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Purification and characterization of lysophospholipase D from rat brain

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Abstract

A lysophospholipase D (lysoPLD) was purified to apparent homogeneity from rat brain nuclear fractions using 1-[^{14}C]palmitoyl-glycerophosphorylcholine as a substrate. The abundance of autotaxin (ATX), a secretory lysoPLD, was also estimated for each fraction. The nuclear fraction had relatively high levels of lysoPLD activity but weak immunoreactivity with an anti-ATX antibody. LysoPLD activity was further purified 5550-fold by sequential chromatography. The final preparation migrated as a single band with a molecular weight of 35,000. Anti-ATX antibodies did not cross-react with the purified enzyme. Moreover, enzyme activity was highest at pH 7.0–7.5 and requires Mg^{2+}. The *K*m and *V*max values for 1-palmitoyl-glycerophosphorylcholine were 176 μM and 0.3 μmol/min/mg, respectively. The purified enzyme hydrolyzed saturated forms of LPC more robustly than unsaturated forms. The enzyme could hydrolyze platelet-activating factor (PAF) to the same extent as 16:0-LPC, and showed a higher activity toward lysoPAF (1-*O*-hexadecyl-2-lyso-glycerophosphorylcholine). These results suggested that the lysoPLD purified from rat brain nuclear fractions in this work is a novel enzyme that hydrolyzes lysoPAF, PAF, and LPC to liberate choline.

Keywords: Lysophospholipase D; Lysophosphatidylcholine; Lysophosphatidic acid; Autotaxin; Rat brain; Lyso-platelet activating factor

1. Introduction

Phosphatidylcholine (PC) is a major phospholipid in the cell membrane and a good substrate for phospholipase A_2 (PLA_2), which is activated when cells are stimulated by hormones and cell proliferating factors [1]. When PC is hydrolyzed by PLA_2, equimolar amounts of free fatty acid and lysophosphatidylcholine (LPC) are produced. The fatty acid at the sn-2 position of PC is primarily composed of unsaturated fatty acids such as arachidonic acid, and the arachidonic acid released by PLA_2 is converted to eicosanoids. The metabolism of arachidonic acid is well characterized [1]; however, the metabolism of LPC has not been fully elucidated. This is at least in part because accumulation of LPC in cells induces cell lysis [2,3]. Hydrolysis of LPC by lysophospholipases was once thought to produce a non-bioactive saturated free fatty acid and glycerophosphorylcholine (GPC). However, LPC itself was recently recognized as a bioactive phospholipid that can induce chemotaxis [4] and enhance kinase activity during signal transduction [5]. With regard to human health, abnormal levels of LPC can induce atherosclerosis [6] and heart arrhythmia [7]. We previously purified and cloned three isoforms of lysophospholipase from rat liver that can hydrolyze fatty acids at the sn-1 position of LPC [8–10]. Besides these lysophospholipases, lysophospholipase C (lysoPLC) hydrolyzes LPC to produce monoacylglycerol and phosphocholine, and lysophospholipase D (lysoPLD) hydrolyzes LPC to produce lysophosphatidic acid (LPA) and choline. LysoPLC was recently cloned from

Abbreviations: ATX, autotaxin; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid; DTT, Dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EDG, endothelial differentiation genes; GPC, glycerophosphorylcholine; GP, glycerophosphate; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; lysoPLC, lysophospholipase C; lysoPLD, lysophospholipase D; lysoPAF, 1-*O*-hexadecyl-2-lyso-GPC (lyso-platelet activating factor); PA, phosphatidic acid; PC, phosphatidylcholine; PLA, phospholipase A; PLD, phospholipase D; PAF, 1-*O*-hexadecyl-2-acetyl-GPC (platelet activating factor); PMSF, phenylmethylsulfonyl fluoride; SPC, sphingosylphosphorylcholine; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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human and mouse kidney [11], based on its similarity to secretory lysoPLD [12,13], a class of enzymes well known to be important for cell biological activities.

