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Biosynthesis of alkyl lysophosphatidic acid by diacylglycerol kinases

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ABSTRACT

Lysophosphatidic acid (LPA) designates a family of bioactive phosphoglycerides that differ in the length and degree of saturation of their radyl chain. Additional diversity is provided by the linkage of the radyl chain to glycerol: acyl, alkyl, or alk-1-enyl. Acyl-LPAs are the predominate species in tissues and biological fluids. Alkyl-LPAs exhibit distinct pharmacodynamics at LPA receptors, potently drive platelet aggregation, and contribute to ovarian cancer aggressiveness. Multiple biosynthetic pathways exist for alkyl-LPA production. Herein we report that diacylglycerol kinases (DGKs) contribute to cell-associated alkyl-LPA production involving phosphorylation of 1-alkyl-2-acetyl glycerol and document the biosynthesis of alkyl-LPA by DGKs in SKOV-3 ovarian cancer cells, specifically identifying the contribution of DGKα. Concurrently, we discovered that treating SKOV-3 ovarian cancer cell with a sphingosine analog stimulates conversion of exogenous 1-alkyl-2-acetyl glycerol to alkyl-LPA, indicating that DGKα contributes significantly to the production of alkyl-LPA in SKOV-3 cells and identifying cross-talk between the sphingolipid and glycerol lipid pathways.

1. Introduction

Lysophosphatidic acid (LPA) designates a family of phosphoglycerides with a phosphate group at the *sn-3* position and a radyl chain at the *sn-1* or *sn-2* position. The aliphatic chain varies in length from 16 to 24 carbons, contains up to six double bonds, and connects to the glycerol backbone via an ester (acyl), an ether (alkyl), or a vinyl ether (alk-1-enyl) linkage.

Acyl-LPA is the most abundant form in plasma, but alkyl-LPA has clinically relevant biological activities. Alkyl-LPA in ovarian cancer ascitic fluid stimulates migration and proliferation of ovarian cancer cells, causing metastasis and overall progression of the disease [1]. Additionally, alkyl-LPA stimulates platelet aggregation: the only documented biological effect where alkyl-LPA is more potent than acyl-LPA [2,3] and a central event in the development of thrombosis [5]. The increased potency in platelet aggregation is because alkyl-LPA binds LPA5 more potently than acyl-LPA [4]. Thus, elucidating the mechanisms of alkyl-LPA synthesis is relevant to our understanding of the pathogenesis of ovarian cancer and thrombosis, ultimately leading to new treatment options for these and related pathologies.

Extracellular LPA is present at about 0.5 μM in plasma [6] and formed by the hydrolysis of lysophosphatidylcholine (LPC) by a plasma lysophospholipase D, autotaxin (ATX). Alkyl lysophospholipids are substrates for ATX, but alkyl-LPA has not been reported in human plasma. Preliminary studies using the LC/MS methods described here indicate that alkyl-LPAs are detected at low levels (less than 5% of total LPA) in human plasma but are not detected in plasma of mice fed normal chow (A.J.M, M.S., and Susan Smyth, unpublished). Whether a diet high in alkyl phosphatidylcholines (PC) generates plasma alkyl-LPC, and thence alkyl-LPA, remains to be tested, and the physiological contribution of ATX to alkyl-LPA generation is presently unknown.

Routes to intracellular alkyl-LPA have been suggested but are not well characterized. The alkylglycerol lipid 1-*O*-hexadecyl-*sn*-2-acetyl glycerol (2-AcMAGE) is de-acetylated by the serine hydrolase KIAA1363, and the resulting 1-*O*-hexadecyl-*sn*-glycerol (MAGE) is phosphorylated by an unknown lipid kinase to yield alkyl-LPA [7]. Furthermore, the variously named multi-substrate lipid kinase (MuLK) or acylglycerol kinase (AGK) phosphorylates monoacylglycerol (MAG) and diacylglycerol (DAG) to produce acyl-LPA and phosphatidic acid (PA), respectively [8,9]. While MuLK/AGK is a candidate for producing alkyl-LPA, the protein

