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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.

Keywords

Alkylglycerol phosphate; alkyl lysophosphatidic acid; diacylglycerol kinase

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 6 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

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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 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, ten 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, 0.2 mg aprotinin) and homogenized with a Dounce homogenizer. Homogenates were centrifuged at 600 × g for 10 minutes. The supernatant was centrifuged at 10,000 × g for 10 minutes. This supernatant was centrifuged at 100,000 × g for one hour.

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 inubation 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 minutes with 10 μM substrate; Type IV (DGKζ), 10 μg of 100,000 × g supernatant, 60 min with 10 μM substrate; Type V (DGKθ), 10 μg of 10,000 × g supernatant for 10 minutes 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 quantification 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 to 24 hours. 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 hours, then OTAA [14] (10 μM) or BSA for one hour [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 minutes, then 1 mL chloroform and 1.3 mL 0.1 M HCl were added. The samples were vortexed for 5 minutes, then centrifuged at 3,000 × g for 10 minutes. 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 minutes, to 30% over the next 6 minutes, to 35% over the next 6 minutes, to 60% over the next 2 minutes, and maintained at 60% for the last 5 minutes. The column was equilibrated to initial conditions in 3 minutes. 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 follows: 423.8/78.8 and 423.8/96.7 for 18:0 ether LPA, 395.7/79.1 and 395.7/96.5 for 16:0 ether LPA, 421.8/78.9 and 421.8/96.9 for 18:1 ether LPA, 437.6/79 for 18:0 ester LPA, 409.6/79 for 18:1 ester LPA and 435.6/79 for 18:1 ester LPA.

2.8. siRNA

Predesigned Mission siRNA for DGKα were purchased from Sigma Aldrich (SASI_Hs01_00072301). Transfection was carried out using Oligofectamine (Invitrogen) according to manufacturer's instructions. After the initial 72-hour transfection, the transfection protocol was repeated with a final concentration of 5 nmol/L siRNA. The cells were incubated for a second 72-hour period. Knockdown of DGKα protein was confirmed by Western blotting 6 days after the initial transfection.

2.9. Reverse-transcriptase PCR

Total RNA was isolated from SKOV-3 cells using TriZol (Invitrogen) and was reverse transcribed into cDNA using (Invitrogen). PCR was done with () and the following primers (gene: primer: expected band size): DGKα forward: 5'-CTCTCAAGCTGAGTGGGTCC-3' reverse: 5'-TTTTACGATCCGGTCCAGAG-3' 515 bp; DGKβ forward: 5'-CATGGTAATGGTGTGCTT GC-3' reverse: 5'-TTGGGCAGTTACCTTCAACC-3' 847 bp; DGKγ forward: 5'-ATTGCCCAGTACCTGGAGTG-3' reverse: 5'-AGGTGGGCAGTAAGATGTGG-3' 562 bp; DGKδ forward: 5'-GGGAAGACAAGCAGAAGTGC-3' reverse: 5'-CCCAGGCTACTGTGAGCTTC-3' 562 bp; DGKη forward: 5'-CAGCCAGCAAAGAAAACCTC-3' reverse: 5'-GCCAGTTCTTGCTCCAAAAG-3' 946 bp; DGKκ forward: 5'-AGAAGCCATCGCTCATCAGT-3' reverse: 5'-ATTTTGTTCAACTCGGTGGC-3' 623 bp; DGKε forward: 5'-TTGGGTTGGGGTACAGGTTA-3' reverse: 5'-CCCATCCCTTCCCATAGT CT-3' 431 bp; DGKζ forward: 5'-TGTCCAAGATCCTCTCCCAC-3' reverse: 5'-GTCCTG GATCTTGGGAGTCA-3' 308 bp; DGKι forward: 5'-CAGGAGGGGAAATGTAAGCA-3' reverse: 5'-GTTCTTCTGGAGGCAAGTCG-3' 769 bp; DGKθ forward: 5'-CGGTGAGAAGAAGCCAGT TC-3' reverse: 5'-AGGCAACGTCCAACACTACC-3' 720 bp.

