown, NJ) using heptane-diethyl etherglacial acetic acid (60:40:3; v/v/v) (35). Authentic standards of triacylglycerol, phospholipids, cholesterol, cholesteryl ester, and unesterified fatty acids were run on the plates to identify the lipids. The plates were sprayed with 0.03% 6-p-Toluidine-2-naphthalene sulfonic acid in 50 mM Tris-HCl buffer (pH 7.4) (w/v), and the lipid bands were visualized under ultraviolet (UV) light. The bands were scraped and used to directly quantify radioactivity by scintillation counting and to prepare FAMEs.

We define “stable” heart lipids as cardiac phospholipids, cardiolipin, triacylglycerol, and cholesterol. To measure the total phospholipid concentration, an aliquot of the total lipid extract was added to a tube and dried in a SpeedVac to prepare for digestion. Total lipid extracts were separated into phospholipid classes by TLC on silica gel 60 plates using chloroform-methanol-glacial acetic acid-water (60:40:1:4; v/v/v/v) to separate cholineglycerophospholipid, phosphatidylserine, phosphatidylinositol, and sphingomyelin (36). Ethanolamineglycerophospholipid and cardiolipin were separated using acetone-petroleum ether (1:3; v/v) followed by chloroform-methanol-glacial acetic acid-water (80:13:8:0.3; v/v/v/v) (37). The bands were scraped and added to the tube. The digestion was carried out by adding 0.5 ml of water and 0.65 ml of perchloric acid (70%) to all material. The scraped and dried extracts were digested at 180°C for 1 h (38). After the sample was cooled to room temperature, 0.5 ml of ascorbic acid (10%; w/v), 0.5 ml of ammonium molybdate (2.5%; w/v), and 3.0 ml of water were added. The mixture was boiled for 5 min to develop color, and after it had cooled, its absorbance was read at 797 nm. Standards for this assay were purchased from Sigma, and phospholipid concentrations were determined using standard curves.

To quantify concentrations of total cholesterol and triacylglycerol, the lipid extract was dried using a SpeedVac, and the residue was dissolved in 0.1% Triton X-100. Total cholesterol was determined with a commercial kit (BioVision Research Products; Mountain View, CA), as was the triacylglycerol concentration (Sigma-Aldrich).

Quantification of radioactivity

Samples for measuring radioactivity were placed in scintillation vials and dissolved in liquid scintillation cocktail (Ready Safe™ plus 1% glacial acetic acid). Their radioactivity was determined using a liquid scintillation analyzer (2200CA, TRI-CARB®; Packard Instruments, Meriden, CT).

FAME preparation

The FAMEs were analyzed by GC and HPLC. Unesterified and esterified fatty acids were methylated with 1% H_2SO_4-methanol for 3 h at 70°C (39, 40). Before the sample was methylated, appropriate quantities of di-17:0 PC (for phospholipids and triacylglycerol) or 17:0 fatty acid (for free fatty acids) were added as internal standards.

GC analysis

Fatty acid concentrations (nmol/g heart wet wt) in heart lipids were determined using a GC (6890N; Agilent Technologies, Palo Alto, CA) equipped with an SP™-2330 fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) (Supelco; Bellefonte, PA) and a flame ionization detector (40). Concentrations were calculated by proportional comparison of peak areas to the area of the 17:0 internal standard.

HPLC analysis

To determine esterified fatty acid radioactivities in heart lipids, FAMEs from the heart lipids were quantified by HPLC by the method of Aveldano, VanRollins, and Horrocks (41) with modifications. Total lipids were alkalinized with KOH solution, and twice extracted with *n*-hexane. The hexane phase was dried and methylated as described above. FAMEs were dissolved in acetonitrile, and the solution was fractionated by reversed-phase column HPLC using a pump (System GOLD 126; Beckman Coulter) outfitted with a UV detector (UV/VIS-151; Gilson, Middleton, WI). The reverse-phase column, Luna 5 μ C18 (2) (5 μM particle size, 4.6 × 250 mm), was obtained from Phenomenex (Torrance, CA). HPLC eluate was collected every 30 s and subjected to liquid scintillation counting to obtain a radioactivity profile. Chromatography was performed using a linear gradient system of water and acetonitrile. The acetonitrile was held at 85% for 30 min, increased to 100% over 10 min, and held again at 100% for 20 min. The flow rate was 1.0 ml/min. The UV detector was set at 205 nm.

Two or three samples were equally pooled to analyze HPLC profiles of FAMEs (see Table 4); the sample number was 4 for the adequate diet group and 3 for the deficient diet group. The percentages of radioactivity in [1-^{14}C]α-LNA, [^{14}C]DHA, and [^{14}C]intermediates in DHA synthesis in heart total lipid fractions were determined from these HPLC profiles.

