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Dietary n-6 PUFA deprivation downregulates arachidonate but upregulates docosahexaenoate metabolizing enzymes in rat brain

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ABSTRACT

Background: Dietary n-3 polyunsaturated fatty acid (PUFA) deprivation increases expression of arachidonic acid (AA 20:4n-6)-selective cytosolic phospholipase A_2 ($cPLA_2$) IVA and cyclooxygenase (COX)-2 in rat brain, while decreasing expression of docosahexaenoic acid (DHA 22:6n-3)-selective calcium-independent $iPLA_2$ VIA. Assuming that these enzyme changes represent brain homeostatic responses to deprivation, we hypothesized that dietary n-6 PUFA deprivation would produce changes in the opposite directions. *Methods:* Brain expression of PUFA-metabolizing enzymes and their transcription factors was quantified in male rats fed an n-6 PUFA adequate or deficient diet for 15 weeks post-weaning. *Results:* The deficient compared with adequate diet increased brain mRNA, protein and activity of $iPLA_2$ VIA and 15-lipoxygenase (LOX), but decreased $cPLA_2$ IVA and COX-2 expression. The brain protein level of the $iPLA_2$ transcription factor SREBP-1 was elevated, while protein levels were decreased for AP-2α and NF-κB p65, $cPLA_2$ and COX-2 transcription factors, respectively. *Conclusions:* With dietary n-6 PUFA deprivation, rat brain PUFA metabolizing enzymes and some of their transcription factors change in a way that would homeostatically dampen reductions in brain n-6 PUFA concentrations and metabolism, while n-3 PUFA metabolizing enzyme expression is increased. The changes correspond to reported *in vitro* enzyme selectivities for AA compared with DHA.

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1. Introduction

The polyunsaturated fatty acids (PUFAs) docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) are highly enriched in brain [1–4], where they are found mainly in the stereospecifically numbered (*sn*)-2 position of membrane phospholipids. *In vitro* studies indicate that DHA and AA can be hydrolyzed selectively from phospholipid by Ca^{2+}-independent phospholipase A_2 ($iPLA_2$ type VI) and Ca^{2+}-dependent cytosolic $cPLA_2$ type IVA, respectively [5–11]. This selectivity is consistent with observations that 15 weeks of dietary n-3 PUFA deprivation in rats increased brain expression (mRNA, protein and/or activity) of $cPLA_2$ IVA, secretory $sPLA_2$ type IIA and COX-2 (which is functionally coupled and co-evolved with $cPLA_2$ [12,13]), while decreasing expression of $iPLA_2$ VIA and COX-1 [14–16]. The enzyme changes corresponded to reduced DHA metabolic loss from brain (prolonged half life) and a reduced brain DHA concentration, but an increased brain concentration of the AA elongation product, docosapentaenoic acid (DPA, 22:5n-6) [17].

In comparison, the brain AA concentration was decreased and the brain DHA concentration was increased in weaned rats fed an n-6 PUFA deficient diet for 15 weeks [17,18]. Assuming that the enzyme changes in the n-3 PUFA deprived rat (see above) reflected homeostatic dampening of brain DHA loss, we hypothesized that dietary n-6 PUFA deprivation would produce changes in the opposite in direction. Accordingly, in the present study we examined brain expression of PLA_2 and downstream oxidative enzymes (COX-1 and 2, and 5-, 12 - and 15-lipoxygenase (LOX)) involved in PUFA metabolism [19,20], and of some of their transcription factors, in rats fed the n-6 PUFA deficient or adequate diet [18] for 15 weeks after weaning. An abstract of part of this work has been published [21].

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-[1-^{14}C] arachidonoyl-*sn*-glycerol-3-phosphorylcholine, purchased from PerkinElmer (Boston, MA, USA), had a specific activity of 60 mCi/mmol. 1-Palmitoyl-2-[1-^{14}C] palmitoyl-*sn*-glycerol-3-phosphorylcholine was purchased from GE Healthcare (Buckinghamshire, UK) and had a specific activity of 53 mCi/mmol. The purity of each exceeded 95%, as determined by thin layer chromatography, scintillation counting and gas chromatography. 1-Palmitoyl-2-arachidonoyl-*sn*-glycerol-3-phosphorylcholine, 1-palmitoyl-2-[1-^{14}C] palmitoyl-*sn*-glycerol-3-phosphorylcholine and phosphatidylinositol-4, 5-

