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A novel lysophosphatidic acid acyltransferase enzyme (LPAAT4) with a possible role for incorporating docosahexaenoic acid into brain glycerophospholipids ☆,☆☆

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ABSTRACT

Glycerophospholipids are important components of cellular membranes, required for constructing structural barriers, and for providing precursors of bioactive lipid mediators. Lysophosphatidic acid acyltransferases (LPAATs) are enzymes known to function in the *de novo* glycerophospholipid biosynthetic pathway (Kennedy pathway), using lysophosphatidic acid (LPA) and acyl-CoA to form phosphatidic acid (PA). Until now, three LPAATs (LPAAT1, 2, and 3) have been reported from the 1-acyl-glycerol-3-phosphate *O*-acyltransferase (AGPAT) family. In this study, we identified a fourth LPAAT enzyme, LPAAT4, previously known as an uncharacterized enzyme AGPAT4 (LPAATδ), from the AGPAT family. Although LPAAT4 was known to contain AGPAT motifs essential for acyltransferase activities, detailed biochemical properties were unknown. Here, we found that mouse LPAAT4 (mLPAAT4) possesses LPAAT activity with high acyl-CoA specificity for polyunsaturated fatty acyl-CoA, especially docosahexaenoyl-CoA (22:6-CoA, DHA-CoA). mLPAAT4 was distributed in many tissues, with relatively high expression in the brain, rich in docosahexaenoic acid (DHA, 22:6). mLPAAT4 siRNA in a neuronal cell line, Neuro 2A, caused a decrease in LPAAT activity with 22:6-CoA, suggesting that mLPAAT4 functions endogenously. siRNA in Neuro 2A cells caused a decrease in 18:0–22:6 PC, whereas mLPAAT4 overexpression in Chinese hamster ovary (CHO)-K1 cells caused an increase in this species. Although DHA is considered to have many important functions for the brain, the mechanism of its incorporation into glycerophospholipids is unknown. LPAAT4 might have a significant role for maintaining DHA in neural membranes. Identification of LPAAT4 will possibly contribute to understanding the regulation and the biological roles of DHA-containing glycerophospholipids in the brain.

1. Introduction

Cellular membranes are comprised of glycerophospholipids, which have many important structural and functional roles for cells, for example maintaining cellular barriers and acting as precursors of lipid signaling molecules [1,2]. Tissues contain distinct contents of glycerophospholipids, such as phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) and cardiolipin (CL), each with different composition of fatty acids [1,3].

Glycerophospholipids are first formed from glycerol-3-phosphate (G3P) through the *de novo* pathway (Kennedy pathway) [4]. G3P is converted to lysoPA (LPA) by glycerol-3-phosphate acyltransferases (GPATs), and are subsequently transformed to PA by LPA acyltransferases (LPAATs) [5]. Once different classes of glycerophospholipids are synthesized from the common intermediate

Abbreviations: PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; LPA, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; AGPAT, 1-acyl-glycerol-3-phosphate *O*-acyltransferase; DHA-CoA, docosahexaenoyl-CoA; DHA, docosahexaenoic acid.

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PA, glycerophospholipids are reconstituted through the remodeling pathway (Lands' cycle) [6]. In the Lands' cycle, glycerophospholipids are degraded to lysophospholipids by phospholipase A_2s (PLA_2s), and are formed back to glycerophospholipids by lysophospholipid acyltransferases (LPLATs) [2,3,7]. Mammalian LPAATs were identified from the 1-acyl-glycerol-3-phosphate *O*-acyltransferase (AGPAT) family, and LPLATs of the remodeling pathway were found from both the AGPAT and the membrane bound *O*-acyltransferase (MBOAT) family [2,7].

The AGPAT family members possess highly conserved motifs [5], and many have been already characterized. LPAAT1, LPAAT2 and LPAAT3 function as LPAATs in the Kennedy pathway [5,8,9]. Other members are reported to function as GPATs, or as LPLATs of the Lands' cycle [5,7].

AGPAT4 (LPAATδ) is a member of the AGPAT family, and has highly homology with LPAAT3 (60%). Although structural and tissue distribution of AGPAT4 is studied in the past [10], little biochemical information has been reported. We newly found that AGPAT4 functions as an LPAAT with high selectivity for polyunsaturated fatty acyl-CoA, especially docosahexaenoyl-CoA (DHA-CoA, 22:6-CoA). From biochemical characteristics identified in this manuscript, we will rename this enzyme as LPAAT4 according to the proposed LPLAT nomenclature [7]. Mouse LPAAT4 (mLPAAT4) was expressed in many tissues, with highest mRNA expression in the brain, particularly rich in docosahexaenoic acid (DHA, 22:6)-containing glycerophospholipids [11]. Although DHA is known to be important for brain functions [11], the mechanism of DHA incorporation into cellular membrane glycerophospholipids is still unknown. LPAAT4 might have an important role for maintaining adequate DHA levels of brain glycerophospholipids.

2. Materials and methods

Please see details in the Supplementary text.