Analysis of long-chain acyl-CoAs

Long-chain acyl-CoAs were extracted from microwaved heart using an affinity chromatography method with slight modification (42). After 5 nmol heptadecanoyl-CoA (17:0-CoA) was added as an internal standard to ~0.5 g of heart, the sample was homogenized in 25 mM KH_2PO_4 (Tissuemizer; Tekmar, Cincinnati, OH). The homogenate was adjusted with isopropanol and acetonitrile to isopropanol-25 mM KH_2PO_4-acetonitrile (1:1:2; v/v/v), then sonicated with a probe sonicator (Model W-225; Misonix, Farmingdale, NY). A small volume (~3% of total) of saturated $(NH_4)_2SO_4$ was added to the homogenate to precipitate protein, then the supernatant was mixed vigorously for 5 min and centrifuged. The sample was diluted with a 1.25-fold volume of 25 mM KH_2PO_4. The solution was passed three times through an oligonucleotide purification cartridge (ABI Master-piece ™, OPC®; Applied Biosystems, Foster City, CA), and the cartridge was washed with 25 mM

KH_2PO_4. Acyl-CoA species were eluted with a small volume of isopropanol-1 mM glacial acetic acid (75:25; v/v).

Extracted acyl-CoAs were separated on a reverse-phase HPLC column (Symmetry, 5 μm particle size, 4.6 mm × 250 mm; Waters Corporation, Milford, MA), using a pump coupled with a UV/VIS detector (System Gold, Model 168; Beckman). Chromatography was performed using a linear gradient system of 75 mM KH_2PO_4 and acetonitrile. At the start, acetonitrile was 44% and held for 1 min, then increased to 49% over 25 min, increased to 68% over 10 min, held at 100% for 4 min, returned to 44% over 6 min, and held for 6 min (52 min total run time). The flow rate was 1.0 ml/min. UV detection was set at 260 nm for integration of concentrations and at 280 nm for identifying acyl-CoAs (260/280 = 4:1) (42). Peaks were identified from retention times of acyl-CoA standards. The acyl-CoA standards for α-LNA, EPA, and DHA were prepared from the UFA and free CoA by an enzymatic method (43). Endogenous acyl-CoA concentrations (nmol/g heart) were calculated by direct proportional comparison with the peak area of the 17:0-CoA internal standard.

In this HPLC system, 14:0-CoA, α-LNA-CoA, and EPA-CoA coelute as a single peak (28). This peak was collected and saponified with 2% KOH/EtOH (wt/v) at 100°C for 45 min and acidified with HCl, and the fatty acids were extracted with *n*-hexane. The fatty acids were converted to FAMEs and separated on HPLC as described above. The FAME derivatives of 14:0 and EPA were completely separated on the HPLC system, but α-LNA-CoA could not be identified. The concentrations of the FAMEs from the acyl-CoA species also were determined by GC, as described above. They were determined by proportional comparison of their GC peak areas to each other.

Calculations

Equations for determining the in vivo kinetics of α-LNA in brain and liver, following a 5 min intravenous infusion of radiolabeled α-LNA to produce a steady-state plasma radioactivity, have been described previously (21, 22, 27, 28). With regard to the heart, following the 5 min [1-^{14}C]α-LNA infusion, incorporation coefficients $k^*_{i(\alpha-LNA)}$ (ml/s/g heart), representing transfer of unesterified [1-^{14}C]α-LNA from plasma into stable heart lipid *i* (phospholipid or triacylglycerol), were calculated as

$$k^*_{i(\alpha-LNA)} = \frac{c^*_{heart,\,i(\alpha-LNA)}(T)}{\int_0^T c^*_{plasma(\alpha-LNA)}dt} \quad \text{(Eq. 1)}$$

where $c^*_{heart,\,i(\alpha-LNA)}(T)$ (nCi/g heart) is heart α-LNA radioactivity in *i* at time T (5 min) after starting tracer infusion, t is time after starting infusion, and $c^*_{plasma(\alpha-LNA)}$ (nCi/ml plasma) is plasma radioactivity of unesterified α-LNA. Coefficients $k^*_{i(\alpha-LNA\rightarrow j)}$ (ml/s/g heart), representing synthesis from α-LNA of *j* = DHA or of its n-3 intermediates, and subsequent incorporation into stable lipid *i*, were calculated as follows

$$k^*_{i(\alpha-LNA\rightarrow j)} = \frac{c^*_{heart,i(j)}(T)}{\int_0^T c^*_{plasma(\alpha-LNA)}dt} \quad \text{(Eq. 2)}$$

where $c^*_{heart,i(j)}$(T) (nCi/g heart) is radioactivity of n-3 PUFA j in stable lipid i at T = 5 min.

Rates of incorporation of unlabeled unesterified α-LNA from plasma into heart lipid were calculated as

$$J_{in,i(\alpha-LNA)} = k^*_{i(\alpha-LNA)}c_{plasma(\alpha-LNA)} \quad \text{(Eq. 3)}$$

where $c_{plasma(\alpha-LNA)}$ is the plasma concentration (nmol/ml) of unlabeled unesterified α-LNA.

In our prior studies on brain and liver, we could calculate rates of incorporation of α-LNA from the precursor tissue α-LNA pool into phospholipids and triacylglycerol, as well as net rates of synthesis of longer chain n-3 PUFA in tissue when taking into account the specific activity of the tissue α-LNA-CoA pool (21, 22). However, because we were unable to measure cardiac α-LNA-CoA specific activity in this study, we did not elaborate analysis.