2.10. Western blot analysis

Transfection efficiency was confirmed by Western blotting. Cultured cells were homogenized in Buffer A, then centrifuged at 10,000 × g for 10 minutes. The supernatant was electrophoresed on a 4–20% sodium dodecyl sulfate polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blotted with: monoclonal mouse anti-α-tubulin (Sigma), polyclonal rabbit anti-DGKα (Proteintech Group), polyclonal rabbit anti-DGK β or anti-DGKγ [17] and IRDye 800CW conjugated goat anti-rabbit IgG or IRDye 680LT conjugated goat anti-mouse IgG (LI-COR Biosciences) according to manufacturer's instructions. The bands were visualized and quantified using the Odyssey Imaging System.

2.11. Statistical analysis

Statistical differences in intracellular production of alkyl-LPA were calculated using a one-way ANOVA or t-test. The ANOVA was followed by a Dunnet's multiple comparison test. Values $p < 0.05$ were considered statistically significant.

3. Results

3.1. DGK activity toward 2-AcMAGE

Knowledge of DGK substrate specificity is limited because the number of available synthetic diradyl glycerols is small and manipulating these substrates in aqueous media is problematic. Only DGKε (Type III) has a reported high selectivity for DAG with an arachidonoyl group at the *sn*-2 position over other DAG species [11,18,19], thus the remaining DGKs were all candidates for generating alkyl-LPA. Additionally, DGKα (Type I) produces 1-O-alkyl-2-acyl-PA and 1,2-diacyl-PA to the same extent in CTLL-2 cells [20], indicating that DGKα does not distinguish between 1-alkyl-2-acyl glycerols and 1,2-diacylglycerols. This suggested to us that at least one DGK isoform could potentially phosphorylate an alkylglycerol lipid.

We expressed mammalian DGKs in HEK-293T cells to test whether DGKs phosphorylate 2-AcMAGE or MAGE, intermediates in the platelet-activating factor and lysophospholipid pathways, respectively, and potentially contribute to alkyl-LPA production through these lipids. We tested: DGK α, β, and γ (Type I) [19]; DGK δ1 and δ2 (Type II, splice variants) [21,22]; DGK ζ and ι (Type IV) [23]; and DGKθ (Type V). Recombinant Type I (DGKα), Type IV (DGK ζ), and Type V (DGKθ) phosphorylated C18:1 DAG and 2-AcMAGE to a greater extent than MAGE (Figure 1A–C). Indeed, the DGKs we tested demonstrated that C18:1 DAG and 2-AcMAGE phosphorylation was as much as 15-fold and 12-fold greater than that of MAGE phosphorylation, respectively. However, none of the DGKs exhibited a pronounced selectivity for 2-AcMAGE over C18:1 DAG in our assays. Under our assay conditions, the Type II DGK splice variants, DGKδ1 and δ2, were less efficient in phosphorylating C8:0 DAG, C18:1 DAG, 2-AcMAGE, MAGE than the other DGK isoforms (data not shown).

3.2. Sphingosine and analogs activate DGK

Our data from broken cell assays indicates that multiple DGK isoforms can phosphorylate 2-AcMAGE, potentially contributing to intracellular alkyl-LPA production through the PA species 2-AcMAGE-P. However, we found that cell-associated alkyl-LPA and 2-AcMAGE-P were not consistently increased when 2-AcMAGE was added to SKOV-3 cells (see below). Therefore, we sought to increase PA production via DGK to determine whether alkyl-LPA production concurrently increased. Our literature search for DGK activators revealed that sphingosine increases DGK activity in intact Jurkat T cells [16] and PA levels in Swiss 3T3 cells [24]. Thus we investigated whether DGK activity is stimulated by sphingosine and the sphingosine analog FTY-720 [25].

We observed that recombinant DGKα incubated with 10 μM 2-AcMAGE and 10 μM sphingosine exhibited a 7- to 9-fold increase in 2-AcMAGE phosphorylation compared with 10 μM 2-AcMAGE alone (Figure 2). Similarly, 10 μM FTY-720 increased 2-AcMAGE phosphorylation approximately 3-fold (Figure 2). The remaining Type I family members, DGKβ and DGKγ, were likewise stimulated by sphingosine and FTY-720. Conversely, DGKζ and DGKθ activity toward 2-AcMAGE was not affected by the presence of either sphingosine or FTY-720 (data not shown).