LPA, which is a bioactive lipid produced from PC via sequential enzymatic reactions or directly from LPC, induces cell proliferation, differentiation and migration [14,15]. LPA binds to specific G-protein-coupled receptors in the endothelial differentiation gene (EDG) family and then activates multiple signal transduction events [16]. Hecht et al. studied the LPA receptor encoded by the ventricular zone gene-1 gene (vzg-1) in neurogenic regions of the developing cerebral cortex [17] and found that the receptor can physically interact with G proteins and mediates multiple cellular responses to LPA [18]. Thus, the pathways for the production of LPA have been extensively studied. Production of PA from PC via phospholipase D (PLD) is important because PA is hydrolyzed by PLA_1 [19]. Although production of PA from PC via PLD in serum has not been reported, it has been clearly shown that LPA is produced directly from LPC in human and bovine sera via a one step enzymatic reaction performed by secretory lysoPLD [12,13]. This enzyme also contains phosphodiesterase activity and is also known as the tumor cell motility-stimulating factor autotaxin (ATX). However, how LPA is produced from LPC in cells and tissues remains to be fully elucidated.

More than 30 years ago, Wykle and Schremmer [20] reported that LPA is produced from LPC via the hydrolyzing activity of lysoPLD. The enzyme activity was found in microsomal fractions prepared from rat brain in the presence of Mg^{2+} and NaF. The same enzymatic activities were detected in microsomal fractions prepared from several other tissues [21–26]. However, the molecular identity of lysoPLD activities in various cells and tissues is not yet known. In this work, we purified lysoPLD to apparent homogeneity from a nuclear rat brain fraction and characterized the enzymatic properties of purified lysoPLD.

2. Materials and methods

2.1. Materials

1-[^{14}C]palmitoyl-GPC (56.0 mCi/mmol) was purchased from Amersham Biosciences Corp. (NJ, USA). LPC (1-myristoyl, 14:0; palmitoyl, 16:0; stearoyl, 18:0; and oleoyl, 18:1), 1-*O*-hexadecyl-2-acetyl-GPC (PAF), 1-*O*-hexadecyl-2-lyso-GPC (lysoPAF), sphingosylphosphorylcholine (SPC) and GPC were obtained from Sigma (MO, USA). LPC (linoleoyl, 18:2) and 1-palmitoyl-2-lyso-glycerophosphate (1-palmitoyl-GP) were obtained from DOOSAN Serdary Research Lab. (Kyungki-Do, Korea) and Avanti Polar Lipids, Inc. (AL, USA), respectively. DEAE Cellulofine A-500 was purchased from Seikagaku Corp. (Tokyo, Japan). Octyl Sepharose 4 Fast Flow, Superdex 200 10/300 GL, HiTrap DEAE FF and Blue Sepharose CL-6B, and POROS HQ were obtained from Amersham Biosciences Corp. and Applied Biosystems (CA, USA), respectively. 3-[(3-Cholamidopropyl) dimethylammonio]propanesulfonic acid (CHAPS), heptyl-thio-glucoside, ethylenediaminetetraacetic acid (EDTA) and 3-(4-hydroxyphenyl)-propionic acid were purchased from Dojindo (Kumamoto, Japan). Dithiothreitol (DTT), peroxidase, choline oxidase and sodium ortho-vanadate (Na_3VO_4) were purchased from Wako (Tokyo, Japan). Silica Gel 60 plates were purchased from Merck (Darmstadt, Germany). All Wister rats (6 weeks old, 170–180 g, male) were obtained from CLEA Japan, Inc. (Tokyo, Japan). A Bio-Rad protein assay kit (Bio-Rad, CA, USA) was used to determine protein concentration using bovine serum albumin (Sigma) as a standard.

