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Review

Threshold changes in rat brain docosahexaenoic acid incorporation and concentration following graded reductions in dietary alpha-linolenic acid

Ameer Y. Taha [a,*], Lisa Chang [b], Mei Chen [b]

[a] Department of Food Science and Technology, College of Agriculture and Environmental Sciences, University of California, Davis, CA, USA
[b] Brain Physiology and Metabolism Section, Laboratory of Neuroscience, National Institute on Aging, National Institutes of Health, Bethesda, MD, USA

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ABSTRACT

Background: This study tested the dietary level of alpha-linolenic acid (α-LNA, 18:3n-3) required to maintain brain ^{14}C-Docosahexaenoic acid (DHA, 22:6n-3) metabolism and concentration following graded α-LNA reduction.

Methods: Fischer-344 (CDF) male rat pups (18–21 days old) were randomized to the AIN-93G diet containing as a % of total fatty acids, 4.6% ("n-3 adequate"), 3.6%, 2.7%, 0.9% or 0.2% ("n-3 deficient") α-LNA for 15 weeks. Rats were intravenously infused with ^{14}C-DHA to steady state for 5 min, serial blood samples collected to obtain plasma, and brains excised following microwave fixation. Labeled and unlabeled DHA concentrations were measured in plasma and brain to calculate the incorporation coefficient, k^*, and incorporation rate, J_{in}.

Results: Compared to 4.6% α-LNA controls, k^* was significantly increased in ethanolamine glycerophospholipids in the 0.2% α-LNA group. Circulating unesterified DHA and brain incorporation rates (J_{in}) were significantly reduced at 0.2% α-LNA. Brain total lipid and phospholipid DHA concentrations were reduced at or below 0.9% α-LNA.

Conclusion: Threshold changes for brain DHA metabolism and concentration were maintained at or below 0.9% dietary α-LNA, suggesting the presence of homeostatic mechanisms to maintain brain DHA metabolism when dietary α-LNA intake is low.

1. Introduction

Brain membrane phospholipids are highly enriched with the polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6), which participate in neurotransmission [1,2], gene transcription and the regulation of brain immunity [3–5]. The brain derives most of its DHA and AA from the plasma unesterified fatty acid pool, which is maintained by liver synthesis—secretion of DHA and AA from their dietary precursors, alpha-linolenic acid (α-LNA, 18:3n-3) and linoleic acid (LA, 18:2n-6), respectively, or from direct dietary incorporation [6–9]. Brain endogenous synthesis of DHA and AA is low, accounting for less than 0.2% and 1% of the unesterified α-LNA and LA that enter the brain, respectively [10,11].

Bourre et al. established that the minimum dietary α-LNA required to maintain maximal tissue DHA concentrations in rats during development and adulthood, is 4% (0.4% energy) and 2.3% (0.26% energy) α-LNA of total fatty acids, respectively, when total fat content is 50–60 g per kg diet [12,13]. Bourre et al. also reported that 0.9% dietary α-LNA of total fatty acids (equivalent to 0.1% energy, or 0.5 g/kg diet) is sufficient to maintain adult rat brain DHA concentrations (Table 6 of the Bourre et al. paper [13]). Typically, rodent diets such as the AIN-93G or AIN-93M diets, are designed to provide 4.6–7.0% dietary α-LNA (of total fatty acids) throughout development and adulthood to ensure maximal DHA liver supply and accretion by the brain and other tissues, and normal brain development and function [14–18]. Total fat content of the AIN-93G diets and other similar dietary formulations ranges between 70–100 g per kg diet [14–16,18].