3.3. DGK expression in SKOV-3 cells

Under our assay conditions, only the Type I DGK family members exhibited increased activity in the presence of sphingosine and FTY-720, thus, we tested whether SKOV-3 cells express Type I DGK family members. The mRNAs encoding seven of the ten known mammalian isoforms were detected by RT-PCR in SKOV-3 cells (Figure 3A). DGKβ, DGKγ (Type I), and DGKκ (Type II) mRNAs were not detected in SKOV-3 cells. We confirmed protein expression of the Type I DGKs, detecting protein of DGKα but not DGKβ or DGKγ (Figure 3B–D).

3.4. Sphingosine analogs promote 2-AcMAGE conversion to alkyl-LPA

We determined whether treatment with 2-AcMAGE and a sphingosine analog increased alkyl-LPA production in SKOV-3 cells using an FTY-720 analog: octyl tetralin-based amino alcohol, (OTAA, (−)-(*S*)-2-amino-2'-((*S*)-6-octyl-1,2,3,4-tetrahydronaphthalen-2-yl)propan-1-ol) [14], which increased recombinant DGKα-mediated phosphorylation of 2-AcMAGE approximately 3.5-fold at 10 μM in broken cell assays (Figure 2). OTAA is not a sphingosine kinase substrate thus, unlike FTY-720 and sphingosine, it is not phosphorylated to produce confounding effects through agonist actions at S1P receptors [14].

OTAA alone tended to stimulate alkyl-LPA production in SKOV-3 cells, but the addition of 2-AcMAGE/OTAA reproducibly increased alkyl-LPA levels to statistical significance (Figure 4A). The addition of MAGE, with or without OTAA, did not significantly increase alkyl-LPA levels. Although significant increases in cell-associated alkyl-LPA were detected after addition of 2-AcMAGE/OTAA, neither extracellular alkyl-LPA nor 2-AcMAGE-P levels changed significantly. The lack of detectable change of the PA species intermediate in the conversion of 2-AcMAGE to alkyl-LPA suggests the species is transient.

These results suggest that alkyl-LPA can be produced in SKOV-3 cells by DGK-mediated phosphorylation of 2-AcMAGE followed by deacetylation of 2-AcMAGE-P. To confirm that DGKα is responsible for the production of alkyl-LPA in SKOV-3 cells, we decreased DGKα expression in SKOV-3 cells by 79–85% using siRNA (Figure 4C). We found significantly less alkyl-LPA in siDGKα-SKOV-3 cells compared with siScramble-SKOV-3 cells treated with 2-AcMAGE/OTAA (Figure 4B). Nevertheless, siDGKα-SKOV-3 cells treated with 2-AcMAGE/OTAA still produced alkyl-LPA, probably due to an incomplete ablation of DGKα levels (Figure 4C) and/or the production of alkyl-LPA by an additional pathway [7].

4. Discussion

In this study, we examined the role of DGKs in the production of alkyl-LPA. We detected DGK activity toward 2-AcMAGE and found that 2-AcMAGE treatment of SKOV-3 cells increased cell-associated alkyl-LPA when DGK activity was stimulated by a sphingosine analog. These data establish a pathway in SKOV-3 cells for the production of alkyl-LPA from the precursor 2-AcMAGE via DGKα.

The data presented here confirmed and extended the results of Yamada *et al.* [16], who reported that sphingosine treatment of Jurkat T cells increased the activity of an 80 kDa DGK with an EF hand motif, and of Zhang *et al.* [24], who reported that PA levels increase in Swiss 3T3 fibroblasts treated with sphingosine. We found that sphingosine and sphingosine analogs stimulate recombinant Type I DGKs and that treatment of SKOV-3 cells with OTAA, a metabolically stable sphingosine analog, promotes intracellular alkyl-LPA accumulation in these cells. Because SKOV-3 cells express predominantly one Type I DGK, DGKα, we surmise that this isoform is responsible for increased alkyl-LPA production in SKOV-3 cells. We confirmed this by treating cells with siDGKα and detecting a significant decrease in intracellular alkyl-LPA levels compared to siScramble-SKOV-3 cells.

The mechanism whereby sphingosine activates Type I DGK has not been fully established [16,24]. Structurally, Type I DGKs differ from the other DGK groups at their N-terminus where they have EF-hand motifs and a recoverin homology domain [11,19], thus sphingosine-dependent DGKα activation may be due to a direct interaction between sphingosine and DGK. Indeed, studies examining truncated DGKα demonstrate that the recoverin homology and EF-hand domains are necessary for sphingosine-dependent DGKα activation, and sphingosine-dependent DGKα activation may require the negatively charged amino acid residues of the EF-hand domain [27].

