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Disturbed brain phospholipid and docosahexaenoic acid metabolism in calcium-independent phospholipase A_2-VIA (iPLA$_2\beta$)-knockout mice

Yewon Cheon [a,*], Hyung-Wook Kim [a], Miki Igarashi [a], Hiren R. Modi [a], Lisa Chang [a], Kaizong Ma [a], Deanna Greenstein [b], Mary Wohltmann [c], John Turk [c], Stanley I. Rapoport [a], Ameer Y. Taha [a]

[a] Brain Physiology and Metabolism Section, National Institute on Aging, National Institutes of Health, Bethesda, MD 20892, USA
[b] Child Psychiatry Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, MD 20892, USA
[c] Medicine Department, Mass Spectrometry Facility, and Division of Endocrinology, Metabolism, and Lipid Research, Washington University School of Medicine, St. Louis, MO 63110, USA

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ABSTRACT

Calcium-independent phospholipase A_2 group VIA (iPLA$_2\beta$) releases docosahexaenoic acid (DHA) from phospholipids *in vitro*. Mutations in the iPLA$_2\beta$ gene, *PLA2G6*, are associated with dystonia-parkinsonism and infantile neuroaxonal dystrophy. To understand the role of iPLA$_2\beta$ in brain, we applied our *in vivo* kinetic method using radiolabeled DHA in 4 to 5-month-old wild type (iPLA$_2\beta^{+/+}$) and knockout (iPLA$_2\beta^{-/-}$) mice, and measured brain DHA kinetics, lipid concentrations, and expression of PLA_2, cyclooxygenase (COX), and lipoxygenase (LOX) enzymes. Compared to iPLA$_2\beta^{+/+}$ mice, iPLA$_2\beta^{-/-}$ mice showed decreased rates of incorporation of unesterified DHA from plasma into brain phospholipids, reduced concentrations of several fatty acids (including DHA) esterified in ethanolamine- and serine-glycerophospholipids, and increased lysophospholipid fatty acid concentrations. DHA turnover in brain phospholipids did not differ between genotypes. In iPLA$_2\beta^{-/-}$ mice, brain levels of iPLA$_2\beta$ mRNA, protein, and activity were decreased, as was the iPLA$_2\gamma$ (Group VIB PLA_2) mRNA level, while levels of secretory sPLA$_2$-V mRNA, protein, and activity and cytosolic cPLA$_2$-IVA mRNA were increased. Levels of COX-1 protein were decreased in brain, while COX-2 protein and mRNA were increased. Levels of 5-, 12-, and 15-LOX proteins did not differ significantly between genotypes. Thus, a genetic iPLA$_2\beta$ deficiency in mice is associated with reduced DHA metabolism, profound changes in lipid-metabolizing enzyme expression (demonstrating lack of redundancy) and of phospholipid fatty acid content of brain (particularly of DHA), which may be relevant to neurologic abnormalities in humans with *PLA2G6* mutations.

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1. Introduction

In vitro studies have demonstrated that the group VI Ca^{2+}-independent phospholipases A_2 (iPLA$_2$, EC 3.1.1.4) hydrolyze docosahexaenoic acid (DHA) from the stereospecifically numbered (*sn*)-2 position of phospholipids [1,2]. This is consistent with reduced brain DHA metabolism in unanesthetized iPLA$_2\beta$-knockout mice [3]. Of known iPLA$_2$ isoforms, iPLA$_2\beta$ is designated *PARK14*, *PNPLA9*, *PLA2G6* or iPLA$_2$-VIA, and iPLA$_2\gamma$ is designated PNPLA8 or iPLA$_2$-VIB. Both isoforms are found post-synaptically in brain [4] and in the cytosol of resting cells [4–7], and can be activated and undergo membrane association by stimuli that induce release of Ca^{2+} from intracellular stores, *e.g.*, muscarinic or serotonergic G-protein-coupled neuroreceptor signaling [3,8–11]. iPLA$_2\beta$, and to a lesser extent iPLA$_2\gamma$, also can hydrolyze arachidonic acid (AA, 20:4n−6) from phospholipids [12–15].

Humans with *PLA2G6* mutations may show progressive regression of cognitive and motor skills, as manifested in the disorders infantile neuroaxonal dystrophy, idiopathic neurodegeneration with brain iron accumulation, dystonia-parkinsonism, and cerebellar cortical atrophy with gliosis [16–19]. In mice, mutations in iPLA$_2\gamma$ or iPLA$_2\beta$ genes cause cognitive deficits and motor abnormalities over time [14,20,21]. iPLA$_2\beta$ knockout mice display neuropathology characterized by swollen axons and vacuoles [20,21], protein misfolding and aggregation [21], and reduced mitochondrial function [14,22] by age 13 months. Other studies have demonstrated a role for iPLA$_2\beta$ in maintaining axonal membrane stability [20] and in regulating fatty acid composition of pancreatic islet β-cell phospholipids [23].