2.2. Enzyme preparations

Rats were anesthetized deeply with diethyl ether, decapitated, and the brains, livers, kidneys, lungs and muscles were removed. All steps for enzyme preparations were carried out at 4 °C. The brains, livers, kidneys, lungs and muscles were minced and homogenized with a Teflon homogenizer in nine volumes of buffer A (0.3 M or 0.25 M sucrose in 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)). Nuclear brain fractions were obtained by centrifugation at 600×*g* for 10 min. The nuclear-rich pellets were resuspended in nine volumes of buffer A. To dissolve lysoPLD in nuclear pellets, 10 mM CHAPS was added to the suspension and sonicated at power level 10 for 15 s with a Microson Ultrasonic cell disruptor (Misonix, NY, USA). The suspensions were then centrifuged at 10,000×*g* for 10 min and the supernatants were used as a source of enzyme.

The mitochondrial fractions were obtained from the supernatants after centrifugation at 600×*g* for 10 min. The supernatants were centrifuged at 8000×*g* for 10 min and the supernatant (S1) and the upper fluffy layer of the pellet were removed. The hard pellets were resuspended in nine volumes of buffer A and used as mitochondrial fractions. The S1 was centrifuged at 105,000×*g* for 60 min. The supernatants were used as the cytosolic fraction and the pellets were resuspended in nine volumes of buffer A and used as a microsomal fraction. To obtain pure nuclei, brains were minced and homogenized in 20 ml of buffer A2 (2.2 M sucrose in 50 mM Tris–HCl, pH 7.4,1 mM EDTA, 1 mM DTT, 0.1 mM PMSF) per g wet weight with a Teflon homogenizer, and homogenates were centrifuged at 40,000×*g* for 60 min. The supernatants were carefully removed and then, the pellets were resuspended in nine volumes of buffer A (pure nuclear fractions).

2.3. Purification procedures of lysoPLD from rat brain

All steps in enzyme purification were carried out at 4 °C. The supernatants resulting from the above-described procedures were applied to a DEAE Cellulofine A-500 column (3×14 cm) equilibrated with buffer B (20 mM Tris–HCl, pH 7.5, 0.1 mM PMSF, 5 mM CHAPS). The enzyme was eluted with a 420 ml linear gradient of NaCl (0–0.6 M) in buffer B. Active fractions were collected and then loaded onto an Octyl Sepharose 4 Fast Flow column (1.8×10 cm) equilibrated with buffer B containing 0.1 M NaCl. The active fractions were obtained with a linear gradient of 54 ml from 0.1 M NaCl, 5 mM CHAPS to 40 mM CHAPS without NaCl in buffer B, then combined and divided into two equal portions. The enzyme activity was stable in −80 °C at this stage for several months. Each portion was separately treated as follows. The divided fraction was diluted with four volumes of distilled water and then loaded onto a Blue Sepharose CL-6B column (1.2×4 cm) equilibrated with Buffer B. The flow-through fractions were used in the subsequent purification step after the addition of 30 mM heptyl-thio-glucoside. The fractions were loaded onto a POROS HQ column (0.46×10 cm) equilibrated with 30 mM heptyl-thio-glucoside with buffer B, and the enzyme fractions were eluted with an NaCl gradient (0–2 M) for 30 min in 20 mM Tris–HCl (pH 7.5) at a flow rate of 0.5 ml/min using a Waters 650 system (Waters Corp., MA, USA). The active fractions were combined to Microcon centrifugal filter devices (Millipore, MA, USA) to concentrate the samples prior to the next gel-filtration procedures. The concentrated enzyme fraction was applied to a Superdex 200 10/300 GL column (1×30 cm) equilibrated with buffer B using a Waters 650. The obtained fraction was applied to a HiTrap DEAE FF (0.7×2.5 cm) equilibrated with buffer B, and then eluted with an NaCl gradient (0–0.6 M) for 15 min in buffer B at a flow rate of 1 ml/min using a Waters 650.

2.4. Isotopic assay for lysoPLD activity

1-[^{14}C]palmitoyl-GPC was mixed with cold 1-palmitoyl-GPC, suspended in distilled water to 5 mM by sonication for 30 s with a Microson Ultrasonic cell disruptor, and used as a substrate. Reaction mixtures containing 0.15 mM 1-palmitoyl-GPC (6000 dpm/nmol), 20 mM Tris–HCl (pH 7.0), 1 mM