2.1. Materials

Fetal bovine serum (FBS) was purchased from Life Technologies (Carlsbad, CA). Ham's F-12 nutrient mixture, minimal essential medium (MEM) and geneticin (G418 Disulfate Aqueous Solution) were obtained from Nacalai Tesque (Kyoto, Japan). 14:0/14:0 PC was purchased from NOF corporation (Tokyo, Japan). G3P and DHA were purchased from Sigma (St. Louis, MO). All other glycerophospholipids, lysophospholipids and acyl-CoAs were purchased from Avanti Polar Lipids (Alabaster, AL). Methanol, chloroform, acetonitrile and ammonium bicarbonate were purchased from Wako (Osaka, Japan).

2.2. Plasmids and vectors

mLPAAT4 (NCBI accession number NM_026644, registered as AGPAT4) cDNA was amplified by PCR with forward 5′-CTAGCTAGCCACCATGGATTACAAGGATGACGATGACAAGGACCTCATCGGGCTGCTGAAGTCCC-3′, and reverse 3′-CCGCTCGAGTCAGTCCGTTTGTTTCCGTTTGTTGTCG-5′ primers using mouse brain as a template. mLPAAT4 H96A with a mutation in AGPAT motif I (NHX_4D motif, histidine was converted to alanine) was constructed by overlap extension PCR with forward 5′-GGTCCTCAATGCCAAGTTTG-3′, and reverse 5′-CAAACTTGGCATTGAGGACC-3′ primers. Mouse GPAT1 (mGPAT1) (NCBI accession number NM_0008149) cDNA was amplified by PCR with forward 5′-CTAGCTAGCCACCATGGATTACAAGGATGACGATGACAAGGAGGAGTCTTCAGTGACAGTTGGC-3′, and reverse 3′-CCGCTTAAGCTACAGCACCACAAAACTC-5′ primers using mouse liver as a template. FLAG epitopes (DYKDDDDK) were attached to the N-terminus. PCR products were ligated into the pCXN2.1 vector [12] and sequenced.

2.3. Transfection and siRNA

Chinese hamster ovary (CHO)-K1 cells (provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan) were transfected with cDNAs using Lipofectamine 2000 (Life Technologies). Microsomal proteins were extracted 48 h post-transfection. For stable overexpression, transfected CHO-K1 cells were selected with 3 mg/ml geneticin for four days, and were maintained in 0.3 mg/ml geneticin. siRNAs of mLPAAT4 (ON-TARGETplus SMARTpoolsiRNA, L-051038-01) and a negative control (D-001810-10) were obtained from Dharmacon. 5 nM siRNA was transfected using Lipofectamine RNAiMAX reagent (Life Technologies) into Neuro 2A cells (ATCC, Manassas, VA).

2.4. LPLAT and GPAT activity measurement

0.5 μg microsomal protein was incubated for 10 min at 37 °C with reaction mixtures containing lysophospholipid and acyl-CoAs (LPLAT activities) or G3P and acyl-CoAs (GPAT activities). Lipids were extracted by Bligh and Dyer method, and were measured using liquid chromatography–mass spectrometry (LC–MS).

2.5. Glycerophospholipid composition analysis

CHO-K1 cells were transfected with pCXN2.1 vector, mLPAAT4 cDNA or mLPAAT4 H96A cDNA, and Neuro 2A cells were transfected with control or mLPAAT4 siRNA. For both cell types, cells were treated with 50 μM DHA in 2% FBS medium 48 h post-transfection, and lipids were extracted at various time points by directly adding methanol to the dishes. Fatty acid composition of PC, PE and PS were analyzed using LC–MS.

2.6. Reversed phase LC–MS

Glycerophospholipids were separated with Acquity Ultraperformance LC (UPLC) system (Waters, Milford, MA), and were detected by TSQ Vantage Triple Stage Quadrupole Mass Spectrometer (Thermo Scientific, Waltham, MA) with a HESI-II electrospray ionization source.

2.7. Quantitative PCR analysis

Quantitative PCRs (LightCycler System; Roche Applied Science, Mannheim, Germany) were performed using FastStart DNA Master SYBR Green I (Roche Applied Science). mRNA levels were normalized by 36B4, a housekeeping gene. Primers used were: mLPAAT4 forward, CAAGATCAATGCCAGACTCTGCT; mLPAAT4 reverse, AAACTTGTGATTGAGGACCACGA; 36B4 forward, CTGAGATTCGGGATATGCTGTTG; and 36B4 reverse, AAAGCCTGGAAGAAGGAGGTCTT.

2.8. Confocal microscopy

CHO-K1 cells stably overexpressing FLAG-tagged mLPAAT4 were used to study subcellular localization. mLPAAT4 was detected using anti-FLAG M2 antibody. ER, mitochondria and Golgi were visualized by anti-PDI antibody (Cell Signaling Technology, Danvers, MA), MitoTracker Red CMXRos (Life Technologies) and anti-GM130 antibody (Abcam, Cambridge, UK), respectively. Confocal microscopy was performed with LSM510 Laser Scanning Microscope (Carl Zeiss, Germany).