Abbreviations: LPA, lysophosphatidic acid; ATX, autotaxin; 2-AcMAGE, 2-acetyl monoalkylglycerol ether, 1-*O*-hexadecyl-*sn*-2-acetyl glycerol; MAGE, monoalkylglycerol ether, 1-*O*-hexadecyl-*sn*-glycerol; MAG, monoacylglycerol; AGK/MuLK, acylglycerolkinase/multiple lipid substrate kinase; DAG, diacylglycerol; DGK, diacylglycerol kinase; PA, phosphatidic acid; PLA_2, phospholipase A_2; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; OTAA, octyl tetralin-based amino alcohol, (−)-(*S*)-2-amino-2′-((*S*)-6-octyl-1,2,3,4-tetrahydronaphthalen-2-yl) propan-1-ol.

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has not been shown to phosphorylate MAGE to form alkyl-LPA [8,9]. Finally, diacylglycerol kinases (DGKs) produce LPA through PA, which is deacylated by a phospholipase A_1 (PLA_1) or phospholipase A_2 (PLA_2) [10]. However, 10 mammalian DGK isoforms (α, β, γ, δ, η, κ, ε, ζ, ι, and θ) [11] exist, and it has not been previously reported whether a particular DGK isoform significantly contributes to intracellular alkyl-LPA production.

We sought to identify a specific mammalian DGK that phosphorylates an alkylglycerol lipid, thereby contributing to the production of cell-associated alkyl-LPA. We observed that DGK activity contributed to the production of alkyl-LPA in SKOV-3 cells in response to exogenous 2-AcMAGE, but not MAGE. Additionally, we discovered that alkyl-LPA levels increased significantly in the presence of alkyl amino alcohols such as sphingosine. Thus our results suggest a pathway whereby Type 1 DGKs generate cell-associated alkyl-LPA.

2. Materials and methods

2.1. Materials

Lipids were from Cayman Chemical Company (Ann Arbor, MI) or Avanti Polar Lipids (Alabaster, AL): 1-*O*-hexadecyl-*sn*-2-acetyl glycerol (2-AcMAGE); 1-*O*-hexadecyl-*sn*-glycerol (MAGE); 1,2-dioleoyl-*sn*-glycerol (C18:0 DAG); sphingosine; and FTY-720.

DNAs encoding (1) DGK α, β, and γ in pcDNA3-FLAG were from Kaoru Goto, (Yamagata University School of Medicine); (2) DGK δ1 and δ2 splice variants in p3x-FLAG-CMV-7.1 were from Fumio Sakane, (Chiba University); (3) DGK ζ and ι in pCMV-HA were from Matthew Topham (University of Utah); and (4) DGKθ in pCMV-SPORT6 was from Thermo Fisher Scientific Open Biosystems.

2.2. Cell culture

SKOV-3 human ovarian cancer cells were from Jill Slack–Davis (University of Virginia) and maintained in an atmosphere of 5% CO_2/95% air in McCoy's 5A medium (Gibco) supplemented with 10% (v/v) charcoal–dextran stripped fetal bovine serum (Gemini BioProducts), and 1% (v/v) penicillin/streptomycin (Gibco).

HEK-293T cells were maintained in an atmosphere of 5% CO_2/95% air in Dulbecco's Modified Eagle Media (Gibco) supplemented with 10% (v/v) charcoal–dextran-stripped fetal bovine serum (Gemini BioProducts), 1% sodium pyruvate, 1% sodium bicarbonate, 1% glutamate, and 1% (v/v) penicillin/streptomycin (Gibco).

2.3. Cell fractionation

At approximately 90% confluency, SKOV-3 or HEK-293T cells were trypsinized and centrifuged. The pellet was resuspended in Buffer A (50 mM HEPES pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 400 μM deoxypyridoxine, 2 mM sodium vanadate, 1 mM DTT, 200 μM PMSF, 0.5 mg soybean trypsin inhibitor, 0.5 mg leupeptin, and 0.2 mg aprotinin) and homogenized with a Dounce homogenizer. Homogenates were centrifuged at 600×*g* for 10 min. The supernatant was centrifuged at 10,000×*g* for 10 min. This supernatant was centrifuged at 100,000×*g* for 1 h.