While molecular details of the mechanisms involved remain to be established, our results identify a node of cross-talk between the sphingolipid and glycerol lipid pathways. Sphingosine kinase inhibitors designed as sphingosine analogs, while decreasing the flux of sphingosine to S1P, might also increase the flux of DAG to PA and thence to alkyl-LPA. These inhibitors, which have been proposed as potential anti-cancer agents [28], might have the unintended effect of increasing one mitogen, alkyl-LPA, along with the intended effect of decreasing S1P. Additional work is necessary to determine if this is the case.

In sum, we have demonstrated that DGKs can significantly contribute to alkyl-LPA production in cells. Specifically, SKOV-3 cells can produce alkyl-LPA from 2-AcMAGE via DGKα. This process can be stimulated by a sphingosine analog that acts via DGKα.

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Abbreviations

LPA	lysophosphatidic acid
ATX	autotaxin
2-AcMAGE	2-acetyl monoalkylglycerol ether, 1-Ohexadecyl-*sn*-2-acetyl glycerol
MAGE	monoalkylglycerol ether, 1-O-hexadecyl-*sn*-glycerol
MAG	monoacylglycerol
AGK/MuLK	acylglycerol kinase / 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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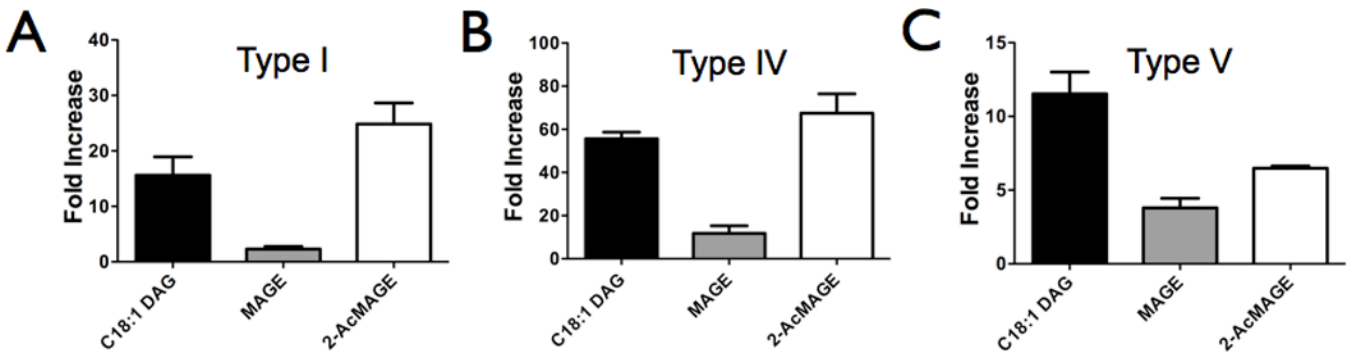

Figure 1. Kinase activity of recombinant mammalian DGKs

Recombinant (A) Type I (DGKα), (B) Type IV (DGKζ), or (C) Type V (DGK θ) from HEK-293T cell homogenate were incubated with γ-[^{32}P]-ATP and C18:1 DAG, 2-AcMAGE, or MAGE (10 μM) and products displayed by TLC. Kinase activity was evaluated as in "Materials and Methods" and normalized to activity in untransfected HEK-293T cells. A 200 μL reaction supplemented with C18:1 DAG yielded an average of 6.4×10^3 CPM for untransfected cells. Data corresponds to average ±SD (n=3).