Abbreviations: α-LNA, alpha-linolenic acid; ANOVA, analysis of variance; AA, arachidonic acid; cPLA2, Ca^{2+}-dependent cytosolic phospholipase A_2; iPLA2, Ca^{2+}-independent phospholipase A_2; CE, cholesteryl ester; ChoGpl, choline glycerophospholipid; COX, cyclooxygenase; DHA, docosahexaenoic acid; EtnGpl, ethanolamine glycerophospholipid; FAMEs, fatty acid methyl esters; GC, gas-chromatography; LA, linoleic acid; LOX, lipoxygenase; PtdIns, phosphatidylinositol; PET, positron emitting tomography; PtdSer, phosphatidylserine; PUFA, polyunsaturated fatty acids; TLs, total lipids; TLC, thin layer chromatography; TG, triacylglyceride

* Correspondence to: RMI North, Department of Food Science and Technology, College of Agriculture and Environmental Sciences, University of California, Davis, CA, USA. Tel.: +1 530 752 7096.
E-mail address: ataha@ucdavis.edu (A.Y. Taha).

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Studies have shown that compared to rats fed a DHA-free, "n-3 PUFA adequate" diet containing 4.6% α-LNA of total fatty acids, rats fed a DHA-free "n-3 PUFA deficient diet" (0–0.2% α-LNA) for 15 weeks or 2–3 generations have reduced plasma and brain DHA concentrations, increased concentrations of n-6 docosapentaenoic acid (n-6 DPA), an elongation product of AA formed in the liver, and behavioral abnormalities [15–18]. Dietary n-3 PUFA deficiency also decreased brain DHA rate of acylation–reacylation (i.e. turnover) within membrane phospholipids [19] and increased elimination half-life from 33 to 90 days [20]. This is consistent with evidence of decreased mRNA, protein or activity of DHA-metabolizing enzymes, including DHA-releasing calcium-independent phospholipase A_2 VIA ($iPLA_2$), cyclooxygenase (COX)-1 and 12-lipoxygenase (LOX) [21,22]. The decrease in brain DHA metabolism is accompanied by a reciprocal increase in AA-metabolizing group IVA calcium-dependent phospholipase A_2 ($cPLA_2$) and COX-2 [21,22]. While brain AA turnover or half-life did not change [23,24], n-6 DPA concentration and turnover increased [25], suggesting that n-6 DPA replaces DHA and may act as a substrate for AA-metabolizing enzymes during chronic n-3 PUFA deficiency.

The decrease in brain DHA metabolism following dietary α-LNA deficiency suggests that homeostatic adaptations spare brain DHA metabolism. A graded dietary α-LNA study that provided 0.2%, 0.8%, 1.7%, 2.6% 3.8% or 4.6% α-LNA of total fatty acids to rats for 15 weeks showed that changes in brain DHA metabolizing enzymes and concentrations occurred at or below 1.7% dietary α-LNA, indicating adaptive changes in brain DHA metabolism [21]. Brain DHA concentration decreased and n-6 DPA concentration increased at 0.8% dietary α-LNA relative to controls (4.6% α-LNA), whereas DHA-metabolizing $iPLA_2$ activity and COX-1 and 12-LOX mRNA decreased at 1.7% α-LNA [21]. Plasma unesterified DHA concentration decreased at 2.7% α-LNA, suggesting that the changes in plasma DHA concentration initiated, rather than followed changes in brain DHA and n-6 DPA concentrations and enzymes [21].

Rapoport and colleagues developed an in vivo kinetic method using radiolabeled fatty acids to quantify the incorporation coefficient (k^*) and rate (J_{in}) of plasma unesterified fatty acids into the brain [26]. Using this method, Contreras et al. reported that k^* for DHA in brain total lipid (TL), ethanolamine glycerophospholipid (EtnGpl) and phosphatidylserine (PtdSer) increased in rats following α-LNA deprivation (n-3 deficiency) for 3 consecutive generations, reflecting an increase in the brain's avidity for DHA [19]. The incorporation rate (J_{in}), reflecting net brain DHA consumption, decreased, however, due to the reduction in circulating unesterified DHA [19].

