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Effects of synthetic sphingosine-1-phosphate analogs on arachidonic acid metabolism and cell death

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Abstract

Sphingolipid metabolites such as sphingosine regulate cell functions including cell death and arachidonic acid (AA) metabolism. D-erythro-C18-Sphingosine-1-phosphate (D-*e*-S1P), a sphingolipid metabolite, acts as an intracellular messenger in addition to being an endogenous ligand of some cell surface receptors. The development of S1P analogs may be useful for studying and/or regulating S1P-mediated cellular responses. In the present study, we found that several synthetic S1P analogs at pharmacological concentrations stimulated AA metabolism and cell death in PC12 cells. D-*erythro-N,O,O*-Trimethyl-C18-S1P (D-*e*-TM-S1P), L-*threo-O,O*-dimethyl-C18-S1P (L-*t*-DM-S1P) and L-*threo-O,O*-dimethyl-3*O*-benzyl-C18-S1P (L-*t*-DMBn-S1P) at 100 μM stimulated [^{3}H]AA release from the prelabeled PC12 cells. L-*t*-DMBn-S1P at 20 μM increased prostanoid formation in PC12 cells. L-*t*-DMBn-S1P-induced AA release was inhibited by D-*e*-sphingosine, but not by the tested PLA_2 inhibitors. L-*t*-DMBn-S1P did not stimulate the activity of cytosolic phospholipase $A_{2\alpha}$ ($cPLA_{2\alpha}$) in vitro and the translocation of $cPLA_{2\alpha}$ in the cells, and caused AA release from the cells lacking $cPLA_{2\alpha}$. These findings suggest that L-*t*-DMBn-S1P stimulated AA release in a $cPLA_{2\alpha}$-independent manner. In contrast, D-*e*-S1P and D-*erythro-N*-monomethyl-C18-S1P caused cell death without AA release in PC12 cells, and the effects of D-*e*-TM-S1P, L-*t*-DM-S1P and L-*t*-DMBn-S1P on cell death were limited. Synthetic S1P analogs may be useful tools for studying AA metabolism and cell death in cells.

Keywords: Sphingosine-1-phosphate; Phospholipase A_2; Arachidonic acid; Prostaglandin; Cell death; PC12 cells

1. Introduction

Metabolites of sphingolipids such as ceramide and D-*erythro*-C18-sphingosine-1-phosphate (D-*e*-S1P) are produced in response to stress and receptor activation in cells [1–4]. Ceramide is produced from sphingomyeline by the activation of sphingomyelinases and by de novo synthesis, and is metabolized to sphingosine by ceramidases. Ceramide intracellularly regulates cell responses such as proliferation and apoptosis [5]. In addition, ceramide 1-phospahte is a putative intracellular modulator of cell functions [5–7]. Sphingosine is phosphorylated by sphingosine kinases to S1P in cells in the endoplasmic reticulum [2,3]. S1P released into extracellular spaces where it acts as an endogenous agonist for the EDG (endothelial differentiation gene) family of G protein-coupled cell surface receptors. In addition, S1P acts as an intracellular messenger important for regulation of calcium homeostasis, various kinases, cell growth and death [2,3,7,8]. It is reported that murine epithelial cells expressing the cystic fibrosis transmembrane regulator protein, a member of the ATP binding cassette family of proteins, exhibit greater uptake of S1P [9]. EDG receptor-dependent responses are

Abbreviations: D-*e*-S1P, D-*erythro*-C18-sphingosine-1-phosphate; L-*t*-S1P, L-*threo*-C18-sphingosine-1-phosphate; D-*e*-MM-S1P, D-*erythro-N*-monomethyl-C18-S1P; D-*e*-TM-S1P, D-*erythro-N,O,O*-trimethyl-C18-S1P; L-*t*-DM-S1P, L-*threo-O,O*-dimethyl-C18-S1P; L-*t*-Bn-S1P, L-*threo*-3*O*-benzyl-C18-S1P; L-*t*-DMBn-S1P, L-*threo-O,O*-dimethyl-3*O*-benzyl-C18-S1P; AA, arachidonic acid; PLA_2, phospholipase A2; $cPLA_2$, cytosolic PLA_2; PG, prostaglandin; EDG, endothelial differentiation gene; GFP, green fluorescent protein; LDH, lactate dehydrogenase; TNFα, tumor necrosis factor α; DMSO, dimethylsulfoxide; DMEM, Dulbeccos modified Eagles medium; FBS, fetal bovine serum; HEK, human embryonic kidney; RHC80275, 1,6-bis(cyclohexyloximinocarbonyl-amino)hexane; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; U0126, 1.4-diamino-2,3-dicyano-1,4-bis[2-amino-phenylthio]-butadiene

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elicited by the exogenous addition of submicromolar concentrations of S1P, but the intracellular effects of S1P are mimicked at greater concentrations [1,3,10]. The development of selective agonists and/or antagonists for EDG receptors may be useful for clarifying the physiological and pathological roles of members of the EDG family [2,4,11]. In addition, synthetic S1P analogs without affinity for EDG family receptors are useful for studying the biological functions of sphingolipid metabolites in cells.