Measurement of cardiac mRNA levels

Rats were decapitated after 15 weeks on a diet, and their hearts were collected and stored at −80°C until mRNA was extracted. Total RNA was isolated from heart using commercial kits (RNeasy Fibrous Tissue Kit; Qiagen, Valencia, CA). cDNA was prepared from total RNA using a high-capacity cDNA Archive kit (Applied Biosystems). mRNA levels of Δ5 desaturase (NM_0534405), Δ6 desaturase (NM_031344), elongase-5 (NM_134382), elongase-2 (AB071986), and acyl-CoA oxidase (NM_017340) were measured with real-time quantitative RT-PCR, using an ABI PRISM 7000 sequence detection system (Applied Biosystems). Specific primers and probes, purchased from TaqMan® gene expression assays (Applied Biosystems), consisted of a 20× mix of unlabeled PCR primers and Taqman minor groove binder probe (FAM™ dye-labeled). Data were analyzed with comparative cycle threshold (44). Data were expressed as the level of the target gene in animals fed the deficient diet, normalized to the endogenous control (β-globulin), and relative to the level in animals fed the adequate diet.

Statistical analysis

Data were expressed as means ± SD. Student's t-tests were used to determine significance of differences between means, taken as $P \leq 0.05$.

RESULTS

Plasma fatty acid concentrations

Table 1 presents mean unesterified and total fatty acid concentrations in plasma from rats fed the n-3 PUFA-adequate and -deficient diets (21, 22). Rats fed the deficient diet had markedly reduced unesterified and total plasma concentrations of α-LNA and DHA, but significantly increased concentrations of unesterified and total AA and its elongation product, docosapentaenoic acid (DPA, 22:5n-6). This pattern of concentration changes in the deprived rats was established within 5 weeks on the diet (40).

Heart stable lipid concentrations

Concentrations of stable lipids (phospholipids, cardiolipin, triacylglycerol, cholesterol) in microwaved heart of rats fed n-3 PUFA-adequate or -deficient diets are shown in Table 2. The mean total phospholipid concentration did not differ significantly between the diet-deficient and diet-adequate rats. Rats on the deficient diet had a 23% reduction in mean heart phosphatidylserine ($P < 0.01$), but concentrations of the other phospholipids, of tri-acylglycerol, and of total cholesterol did not differ significantly from control.

Heart fatty acid concentrations

Table 3 shows mean esterified fatty acid concentrations in heart phospholipid and triacylglycerol in the diet-adequate and diet-deficient rats. In the deficient compared with adequate group, heart DHA was reduced significantly, by 92% and 93%, in phospholipid and tri-acylglycerol, respectively, whereas total n-3 PUFAs were reduced by 93% and 91%, respectively. Heart total n-6 PUFA concentrations were increased respectively by 22% and 26%, with the largest proportional increases (15- and 8-fold in phospholipid and triacylglycerol, respectively) occurring for DPA (22:5n-6).

Distribution of heart radioactivity

After the 5 min [1-^{14}C]α-LNA infusion, total heart (total lipid plus aqueous) radioactivity did not differ significantly between n-3 PUFA diet-adequate (1,724 ± 283 nCi/g heart) and -deficient (1,857 ± 306 nCi/g heart) rats. Most radioactivity was in the aqueous phase, and dietary deprivation did not affect this fraction (Fig. 1A). Radioactivity equaled 1,399 ± 219 nCi/g (81.9% of net radioactivity) and 1,528 ± 270 nCi/g (82.2% of net radioactivity), respectively, in the aqueous phase of the adequate and deficient groups; and 329 ± 60 nCi/g heart (18.1%) and 325 ± 112 nCi/g heart (17.8%), respectively, in total heart lipids. Dietary deprivation also did not affect radioactivity in stable heart lipids (Fig. 1B). Radioactivity equaled 248 ± 103 nCi/g heart (13.5%) and 250 ± 44.8 nCi/g heart (13.5%) in triacylglycerol of the adequate and deficient groups, respectively; 53.7 ± 10.1 nCi/g heart (3.2%) and 54.0 ± 10.3 nCi/g heart (3.0%), respectively, in phospholipid; and 22.9 ± 5.2 nCi/g heart (1.4%) and 25.8 ± 12.4 nCi/g heart (1.3%), respectively, in cholesterol.

Radioactivity composition of esterified fatty acid in heart

In most mammalian tissues, α-LNA can be elongated and desaturated to 24:6n-3 by the following steps: 18:3→ 18:4→20:4→20:5→22:5→24:5→24:6, after which 24:6n-3 is

shortened to DHA (22:6n-3) by one round of β-oxidation in peroxisomes (14). An additional pathway of α-LNA conversion has been reported, in which 20:3n-3, 22:4n-3, and 24:4n-3 intermediates are converted to 24:5n-3 and then to DHA in the usual manner (45). In the tracer-infused rats, esterified [1-^{14}C]α-LNA and several [^{14}C]n-3 PUFA intermediates (18:4, 20:4, 20:5, 22:5, and 20:3, but not 22:6n-3) along the pathways of conversion of α-LNA to DHA could be detected in the heart (Table 4). The percent [1-^{14}C]α-LNA in total lipid was 97.9% (17.5% of net heart radioactivity) and 97.8% (17.8% of net radioactivity), respectively, in the diet-adequate and diet-deficient group, which means that conversion of α-LNA to longer chain intermediates was very limited. Radioactivity due to elongation products equaled 0.3% of net heart radioactivity in both the diet-adequate and diet-deficient groups, and was unaffected by deprivation.