In the present study, we used the Rapoport in vivo kinetic method [26] to measure radiolabeled DHA incorporation into brain following graded dietary α-LNA reductions below 4.6% α-LNA, considered to be an n-3 PUFA adequate amount [14] that exceeds by five-fold the minimum α-LNA amount established by Bourre et al. required to maintain adult rat brain DHA composition (0.9% α-LNA of total fatty acids) [13]. Similar to the study by Kim et al. [21], rats received for 15 weeks the AIN-93G diet containing the recommended amount of n-6 LA, but varying levels of α-LNA, which amounted to 4.6% (Adequate), 3.8%, 2.7%, 1.8%, 0.9% and 0.2% (deficient) of total fatty acids. The rats were injected with [1-^{14}C] DHA in order to measure adaptive changes to k^* and J_{in} in response to dietary α-LNA reduction. In view of the Kim et al. finding, that changes in DHA metabolizing enzymes occurred at threshold levels above 0.9% α-LNA [21], we hypothesized that adaptive increases in k^* and reductions in J_{in} will occur at or greater than 0.9% dietary α-LNA, to maintain brain DHA concentrations.

Upon completion of the study, the fatty acid composition of the diets, which were obtained from Dyets Inc., was measured by gas-chromatography. Surprisingly, the measurements revealed that the 1.8% α-LNA diet contained 5.1% α-LNA. Thus, data related to this group was removed from the analyses on the basis that the diet composition was not accurate.

Contrary to the hypothesis, we found that k^* increased and J_{in} decreased only at 0.2% α-LNA.

2. Material and methods

2.1. Animals and diets

Experiments were conducted following the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health Publication No. 86-23) and were approved by the Animal Care and Use Committee of *Eunice Kennedy Shriver* National Institute of Child Health and Human Development. Male Fischer-344 (CDF) rat pups aged 18–21 days and their surrogate mothers were purchased from Charles River Laboratories (Portage, MI, USA). Upon arrival, the pups were weaned from their surrogate mothers and randomly allocated to 6 diets containing 4.6% (control, n-3 PUFA adequate), 3.8%, 2.7%, 1.8%, 0.9% and 0.2% (n-3 PUFA deficient) α-LNA of total fatty acids (7–9 rats per dietary group). The calculated percent energy equivalent of the 4.6%, 3.8%, 2.7%, 1.8%, 0.9% and 0.2% α-LNA rodent diets, based on prior quantitative fatty acid gas-chromatography (GC) analysis done on the 4.6% α-LNA diet, is 0.88%, 0.74%, 0.43%, 0.18%, 0.03% and 0.002%, respectively. The macronutrient composition of the diets which were prepared by Dyets Inc. (Bethlehem, PA, USA) based on the AIN-93G formulation [14], is presented in Supplementary Table 1. The fatty acid composition of the diets (analytical details in following section) is shown in Supplementary Table 2. The diets were isocaloric and contained 10% fat. α-LNA composition was altered by substituting coconut oil with flaxseed oil which is high in α-LNA, while maintaining safflower oil content (main source of dietary LA).

The rats were maintained on the diets for 15 weeks, with their food being replaced every 3–4 days. Water and food were provided ad libitum. Animals were housed in an animal facility that had regulated temperature, humidity and a 12 h light/dark cycle. They were initially housed in groups of 4–6 per cage and then 3–4 per cage a few weeks later to accommodate for cage space.

2.2. Diet fatty acid analysis

The fatty acid composition of the diets was analyzed, approximately two years after the study ended. Diets were stored at 4 °C during and after the study. A few pellets from each diet were crushed using a pestle and mortar. A portion of the crushed pellets was weighed to approximately 0.2 g and transferred to glass vials ($n=3$ replicates per diet). To each sample, 400 μl toluene, 3 ml methanol and 600 μl of 8% hydrochloric acid in methanol were added. Samples were vortexed after adding each reagent. The samples were heated at 90 °C for one hour and cooled thereafter for 10–15 min at room temperature. Water (1 ml) and hexane (1 ml) were added to each vial. The mixture was vortexed and the phases allowed to separate for 15–30 min. A portion of the top hexane layer (200 μl) from each sample was transferred to microcentrifuge tubes containing ~20 mg of sodium sulfate, to absorb residual water. The tube was inverted a few times and its contents allowed to settle for 10 min. The top hexane layer was transferred to gas-chromatography (GC) vials. Fatty acid methyl esters (FAMEs) were analyzed by injecting 1 μl of the top hexane layer into a Varian GC (see below section on FAME analysis by GC).