Arachidonic acid (AA) is another component of membrane lipids that can directly activate signaling pathways or do so through its metabolites after activation by enzymes such as the cyclooxygenases. AA and the resulting eicosanoids such as prostaglandins (PGs) play a regulatory role in cell functions in cells including neuronal cells [12–14]. Since AA production is the rate-limiting step in eicosanoid biosynthesis, the regulation of phospholipase A_2 (PLA_2) is important in the process [15]. Sphingolipid metabolites such as ceramide regulate PLA_2 activity and/or AA release in various cells [16–19]. Recently, it is reported that activation of ceramide kinase and the resulting ceramide 1-phospahte accumulation causes AA release and prostanoid synthesis in cells [6]. We reported that D-*erythro*-sphingosine inhibits cytosolic $PLA_{2\alpha}$ ($cPLA_{2\alpha}$) activity in vitro and AA release from cells [20]. These findings suggest that sphingolipid metabolites intracellulary regulate PLA_2 activity and AA metabolism. Although activation of EDG family receptors with D-*e*-S1P stimulated AA release in A549 human lung adenocarcinoma cells [21], S1P was unable to promote AA release in several cells expressing EDG family receptors such as endothelial cells and fibroblasts [22,23]. The role of intracellular D-*e*-S1P in AA metabolism has not been established. In the present study, we examined the effects of several synthetic analogs of S1P on AA metabolism in PC12 cells (a rat pheochromocytoma cell line), L929 cells (a murine fibrosarcoma cell line) and its variant C12 cells lacking $cPLA_{2\alpha}$. The effects of S1P analogs on cell death were also examined. D-*erythro*-*N*,*O*,*O*-Trimethyl-C18-S1P (D-*e*-TM-S1P) and L-*threo*-*O*,*O*-dimethyl-3*O*-benzyl-C18-S1P (L-*t*-DMBn-S1P) stimulated AA release and prostanoid formation in an $cPLA_{2\alpha}$-independent manner, and D-*e*-S1P, D-*erythro*-*N*-monomethyl-C18-S1P (D-*e*-MM-S1P) and L-*threo*-C18-S1P (L-*t*-S1P) caused PC12 cell death without AA release. The relation between AA release and cell death is discussed.

2. Experimental procedures

2.1. Materials

[5,6,8,9,11,12,14,15-^{3}H]AA (215 Ci/mmol, 7.96 TBq/mmol) and [7,8-^{3}H]noradrenaline (40 Ci/mmol, 1.48 TBq/mmol) were obtained from Amersham (Buckinghamshire, UK), and 1-palmitoyl-2-[^{14}C]-arachidonyl phosphatidylcholine (48 mCi/mmol, 1776 MBq/mmol) was obtained from Perkin-Elmer (Boston, MA, USA). D-*erythro*-C18-S1P (D-*e*-S1P) and L-*t*-S1P, 1,6-bis(cyclohexyloximinocarbonyl-amino)hexane (RHC80275) and z-Val-Ala-Asp(OMe)-fluoromethylketone (a pan-caspases inhibitor) were acquired from Biomol Res. Lab. (Plymouth Meeting, PA, USA). D-*e*-C13-S1P, D-*e*-C8-S1P, D-*e*-MM-S1P, D-*e*-TM-S1P, L-*t*-S1P, L-*threo*-*O*,*O*-dimethyl-C18-S1P (L-*t*-DM-S1P), L-*threo*-3*O*-benzyl-C18-S1P (L-*t*-Bn-S1P) and L-*t*-DMBn-S1P were prepared by standard methods. The formulae of the tested synthetic S1P analogs are shown in Fig. 1. D-*e*-Sphingosine, D-*e*-dihydrosphingosine, D-*e*-dimethylsphingosine, ionomycin and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580) were purchased from Sigma (St. Louis, MO, USA). Arachidonyl trifluoromethyl ketone and bromoenol lactone were acquired from Research Biochemcals (Natick, MA, USA) and Cayman Chemicals (Ann Arbor, MI, USA), respectively, and nerve growth factor (2.5S) and dithiothreitol were obtained from Wako (Osaka, Japan). Recombinant human tumor necrosis factor α (TNFα) was purchased from Pepro Tech (London, UK), and 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole (Gö6976) and 1.4-diamino-2,3-dicyano-1,4-bis[2-amino-phenylthio]-butadiene (U0126) were purchased from Calbiochem (La Jolla, CA, USA) and Promega (Woods Hallow, WI, USA), respectively. The tested S1P analogs and inhibitors such as RHC80275 and bromoenol lactone were dissolved in dimethyl sulfoxide (DMSO). The effects of vehicles on responses are described in their respective results.