2.4. Kinase assay

Homogenates were assayed in Buffer A supplemented with ATP (200 μM), [γ-^{32}P]-ATP, and substrate at 37 °C for the specified incubation period. Assay conditions for DGK family members were designed to measure initial reaction rates under zero order conditions as established in preliminary studies using recombinant protein from HEK-293T cell homogenates. The assay conditions are: Type I (DGKα), 3 μg of 100,000×*g* supernatant, 5 min with 10 μM substrate; Type IV (DGKζ), 10 μg of 100,000×*g* supernatant, 60 min with 10 μM substrate; and Type V (DGKθ), 10 μg of 10,000×*g* supernatant for 10 min with 10 μM substrate [12,13]. The reaction was stopped with ice-cold HCl (0.1 M). Lipids were extracted using KCl (1.5 M) and chloroform:methanol (2:1 v/v). The organic-soluble material was analyzed by normal thin layer chromatography (TLC) developed in 1-butanol:glacial acetic acid:-water (3:1:1 v/v/v). Autoradiography revealed the radiolabeled compounds. The material was scraped from the plate for quantitation by liquid scintillation counting.

2.5. Overexpression of DGK isoforms

HEK-293T cells were transfected with DGK cDNA using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Protein concentration was measured using the BCA method (Fischer Scientific). Homogenates of HEK-293T cells expressing DGK were compared to control untransfected HEK-293T cells.

2.6. Cell assay

SKOV-3 cells were seeded at approximately 85% confluency and allowed to attach for 12–24 h. 2-AcMAGE (10 mM) was dissolved in DMSO; octyl tetralin-based amino alcohol (OTAA, (−)-(*S*)-2-amino-2′-((*S*)-6-octyl-1,2,3,4-tetrahydronaphthalen-2-yl)propan-1-ol) (1 mM) [14] was dissolved in 0.1% BSA. SKOV-3 cells were incubated with 2-AcMAGE (10 μM) or DMSO for 2 h, then OTAA [14] (10 μM) or BSA for 1 h [15,16]. The DMSO concentration did not exceed 0.001%. The media was collected and the cell monolayers were washed with ice-cold PBS. Cells were scraped twice into a final volume of 2 mL ice-cold methanol. The suspension was transferred to a borosilicate glass tube and supplemented with 1 mL chloroform, 0.5 mL 0.1 M HCl, and the internal standard C17:0 LPA. The samples were vortexed for 5 min, then 1 mL chloroform and 1.3 mL 0.1 M HCl were added. The samples were vortexed for 5 min, then centrifuged at 3000×*g* for 10 min. The lower (organic) phase was transferred to a glass vial and evaporated under N_2 gas. Dried samples were dissolved in 1 mL chloroform:methanol (1:4 v/v). A 0.1 mL aliquot was removed for phosphate determination. The remaining sample was dried under N_2 gas, resuspended in 0.1 mL methanol, vortexed, and transferred to autosampler vials for LC/MS analysis.

2.7. LC/MS analyses

LPA species were separated with Eclipse XDB C8, 5 micron, 4.6 × 150 mm (Agilent) column. The mobile phase had solvent A (75:25 methanol:water, 0.5% formic acid, 0.1% ammonium formate) and solvent B (80 (99/1 = methanol/water, 0.5% formic acid, 0.1% ammonium formate):20 (chloroform)). For the combined analysis of ether- and ester-linked LPAs, chromatographic separation was achieved with 20% solvent B gradually increased to 25% over 6 min, to 30% over the next 6 min, to 35% over the next 6 min, to 60% over the next 2 min, and maintained at 60% for the last 5 min. The column was equilibrated to initial conditions in 3 min. The flow rate was 0.5 mL/min with a column temperature of 30 °C. The sample injection volume was 10 μL. The mass spectrometer was operated in the negative electrospray ionization mode with optimal ion source settings determined by synthetic standards of 16:0, 18:1 and 18:0 ether and ester LPA with a declustering potential of −90 V, entrance potential of −10 V, collision energy of −68 V, collision cell exit potential of −1 V, curtain gas of 30 psi, ion spray voltage of −4500 V, ion source gas of 40 psi and temperature of 550 °C. MRM transitions monitored were as