Calculated incorporation coefficients and rates

Detectable radioactivity due to esterified [1-^{14}C]α-LNA and longer chain [^{14}C]n-3 PUFAs in total heart lipids is presented in Table 5, and was calculated using data from Fig. 1 and Table 4. Esterified 18:4n-3, 24:5n-3, 24:6n-3, and 22:6n-3 (DHA) concentrations could not be detected. Previous reports in these same rats (21, 22) showed that mean integrated plasma radioactivity during the 5 min tracer infusion was due entirely to [1-^{14}C]α-LNA, and equaled 452,235 ± 75,337 nCi/ml plasma/s and 500,384 ± 77,308 nCi/ml plasma/s in diet-adequate and diet-deprived rats, respectively. Incorporation and conversion-incorporation coefficients $k^*_{i(\alpha - LNA)}$ and $k^*_{i(\alpha - LNA \rightarrow j)}$ were calculated after inserting individual experimentally determined integrals and lipid radioactivities into Equations 1 and 2, respectively, and are shown in the second pair of data columns in Table 5. Dietary n-3 PUFA deprivation did not significantly alter either set of coefficients for total lipid (Table 5), or for individual phospholipid or triacylglycerol moieties (data not shown).

The unesterified unlabeled plasma α-LNA concentration before tracer infusion equaled 27 ± 6.0 nmol/ml and 1.0 ± 0.45 nmol/ml in the dietary n-3 PUFA-adequate and -deficient rats, respectively (22). Inserting these concentrations from individual experiments into Equation 3 yielded incorporation rates $J_{in,i(\alpha - LNA)}$ in total heart lipid for unesterified α-LNA equal to 171 ± 57 nmol/s/g × 10^{-4} and 6.2 ± 2.3 nmol/s/g × 10^{-4}, respectively, for the two groups (last pair of columns in Table 5).

mRNA levels of conversion enzymes

mRNA levels of desaturases, elongases, and acyl-CoA oxidase were analyzed in hearts from diet-deficient and diet-adequate rats (Fig. 2). The levels of elongase-5 and Δ6 and Δ5 desaturases were not changed significantly by deprivation; acyl-CoA oxidase mRNA was decreased significantly, whereas mRNA for elongase-2 could not be identified.

DISCUSSION

In this study, we examined α-LNA incorporation, oxidation, and conversion rates to longer chain n-3 PUFAs in the heart of unanesthetized adults rats fed, for 15 weeks after weaning, an n-3 PUFA-adequate or -deficient diet. A mathematical model was used to calculate kinetic parameters from the cardiac distribution of [1-^{14}C]α-LNA and its oxidation and

elongated n-3 PUFA products after 5 min of an intravenous infusion. Figure 3 summarizes the primary results and shows that α-LNA was not converted to n-3 PUFAs longer than DPA (22:5n-3), including DHA, due to the absence of elongase-2.

Approximately 82% of [1-^{14}C]α-LNA in the heart after 5 min of intravenous infusion represented aqueous β-oxidation products, whereas about 18% was esterified in stable lipids in both dietary groups (Fig. 1). To the extent that labeled CO_2 was produced and lost, the fractional oxidation would be higher. Less than 1% of heart label represented longer chain n-3 PUFAs, and DHA could not be detected. Dietary deprivation did not affect cardiac incorporation, conversion coefficients, or β-oxidation of α-LNA, but did decrease unlabeled esterified concentrations of α-LNA, EPAn-3, DPAn-3, and DHA (by more than 90%), while increasing unlabeled esterified AA, DTAn-6, and DPAn-6 concentrations. Deprivation did not affect the heart acyl-CoA concentrations that could be measured. mRNA levels of elongase-5 and Δ6 and Δ5 desaturases were not changed significantly by deprivation; acyl-CoA oxidase mRNA was decreased significantly, and mRNA for elongase-2 could not be identified.

Under normal physiological conditions, approximately 60–90% of the heart's ATP is generated by β-oxidation of fatty acids within mitochondria (46), following entry of the fatty acid from the acyl-CoA pool via carnitine-*o*-palmitoyltransferase I (CPT-I; EC 2.3.1.21) (47, 48). In one study of rat liver mitochondria, of the long-chain fatty acids tested, α-LNA had the highest rate of transfer by CPT-1, and DHA and 18:0 had the lowest rates (49). Showing that α-LNA taken up in heart was largely β-oxidized is consistent with these observations and with studies reporting a high β-oxidation rate of α-LNA in the whole animal (50). In the rats of this study, about 70% and 30% of α-LNA taken up by brain and liver, respectively, underwent β-oxidation after 5 min (21, 22, 27, 28).