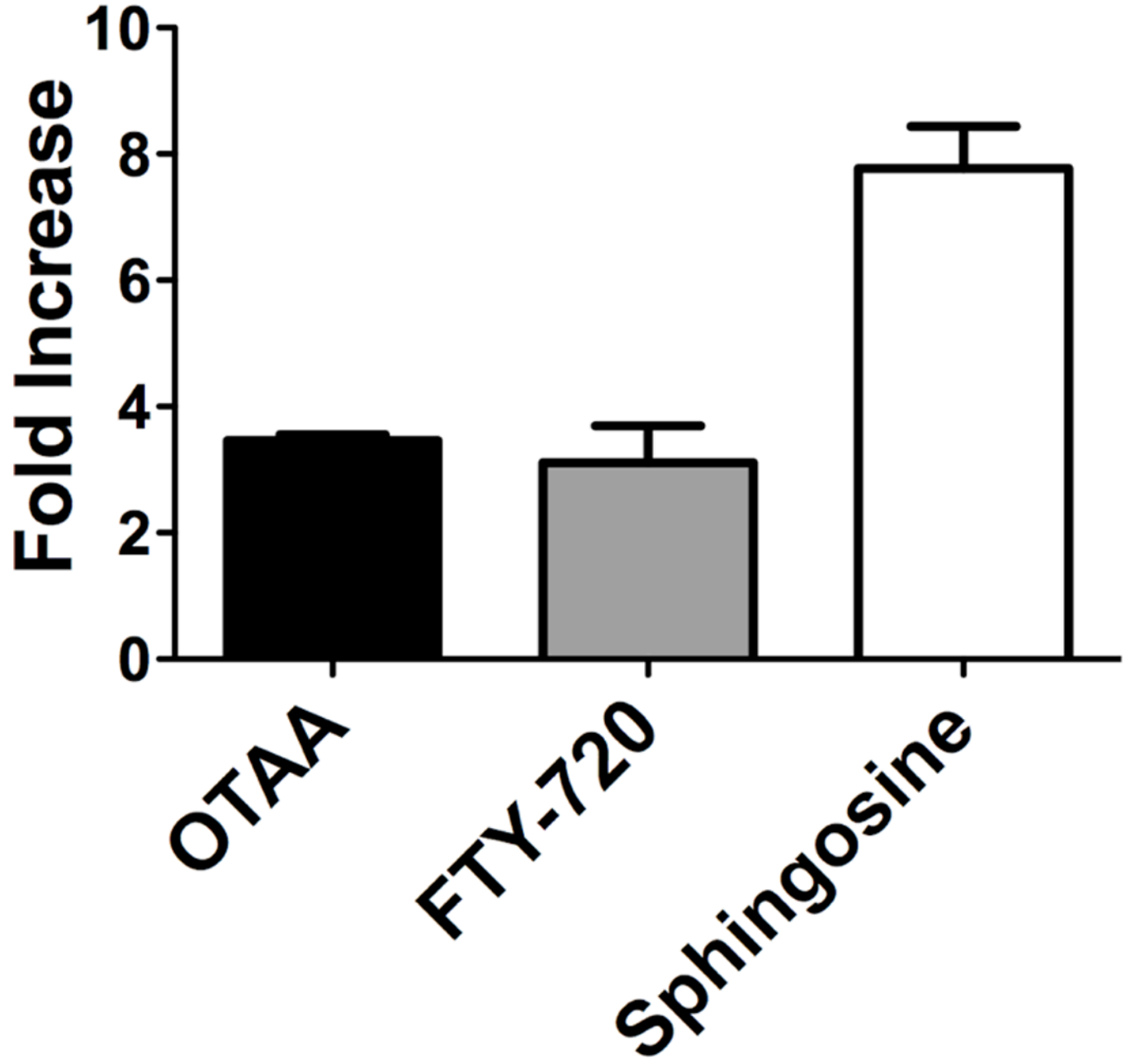

Figure 2. Sphingosine and sphingosine analogs on kinase activity of recombinant DGKα

Recombinant DGKα from HEK-293T cell homogenate was incubated with γ-[^{32}P]-ATP, 10 μM 2-AcMAGE, and sphingosine or sphingosine analogs: FTY-720 or OTAA (10 μM). Kinase activity was evaluated as in "Materials and Methods" and normalized to activity in untransfected HEK-293T cells. A 200 μL reaction supplemented with 2-AcMAGE and OTAA yielded an average of 1.6×10^3 CPM. Data corresponds to average ±SD (n=3).