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; PUFA, polyunsaturated fatty acid; $cPLA_2$, cytosolic phospholipase A_2; $sPLA_2$, secretory PLA_2; $iPLA_2$, Ca^{2+}-independent PLA_2; COX, cyclooxygenase; SREBP, sterol regulatory element binding protein; LOX, lipoxygenase; NF-κB, nuclear factor-κB; AP, activator protein; *sn*, stereospecifically numbered

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bisphosphate were purchased from Avanti (Alabaster, AL, USA). Protease inhibitor cocktail was purchased from Roche (Indianapolis, IN, USA). Antibodies against group IVA cPLA$_2$, group IIA sPLA$_2$, group VIA iPLA$_2$, COX-1, COX-2, 5-LOX, 12-LOX, 15-LOX, nuclear factor-kappa B (NF-κB) p65, NF-κB p50, activator protein (AP)-2α and AP-2 β were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA), and antibodies against sterol regulatory binding protein (SREBP)-1 and -2 were from Abcam (Cambridge, MA, USA). β-Actin antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA) and appropriate horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling (Beverly, MA, USA). A high capacity cDNA reverse transcription kit, Taqman® gene expression master mix, and specific primers for real time RT-PCR were purchased from Applied Biosystems (Foster city, CA, USA).

2.2. Animals

The protocol was approved by the Animal Care and Use Committee of the *Eunice Kennedy Schriver* National Institute of Child Health and Human Development and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23). Fischer-344 (CDF) male rat pups (19 days old) and their surrogate mothers, purchased from Charles River Laboratories (Portage, MI, USA), were housed in an animal facility with regulated temperature, humidity, and a 12 h light/12 h dark cycle. The pups were allowed to nurse until 21 days old. Lactating rats had free access to water and rodent chow formulation NIH-31 18-4, which contained 4% (wt/wt) crude fat (Zeigler Bros., Gardners, PA, USA) and whose fatty acid composition has been reported [18]. Briefly, α-linolenic acid (18:3n-3), eicosapentaenoic acid (20:5n-3) and DHA contributed 5.1%, 2.0% and 2.3% of total fatty acid, respectively, whereas linoleic acid (18:2n-6) and AA contributed 47.9% and 0.02%, respectively.

After weaning, pups were divided randomly into n-6 PUFA adequate ($n=10$) and deficient ($n=10$) diet groups as described below. They had free access to food and water, their food being replaced every 2 or 3 days. After 15 weeks on a chosen diet, a rat was asphyxiated by CO_2 inhalation and decapitated. The brain was excised rapidly and frozen in 2-methylbutane with dry ice at −50 °C, then stored at −80 °C until use. Animals were provided food until sacrifice.

2.3. Dietary composition

The n-6 PUFA adequate and deficient diets (Supplementary Table 1) were prepared by Dyets Inc. (Bethlehem, PA, USA), based on the AIN-93 G formulation [22,23], and contained 10% fat [24]. The adequate diet contained hydrogenated coconut oil (6 g/100 g diet), safflower oil (3.23 g/100 g) and flaxseed oil (0.77 g/100 g) (Supplementary Table 1) [17,25,26]. The deficient diet contained hydrogenated coconut oil (8.73 g/100 g), flaxseed oil (0.77 g/100 g), and olive oil (0.5 g/100 g), but no safflower oil (Supplementary Table 1).

Fatty acid concentrations (μmol/g food or percent of total fatty acid) of the two diets have been reported [24] and are shown in Table 1. The n-6 PUFA adequate diet contained linoleic acid at 52.1 μmol/g diet (27.6% of total fatty acid), whereas the deficient diet contained linoleic acid at 4.2 μmol/g (2.3% of total fatty acid), 10% of the suggested minimum requirement for rodents (42.8 μmol/g) [27]. Both diets contained α-linolenic acid 8.5–8.9 μmol/g (4.5–4.8% of total fatty acid), close to the minimum requirement for dietary n-3 PUFA adequacy in rodents [28,29], and oleic acid (18:1n-9) at 13.6–14.4 μmol/g (7.3–7.7% of total fatty acids). Other n-3 and n-6 PUFAs were absent from both diets.

Table 1
Fatty acid composition of n-6 PUFA adequate and deficient diets.