The calculated incorporation rate of unesterified α-LNA from plasma into total heart lipid of rats fed the n-3 PUFA-adequate diet equaled 171 ± 57 nmol/s/g × 10^{-4} (61.9 nmol/h/g) (Table 5). In comparison, the liver incorporation rate in the same rats equaled 709 ± 244 nmol/s/g × 10^{-4} and the brain rate equaled 10.4 ± 3.9 nmol/s/g × 10^{-4} (21, 22). The calculated heart incorporation coefficient $k^*_{i(\alpha-LNA)}$ equaled 6.3 ± 1.8 ml/s/g ×10^{-4}, compared with 26.1 ± 7.1 and 7.16 ± 2.09 ml/s/g ×10^{-4} in liver triacylglycerol and phospholipid, respectively; and with 0.460 ± 0.105 ml/s/g ×10^{-4} in total brain lipid. Thus, tissue ordering for α-LNA incorporation coefficients and rates is liver > heart > brain.

Although α-LNA can be elongated to DHA in a number of mammalian tissues, including liver and brain (14–18, 21, 22), there was no evidence of its elongation in the heart at 5 min, when elongation to 20:4n-3, 20:5n-3, and 20:3n-3 could be demonstrated (Table 4). Rat heart expresses Δ6 and Δ5 desaturases and elongase-5, whereas elongase-2 has not been detected (15–17, 24) and its mRNA was not detected. Elongase-2 is required for elongation of 22:5n-3 to 24:5n-3, the precursor for DHA (17), and its absence in the heart probably explains why [^{14}C] DHA was not found there (Table 4).

The baseline esterified cardiac fatty acid and acyl-CoA concentrations are comparable to published concentrations (51, 52). Whereas the 15 week n-3 PUFA deprivation reduced

heart esterified DHA (Table 3), it reduced brain phospholipid DHA by 37% and liver esterified DHA by 92% in the same rats (21, 22). Total plasma DHA was reduced from 158 nmol/ml plasma to 14 nmol/ml plasma (by 91%) and unesterified plasma DHA was reduced from 6.5 nmol/ml plasma to 0.3 nmol/ml plasma (by 95%) (Table 1); these low values were approximated in rats after only 5 weeks on the n-3 PUFA-inadequate diet (40).

The measurable synthesis-incorporation (conversion) coefficients $k^*_{i(\alpha - LNA \rightarrow j)}$ of unesterified α-LNA to longer chain n-3 PUFAs within heart total stable lipids were not altered significantly by the n-3 PUFA-deficient diet (Table 5), consistent with the unchanged mRNA levels of elongase-5 and of Δ6 and Δ5 desaturases (Fig. 2). We did not determine the activities of these enzymes. $k^*_{i(\alpha - LNA \rightarrow j)}$ of α-LNA in the diet-adequate rats equaled 0.018 ± 0.005 ml/s/g × 10^{-4} for EPA (20:5n-3), but it could not be measured for DHA. In the liver of the same rats, $k^*_{i(\alpha - LNA \rightarrow j)}$ equals 1.05 ± 0.29 and 0.107 ± 0.029 ml/s/g ×10^{-4} for EPA and DHA, respectively, whereas in brain, it equals 0.029 ± 0.007 and 0.006 ± 0.002 ml/s/g ×10^{-4} for EPA and DHA, respectively. Thus, all of heart DHA, in the absence of dietary DHA but with α-LNA as the only dietary n-3 PUFA, is derived from circulating DHA synthesized from α-LNA in the liver.

Unlike the brain, into which fatty acids within circulating lipoproteins do not enter to a measurable extent (40, 53, 54), fatty acids esterified in triacylglycerols and phospholipids of circulating lipoproteins make important contributions to cardiac composition, and can gain access through very low density lipoprotein receptors or the action of lipoprotein lipase (55, 56). In an extended kinetic model, we can take this contribution into account by calculating the ratio of specific activities of α-LNA-CoA to that of plasma α-LNA (21, 22, 57), but were unable to do so in this study. Thus, we could not calculate the actual elongation rates of the n-3 PUFAs from α-LNA.

n-3 PUFA dietary deficiency led to marked (8- to 15-fold) accumulation of DPAn-6, an AA elongation product, in stable heart lipid (Table 3). Because the rat heart cannot synthesize DPAn-6 from AA, owing to the absence of elongase-2 (also explaining its inability to synthesize DHA from α-LNA, see above), heart DPAn-6 must have been derived from the blood after being synthesized and delivered to the blood by the liver in the diet-deficient animals. DPAn-6 could not be detected in plasma in the diet-adequate rats. In the same rats, liver desaturases and elongases are upregulated by the n-3 PUFA-deficient diet, accounting for the appearance of DPAn-6 in plasma (Table 1) (20, 22).

Dietary α-LNA has been investigated with regard to prevention of cardiovascular disease (58–60). Dietary α-LNA can reduce cardiac arrhythmias and heart rate in rats, although less effectively than dietary EPA or DHA (58, 61). We do not know the rate of DHA consumption by the heart, but now know that liver conversion of dietary α-LNA to DHA can maintain the heart DHA concentration in the absence of dietary DHA (31, 32). Liver enzyme changes associated with aging or disease (62, 63) might decrease DHA synthesis from α-LNA and increase risk for heart disease. Furthermore, the advantage of including EPA in dietary n-3 PUFA supplementation to maintain heart function may be that EPA conversion to

DHA requires only Δ6 desaturase and acyl-CoA oxidase (17, 19, 20), which are present in heart, but not the absent elongase-2.