In view of the involvement of iPLA$_2\beta$ in DHA hydrolysis from phospholipids [1,2] and the reduced plasma DHA incorporation and

Abbreviations: AA, arachidonic acid; ChoGpl, choline glycerophospholipid; COX, cyclooxygenase; cPLA$_2$, cytosolic phospholipase A_2 (Group IVA PLA_2); DHA, docosahexaenoic acid; DHA-CoA, docosahexaenoyl-CoA; EtnGpl, ethanolamine glycerophospholipid; FAME, fatty acid methyl ester; GC, gas chromatography; iPLA$_2$, Ca^{2+}-independent phospholipase A_2 (Group VIA PLA_2); LOX, lipoxygenase; PUFA, polyunsaturated fatty acid; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; sPLA$_2$, secretory phospholipase A_2; *sn*, stereospecifically numbered; TLC, thin layer chromatography

* Corresponding author at: Brain Physiology and Metabolism Section, National Institute on Aging, National Institutes of Health, 9000 Rockville Pike, Bldg. 9, 1S126, Bethesda, MD 20892, USA. Tel.: +1 301 496 3567; fax: +1 301 402 0074.
E-mail address: cheony@mail.nih.gov (Y. Cheon).

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signaling in brains of iPLA$_2$β knockout mice [3], it is possible that neuropathology and altered behavior that arise from mutations or deficiencies in iPLA$_2$β are related to disturbed brain DHA metabolism. DHA has been reported to modulate gene transcription and membrane fluidity, to act as a signaling molecule during neurotransmission, to serve as a precursor of antiinflammatory resolvins and neuroprotectins, to influence rodent behavior, to act as an antioxidant, and to alter ion channel activities [1,3,20,24–31].

To further characterize brain DHA metabolism in mice with genetic deficiency of iPLA$_2$β, here we used our *in vivo* kinetic infusion model [32–35] to quantify DHA incorporation and turnover in brain phospholipids and to determine fatty acid concentrations of brain phospholipids and lysophospholipids of iPLA$_2\beta^{-/-}$ and wild type iPLA$_2\beta^{+/+}$ mice at age 4–5 months. We also examined brain expression of enzymes involved in polyunsaturated fatty acid (PUFA) metabolism, including iPLA$_2$β, iPLA$_2$γ, cytosolic cPLA$_2$ (Group IVA PLA$_2$), secretory sPLA$_2$ (Group V PLA$_2$), cyclooxygenase (COX)-1, COX-2, 5-lipoxygenase (LOX), 12-LOX and 15-LOX. Widespread neuropathologic changes develop by age 13 months in iPLA$_2\beta^{-/-}$ mice, so we chose to study younger mice in order to reduce the impact that such neuropathologic abnormalities might have on brain PUFA metabolism, but even at age 4 months, iPLA$_2\beta^{-/-}$ mice exhibit tubulovesicular membranes and small vacuoles with edema in brain [14,20–22].

2. Methods and materials

2.1. Animals

The study was conducted following the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (Publication no. 86-23) and was approved by the Animal Care and Use Committee of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development. Male iPLA$_2\beta^{-/-}$ mice and their littermate iPLA$_2\beta^{+/+}$ controls, derived from a C57BL/6J genetic background [36], were maintained in an animal facility where temperature, humidity, and light cycle were regulated, with free access to water and a diet (Rodent NIH-07) that contained (as percent of total fatty acid concentration), 30.6% saturated, 22.5% monounsaturated, 47.1% linoleic, 4.9% α-linolenic (α-LNA), 0.2% AA, 1.6% eicosapentaenoic (EPA), and 2.2% DHA [3]. Five mice of each genotype underwent surgical procedures, tracer infusion, and microwave fixation for determining brain DHA turnover and concentration. Six mice of each genotype were asphyxiated by CO_2 inhalation and decapitated, and the brains were excised and rapidly frozen in 2-methylbutane with dry ice (at −50 °C) and stored at −80 °C for subsequent analyses.