Fatty acid	n-6 PUFA adequate diet		n-6 PUFA deficient diet	
	μmol/g food	% of total fatty acid	μmol/g food	% of total fatty acid
12:0	54.6 ± 3.3	29.0	81.1 ± 20.7	43.8
14:0	23.5 ± 1.4	12.5	34.6 ± 9.0	18.7
14:1n-5	0.06 ± 0.01	0.03	0.06 ± 0.02	0.03
16:0	18.2 ± 1.0	9.7	20.6 ± 5.3	11.1
16:1n-7	0.08 ± 0.01	0.04	0.10 ± 0.03	0.1
18:0	17.1 ± 1.0	9.0	22.0 ± 5.8	11.9
18:1n-9	14.4 ± 0.8	7.7	13.6 ± 3.5	7.3
18:2n-6	52.1 ± 7.6	27.6	4.2 ± 1.1	2.3
18:3n-3	8.5 ± 0.5	4.5	8.9 ± 2.4	4.8
Saturated	113.5 ± 6.6	60.1	158.2 ± 40.8	85.5
Monounsaturated	14.6 ± 0.8	7.7	13.7 ± 3.5	7.4
n-6 PUFA	52.1 ± 7.6	27.6	4.2 ± 1.1	2.3
n-3 PUFA	8.5 ± 0.5	4.5	8.9 ± 2.4	4.8
n-6/n-3	6.1		0.5	

Values are mean ± SD ($n=3$).

2.4. Preparation of cytoplasmic and nuclear extracts for Western blotting

Cytoplasmic and nuclear proteins were prepared with a compartmental protein extraction kit (Chemicon, Temecula, CA, USA), according to the manufacturer's protocol. Prepared fractions were kept at −80 °C until used for Western blotting (see below). Protein concentrations of cytoplasmic and nuclear fractions were measured by the Bradford assay (Bio-Rad) [30]. Expression of group IVA cPLA$_2$, group IIA sPLA$_2$, group VIA iPLA$_2$, COX-1, COX-2, 5-LOX, 12-LOX and 15-LOX was determined in the cytoplasmic fraction, whereas expression of NF-κB p65, NF-κB p50, AP-2α, AP-2β, SREBP-1, SREBP-2, and lamin B was determined in the nuclear fraction.

2.5. Western blot analysis

Proteins from the cytoplasmic (50 μg) and nuclear (50 μg) extracts were separated on 4–20% SDS-polyacrylamide gels (PAGE) (Bio-Rad). Following SDS-PAGE, the proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Protein blots were incubated overnight at 4 °C in TBS buffer containing 5% nonfat dried milk and 0.1% Tween-20, with specific primary antibodies (1:1000 dilution) to: group IVA cPLA$_2$, group IIA sPLA$_2$, group VIA iPLA$_2$, COX-1, COX-2, 5-LOX, 12-LOX , 15-LOX, NF-κB p65, NF-κB p50, AP-2α, AP-2β, SREBP-1, SREBP-2, β-actin and lamin B. Protein blots were incubated with appropriate HRP-conjugated secondary antibodies (Cell Signaling, Beverly, MA, USA) and visualized using a chemiluminescence reaction (Pierce, Rockford, IL, USA) on BioMax X-ray film (Eastman Kodak, Rochester, NY, USA). Optical densities of immunoblot bands were measured with Alpha Innotech Software (Alpha Innotech, San Leandro, CA, USA) and were normalized to the optical density of β-actin (Sigma-Aldrich) to correct for unequal loading. All experiments were carried out with 10 independent samples per group. Values are expressed as percent of control.

2.6. RNA isolation and real time RT-PCR

Total RNA was isolated from brain using commercial kits (RNeasy Lipid Tissue Kit; Qiagen, Valencia, CA). cDNA was prepared from total RNA using a high-capacity cDNA Archive Kit (Qiagen). mRNA levels of cPLA$_2$ IVA (Rn 00591916_m1), sPLA$_2$ IIA (Rn 00580999_m1), iPLA$_2$ VIA (Rn 01504424_m1), COX-1 (Rn 00566881_m1), COX-2 (Rn 00568225_m1), 5-LOX (Rn 00563172_m1), 12-LOX (Rn 01461082_m1), and 15-LOX (Rn 00696151_m1) were measured by real time quantitative RT-PCR, using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The fold change in gene expression was determined by the $\Delta\Delta C_T$ method [31]. Data are expressed as the relative level of the target gene in the n-6 PUFA deficient group normalized to the endogenous control (β-globulin, Rn_00560865_m1) and relative to the level in the n-6 PUFA adequate