In summary, the heart itself does not synthesize DHA from circulating α-LNA, owing to the absence of elongase-2, but must derive its DHA from the blood. When the rate of DHA synthesis is reduced by dietary n-3 PUFA deprivation, heart DHA falls in rough proportion to plasma DHA, reaching 10% of the normal level after 15 weeks of deprivation, whereas DPAn-6 accumulates in both plasma and heart. Most α-LNA taken up by heart is oxidized.

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Abbreviations

AA	arachidonic acid (20: 4n-6)
CPT	carnitine-*o*-palmitoyltransferase
DPA	docosapentaenoic acid (22:5)
DHA	docosahexaenoic acid (22:6n-3)
EPA	eicosapentaenoic acid (20:5n-3)
FAME	fatty acid methyl ester
HPLC	high-performance liquid chromatography
LA	linoleic acid (18:2n-6)
α-LNA	α-linolenic acid (18:3n-3)

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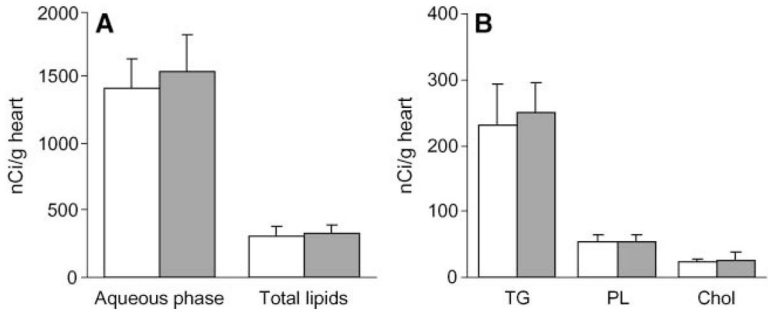

Fig. 1.

Radioactivity in heart aqueous fraction and lipids in dietary n-3 PUFA-adequate and -deficient rats after intravenous infusion of [1-^{14}C]α-LNA for 5 min. A: Radioactivity of total lipid and aqueous phases in both groups. B: Radioactivity in individual lipids. TG, triacylglycerol; PL, phospholipid; Chol, cholesterol. Open bars represent the n-3 PUFA-adequate group; closed bars represent the deprived group. Values are means ± SD (n = 10 and 7 in adequate and deficient group, respectively).

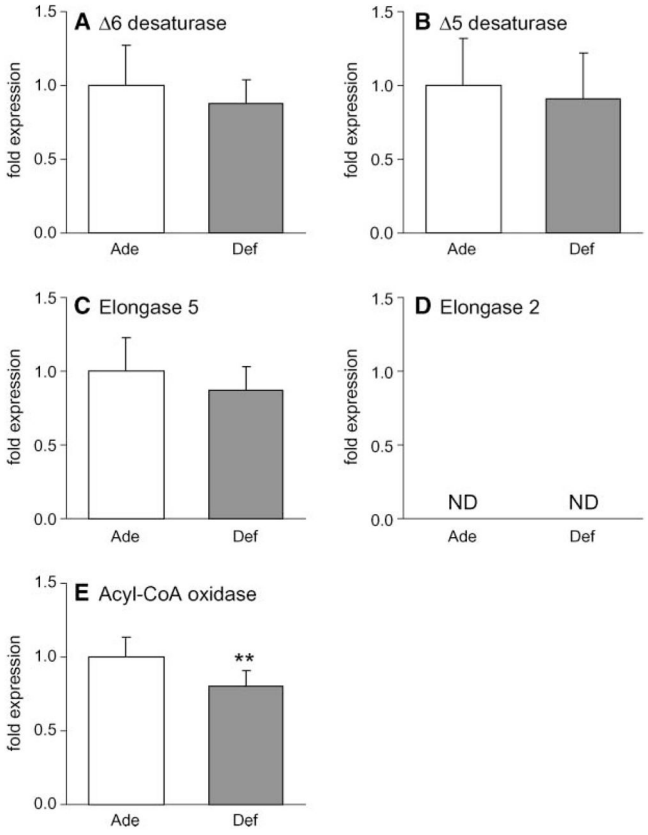

Fig. 2.

mRNA levels of Δ6 desaturase (A), Δ5 desaturase (B), elongase-5 (C), elongase-2 (D), and acyl-CoA oxidase (E) in heart of rats fed n-3 PUFA-adequate and n-3 PUFA-deficient diets. ND, not detected. Values are means ± SD (n = 10 for each group). ** $P < 0.01$, differs significantly from mean in adequate group.