2.2. Surgical procedures and tracer infusion

At age 4–5 months, mice were anesthetized with 1–3% halothane, and polyethylene catheters were inserted into the femoral artery and vein [33]. Recovery from anesthesia was allowed to occur (3 h, 25 °C) with animal hindquarters loosely wrapped and taped to a wooden block. During recovery, body temperature was maintained at 37 °C with a rectal probe and a heating element (Indicating Temperature Controller; Yellow Springs Instrument, Yellow Springs, OH, USA). After recovery, unanesthetized mice were infused (5 min) intravenously with HEPES buffer (130 μl, pH 7.4) containing fatty acid-free bovine serum albumin (50 mg/ml, Sigma, St. Louis, MO, USA) and [1-^{14}C]DHA (5 μCi, 53 mCi/mmol, 90% pure, Moravek Biochemicals, Brea, CA, USA) at a rate of 0.0223 $(1+e^{-0.032t})$ ml/min, (t = sec), using a computer-controlled infusion pump (No. 22; Harvard Apparatus, South Natick, MA, USA) to achieve steady-state plasma specific activity within 1 min [37]. During infusion, timed arterial blood samples (15 μl) were collected in polyethylene-heparin lithium fluoride-coated Beckman centrifuge tubes at various intervals (0, 0.25, 0.5, 1.0, 1.5, 3.0, and 4.0 min) and a final collection (150 μl) was performed at 4.9 min. Plasma was separated by centrifugation (13,000 rpm, 1 min) and radioactivity determined by liquid scintillation counting. Unlabeled DHA concentrations of the final (4.9 min) sample were measured by gas chromatography (GC). At 5 min, animals were anesthetized (sodium pentobarbital, 50 mg/kg, i.v.) and subjected to head-focused microwave irradiation (5.5 kW, 0.9 s, 75% power output; Cober Electronics, Norwalk, CT, USA) to stop brain lipid metabolism [38,39]. Brains were excised, dissected sagittally, and stored (−80 °C).

2.3. Plasma and brain lipid extraction and separation

Total lipids were extracted from plasma (50 μl) after adding heptadecanoic acid (17:0) as an internal standard, and from one cerebral hemisphere (~0.2 g) as reported [40]. Lipid extracts were separated by thin layer chromatography (TLC) on Silica Gel 60A plates (Whatman, Clifton, NJ, USA) [41]. Neutral lipid subclasses including unesterified fatty acids were separated using a mixture of heptane/diethylether/glacial acetic acid (60/40/3 v/v/v), alongside authentic standard phospholipids, cholesterol, free fatty acids, triacylglycerols, and cholesteryl esters standards to identify the bands. Phospholipid classes (EtnGpl, ethanolamine glycerophospholipid; ChoGpl, choline glycerophospholipid; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine) were separated in chloroform/methanol/H_2O/glacial acetic acid (60/50/4/1 v/v/v) and identified by comparison with standards in separate lanes. Lysophospholipids were analyzed in chloroform/methanol/acetic acid/acetone/water (35/25/4/14/2 v/v/v/v/v). This method achieves separation of lysophosphatidylcholine (lysoPC), and of co-migrating lysophosphatidylinositol (lysoPI) and lysophosphatidylethanolamine (lysoPE). Plates were sprayed with 0.03% (w/v) 6-*p*-toluidine-2-naphthalene sulfonic acid (Acros, Fairlawn, NJ, USA) in 50 mM Tris–HCl buffer (pH 7.4), and the lipid bands were visualized with UV light. Each band was scraped from the plate, and the silica gel containing the target analyte was used to quantify radioactivity of phospholipid classes by liquid scintillation counting, to prepare fatty acid methyl esters (FAMEs) by transmethylation of neutral lipids, phospholipids, and lysophospholipids (see below), to quantify plasmalogen concentrations, and to measure phospholipid and lysophospholipid phosphorous concentrations.

2.4. FAME preparation and GC analysis

After adding appropriate quantities of internal standard (17:0/17:0-PC), FAMEs were formed from brain lipids and plasma esterified lipids in silica gel scraped from TLC plates by acid methanolysis (1% H_2SO_4 in methanol, 70 °C, 3 h). FAMEs were then analyzed by GC (SP™-2330 fused silica capillary column, 30 m × 0.25 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA, USA) and detected by flame ionization (Model 6890N detector; Agilent Technologies, Palo Alto, CA, USA). Initial column temperature was 80 °C, followed by a gradient (10 °C/min) to 150 °C and then a gradient (6 °C/min) to 200 °C, where temperature was held for 10 min, and then increased to 240 °C (38 min total run time). Peaks were identified by comparison to the retention times of FAME standards (Nu-Chek-Prep, Elysian, MN, USA). Fatty acid concentrations (nmol/μmol brain total phosphorous or nmol/ml plasma) were calculated by proportional comparison of GC peak areas to that of the 17:0 internal standard.

2.5. Quantification of radioactivity

Samples were placed in scintillation vials and dissolved in liquid scintillation cocktail (ReadySafe™ plus 1% glacial acetic acid), and