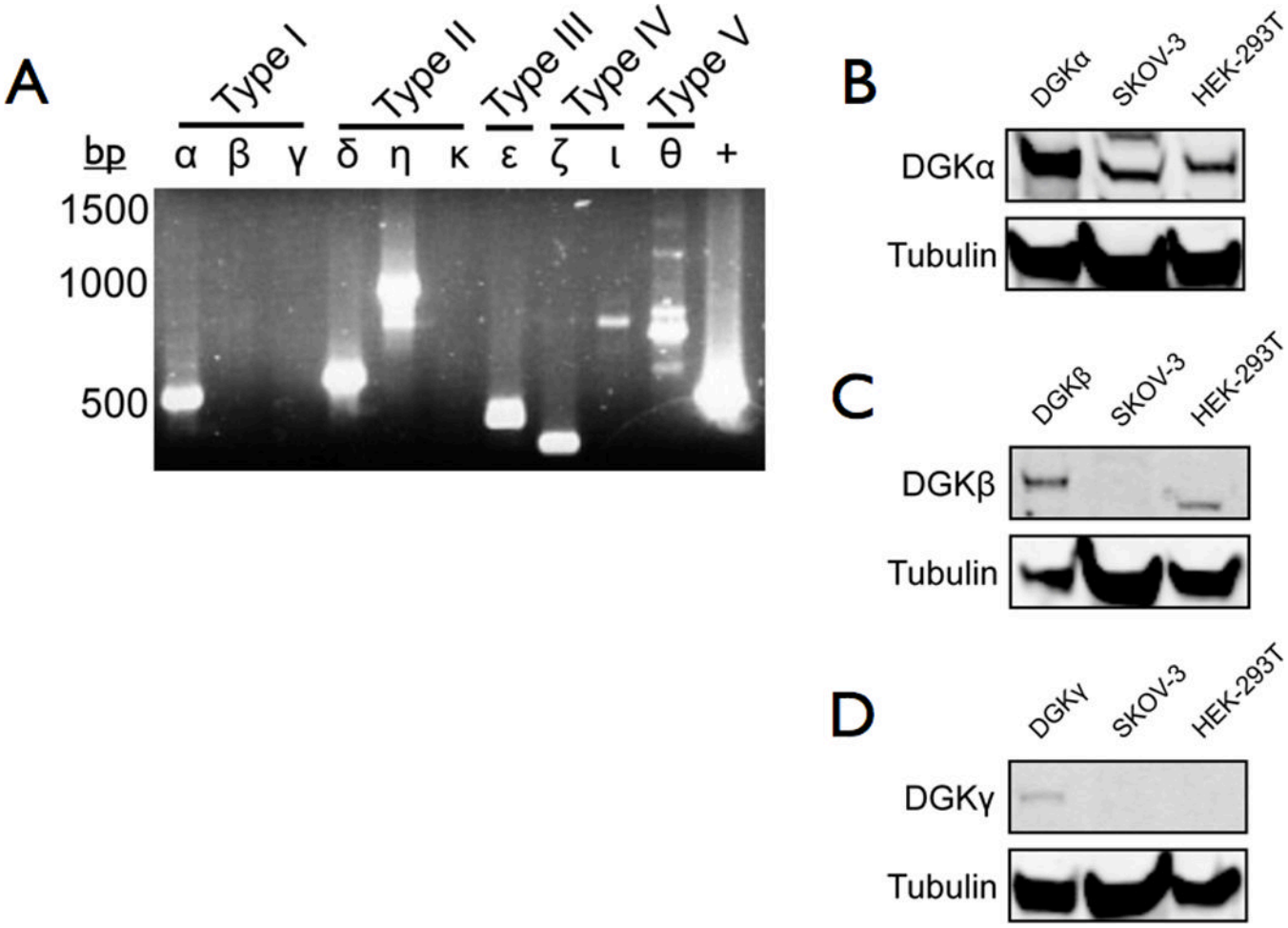

Figure 3. DGK expression by SKOV-3 cells

(A) RT-PCR analysis of DGK isoforms in SKOV-3 cells. (B–D) Western blot analysis for DGKα, DGKβ, and DGKγ, with α-tubulin. Shown are recombinant DGKα, DGKβ, or DGKγ from the 100,000 × g supernatant of transfected HEK-293T cells; SKOV-3 cells; and untransfected HEK-293T cells.