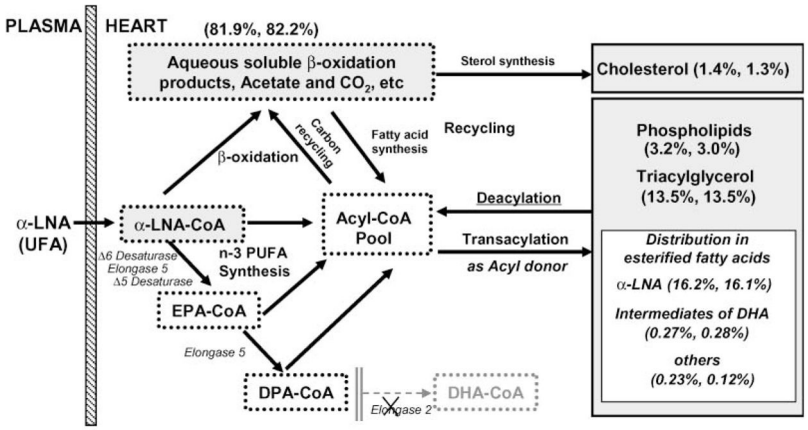

Fig. 3.

Scheme of plasma-derived α-LNA uptake and metabolic pathways in rat heart as determined following 5 min intravenous infusion of [1-^{14}C]α-LNA. Heart compartment contents of [1-^{14}C]α-LNA were calculated by dividing the radioactivity in each compartment by the net total heart radioactivity (excluding unesterified plasma fatty acid radioactivity) and expressing the values as a percentage. The left number in parentheses is for the n-3 PUFA-adequate group, the right for the deprived group. Arrows show metabolic pathways.

TABLE 1

Fatty acid composition of plasma lipids in dietary n-3 PUFA-adequate and -deficient rats

	Unesterified Fatty Acid		Total Fatty Acid	
Fatty Acid	**Adequate**	**Deprived**	**Adequate**	**Deprived**
		nmol/ml plasma		
14:0	57 ± 11	64 ± 7.6	111 ± 35	132 ± 39
16:0	459 ± 108	488 ± 60	1,677 ± 441	1,676 ± 571
16:1n-7	84 ± 25	95 ± 15	188 ± 78	235 ± 99
18:0	96 ± 20	95 ± 8.5	1,118 ± 125	1,069 ± 141
18:1n-9	240 ± 66	244 ± 36	688 ± 264	722 ± 333
18:1n-7	49 ± 12	56 ± 18	201 ± 62	230 ± 96
18:2n-6	259 ± 49	253 ± 36	1,102 ± 260	1,023 ± 368
18:3n-3	27 ± 6.0	1.0 ± 0.45[c]	51 ± 15	3.6 ± 1.2[c]
20:4n-6	25 ± 4.8	34 ± 5.9[c]	1,361 ± 204	1,597 ± 141[b]
20:5n-3	3.3 ± 1.1	ND	33 ± 11	ND
22:5n-6	ND	8.7 ± 1.2	ND	13 ± 38
22:5n-3	3.6 ± 1.3	ND	19 ± 3.1	ND
22:6n-3	6.5 ± 2.6	0.23 ± 0.10[c]	151 ± 35	14 ± 5.9[c]
Total	1,312 ± 292	1,338 ± 171	6,719 ± 1,439	6,899 ± 1,733
Total n-6	285 ± 53	295 ± 41	2,463 ± 388	2,756 ± 529
Total n-3	40.7 ± 9.7	1.3 ± 0.4[c]	246 ± 53	17 ± 5.1[c]
Total saturated	613 ± 134	647 ± 73	2,906 ± 585	2,877 ± 718
Total monounsaturated	374 ± 101	395 ± 61	1,077 ± 398	1,187 ± 527
18:3n-3[a]	53 ± 5.3	33 ± 8.4		

Adapted from (23). Values are means ± SD (n = 10 and 7 for diet-adequate and diet-deficient groups, respectively). ND, not detected.

[a] Unesterified 18:3n-3 concentration in plasma after 5 min of intravenous [1-^{14}C]α-LNA.

[b] $P < 0.01$, differs significantly from mean in diet-adequate group.

[c] $P < 0.001$, differs significantly from mean in diet-adequate group.

TABLE 2

Concentrations of stable heart lipids in rats fed n-3 PUFA-adequate and -deficient diets

	Concentration	
Lipids	**Adequate**	**Deficient**
	μmol/g heart	
Total phospholipid	34.6 ± 4.3	33.7 ± 5.2
Ethanolamineglycerophospholipid	13.7 ± 2.9	12.3 ± 1.7
Cholineglycerophospholipid	14.6 ± 2.2	14.1 ± 0.4
Phosphatidylserine	0.98 ± 0.17	0.75 ± 0.07[a]
Phosphatidylinositol	2.1 ± 0.3	2.0 ± 0.1
Sphingomyelin	1.3 ± 0.5	1.1 ± 0.4
Cardiolipin	2.8 ± 0.8	3.3 ± 1.4
Triacylglycerol	2.7 ± 0.6	3.0 ± 0.9
Total cholesterol	8.5 ± 2.8	7.9 ± 1.7

Values are means ± SD (n = 10 and 7 for adequate and deficient groups, respectively).

[a] $P < 0.01$, differs significantly from mean in adequate group.

TABLE 3

Esterified fatty acid concentrations in heart phospholipid and triacylglycerol from n-3 PUFA-adequate and -deficient rats

	Phospholipid		Triacylglycerol	
Fatty Acid	**Adequate**	**Deficient**	**Adequate**	**Deficient**
	nmol/g heart			
14:0	186 ± 55	198 ± 36	280 ± 103	367 ± 130
16:0	6,731 ± 942	6,327 ± 340	1,796 ± 485	2,150 ± 536
16:1n-7	322 ± 188	286 ± 28	212 ± 71	261 ± 85
18:0	13,344 ± 1,430	13,350 ± 478	470 ± 93	546 ± 96
18:1n-9	2,011 ± 444	2,216 ± 115	1,229 ± 283	1,433 ± 366
18:1n-7	1,881 ± 621	1,686 ± 108	347 ± 89	437 ± 96[a]
18:2n-6	12,140 ± 2,304	11,211 ± 824	1,290 ± 294	1,490 ± 400
18:3n-3	118 ± 44	ND	89 ± 25	9.0 ± 1.8[b] −90%
20:4n-6	9,793 ± 1,110	11,466 ± 414[b] +17%	157 ± 27	230 ± 34[b] +46%
20:5n-3	55 ± 8	ND	ND	ND
22:4n-6	375 ± 42	1,010 ± 87[b] +269%	29 ± 5	74 ± 13[b] +103%
22:5n-6	264 ± 29	3,989 ± 413[b] +1,510%	8.6 ± 2.3	72 ± 13[b] +837%
22:5n-3	1,062 ± 108	60 ± 5[b] −94%	59 ± 9	2.8 ± 0.5[b] −95%
22:6n-3	4,433 ± 426	344 ± 28[b] −92%	70 ± 15	5.1 ± 0.8[b] −93%
Total	52,716 ± 6,141	52,144 ± 1,661	6,039 ± 1,431	7,077 ± 1,712
Total saturated	20,262 ± 2,374	19,876 ± 752	2,547 ± 661	3,063 ± 755
Total monounsaturated	4,214 ± 714	4,188 ± 227	1,789 ± 434	2,131 ± 545
Total n-6	22,572 ± 3,196	27,676 ± 862[b] +122%	1,485 ± 316	1,866 ± 436[a] +126%
Total n-3	5,668 ± 514	404 ± 30[b] −93%	218 ± 38	17 ± 3[b] −91%
n-6/n-3	4.0	68.5	6.8	110

Values are means ± SD (n = 10 and 7 for diet-adequate and diet-deficient groups, respectively). ND, not detected.

[a] $P < 0.05$, differs significantly from mean in adequate group.

[b] $P < 0.001$, differs significantly from mean in adequate group.

TABLE 4

Radioactivity of esterified fatty acids in heart total lipids

	Percent Total Esterified Fatty Acid Radioactivity in Heart	
Fatty Acid	**Adequate**	**Deficient**
18:3n-3	97.9 ± 0.3	97.8 ± 0.3
18:4n-3	ND	ND
20:4n-3	0.40 ± 0.10	0.40 ± 0.07
20:5n-3	0.65 ± 0.12	0.67 ± 0.10
22:5n-3	0.28 ± 0.04	0.31 ± 0.04
24:5n-3	ND	ND
24:6n-3	ND	ND
22:6n-3	ND	ND
20:3n-3	0.31 ± 0.07	0.30 ± 0.03
Unknown	0.47 ± 0.13	0.48 ± 0.13

Values are means ± SD (n = 4 and 3 for diet-adequate and diet-deficient groups, respectively). Detection level was > 0.1% of the applied radioactivity. ND, not detected.

TABLE 5

Calculated incorporation coefficients and influx rates of α-LNA into heart stable lipids, and conversion-incorporation coefficients into esterified elongated n-3 PUFA products, in diet-adequate and diet-deficient rats

	Radioactivity		$k^*_{i(\alpha - LNA)}$ [a] or $k^*_{i(\alpha - LNA \rightarrow j)}$ [b]		$J_{in,i(\alpha\text{-}LNA)}$	
Total Esterified	**Adequate**	**Deficient**	**Adequate**	**Deficient**	**Adequate**	**Deficient**
	nCi/g heart		*ml/s/g × 10⁻⁴*		*nmol/s/g × 10⁻⁴*	
18:3n-3	279 ± 66	297 ± 53	6.3 ± 1.8	6.0 ± 1.1	171 ± 57	6.2 ± 2.3[a]
20:4n-3	1.2 ± 0.3	1.2 ± 0.2	0.026 ± 0.007	0.024 ± 0.005		
20:5n-3	1.8 ± 0.4	2.0 ± 0.4	0.042 ± 0.012	0.041 ± 0.008		
22:5n-3	0.81 ± 0.19	0.95 ± 0.17	0.018 ± 0.005	0.019 ± 0.004		
20:3n-3	0.88 ± 0.21	0.90 ± 0.16	0.020 ± 0.006	0.018 ± 0.003		

Values are means ± SD (n = 10 and 7 for diet-adequate and -deficient groups, respectively).

[a] $P < 0.001$, differs significantly from mean in adequate group.