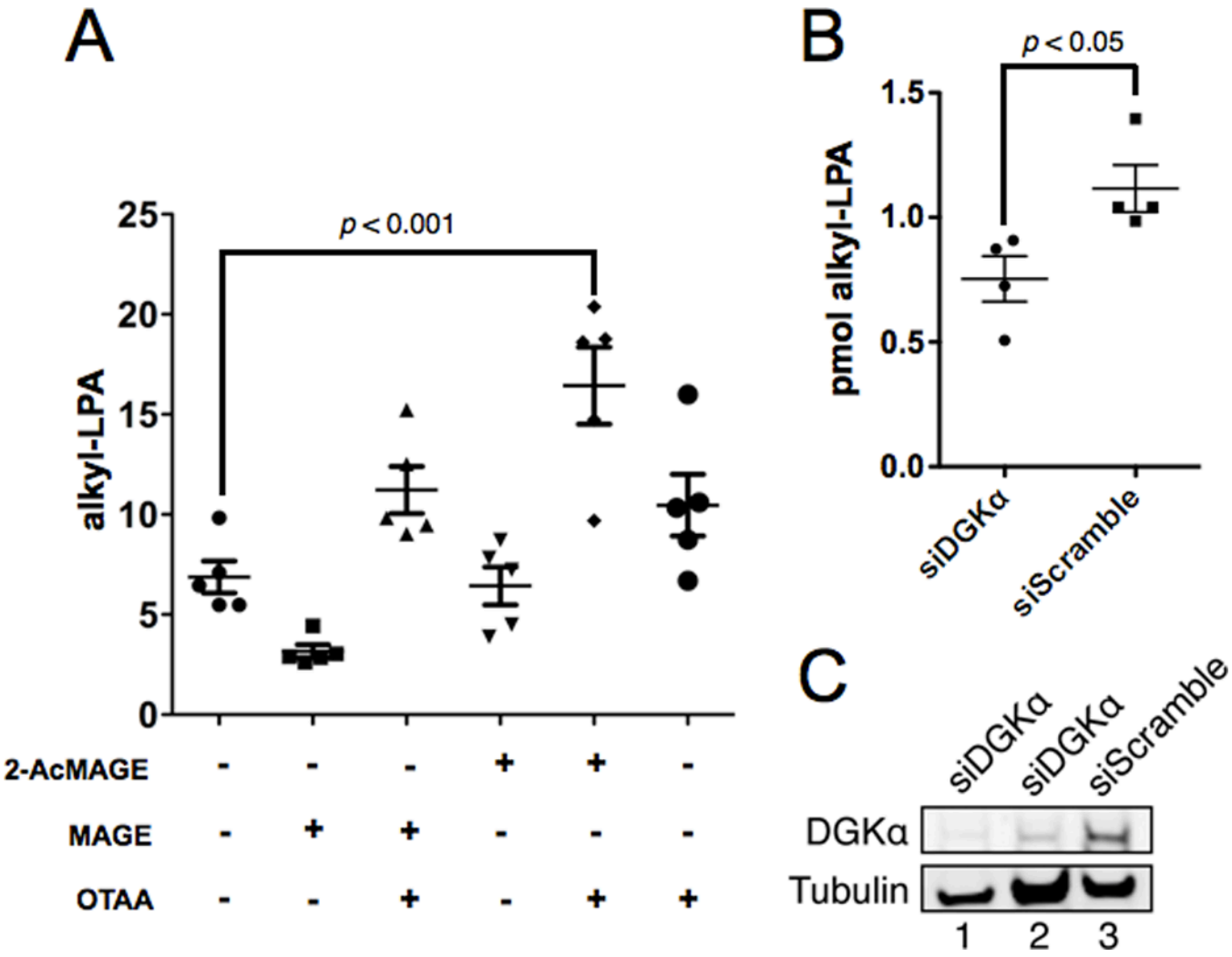

Figure 4. Measurement of alkyl-LPA in SKOV-3 cells

(A) SKOV-3 cells and (B) SKOV-3 cells treated with either DGKα siRNA (siDGKα cells) or a scrambled probe (siScramble cells) were incubated with 2-AcMAGE (10 μM), MAGE (10 μM), or DMSO for 2 hours, then OTAA (10 μM) or BSA for 1 hour. The data shown are individual measurements from quadruplicate determinations and represents the cell-associated alkyl-LPA levels normalized to C17 LPA. (A) Statistically significant differences of intracellular production of alkyl-LPA were determined by one-way ANOVA. Differences compared to DMSO/BSA treated cells were calculated with Dunnet's multiple comparison test. Data correspond to the average ±SD (n=5). (B) Statistically significant differences of intracellular production of alky LPA were determined by student's t-test. Data corresponds to the average ±SD (n=4). (C) Expression of DGKα in siDGKα-SKOV-3 and siScramble-SKOV-3 cells assessed by Western blotting. Lanes 1 and 2 represent two separate batches treated with the same siDGKα probe. Lane 3 is cells treated with siScramble. DGKα levels were normalized to tubulin.