2.2. Cell cultures and measurement of [^{3}H]AA release from PC12, L929 and C12 cells

PC12 cells were cultured on collagen-coated dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS) and 5% horse serum, as previously described [24]. L929 cells and its variant C12 cells were cultured in DMEM supplemented with 5% heat-inactivated FBS [25]. [^{3}H]AA release from the prelabeled cells was determined as previously described [24]. In brief, subconfluent cells on dishes were incubated with DMEM (0.2% FBS) and 0.33 μCi/mL (12.2 kBq/mL) [^{3}H]AA for 24 h. The cells were washed and suspended in modified Tyrode HEPES buffer (137 mM NaCl, 5 mM KCl, 5 mM glucose, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES, pH 7.4). Cell suspensions (30–50 μg of protein) were incubated with the indicated reagents for 30 min at 37 °C in the presence of 0.1% fatty acid-free bovine serum albumin (Sigma A-7511). In some experiments, cell suspensions were prepared with $CaCl_2$-free buffer, and [^{3}H]AA release was measured in $CaCl_2$-free buffer containing 0.2 mM EGTA. The total volume was 200 μl and the reaction was terminated by the addition of 500 μl of ice-cold, Ca^{2+}-free,

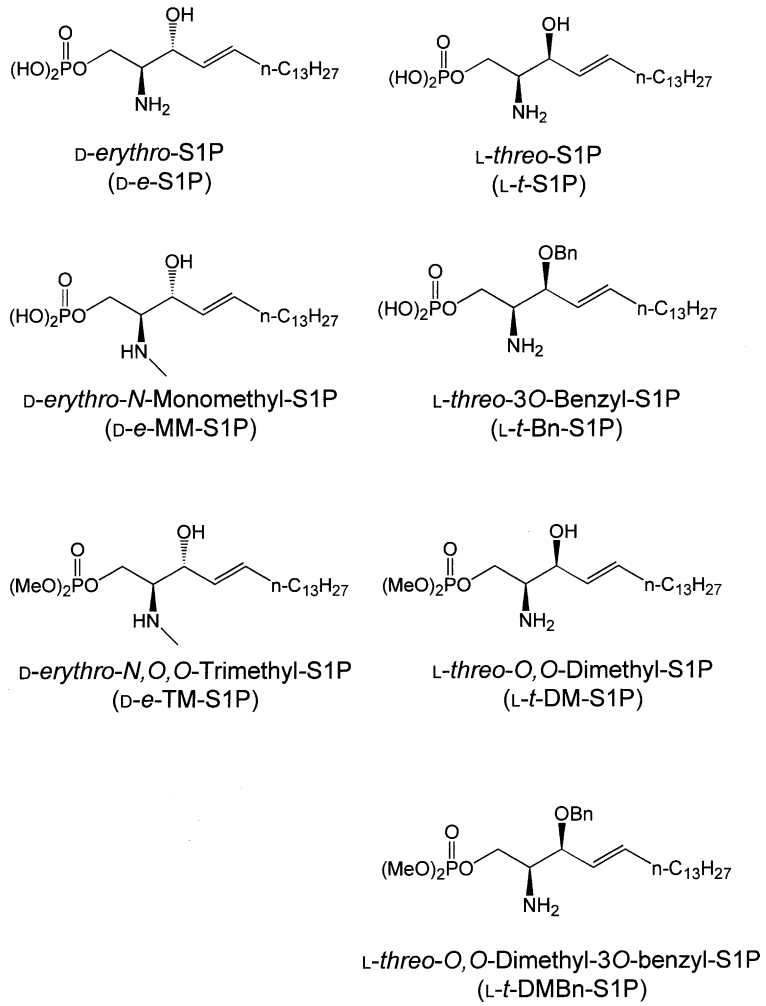

Fig. 1. Formulae of tested S1P analogs.

Mg^{2+}-free Tyrode buffer containing 5 mM EDTA and EGTA followed by centrifugation (5000 × *g*; 30 s) at 4 °C. The ^{3}H content of the supernatant was estimated, and values were calculated as percentages relative to the total incorporation of [^{3}H]AA (15,000–20,000 dpm per tube). The vehicle containing 1% DMSO had no effect on AA release.

2.3. Measurement of PGE_2 and $PGF_{2\alpha}$ formation in PC12 cells

PC12 cell suspensions were incubated with DMEM containing 0.1% fatty acid-free albumin at 37 °C in the presence of the indicated reagents. The contents of PGs in the medium after centrifugation (1000 × *g*; 30 s, 4 °C) were determined using respective enzyme immunoassay kits (Cayman, Ann Arbor, MI, USA). Although the medium containing S1P analogs at 100 μM without PC12 cells slightly cross-reacted with the kits, the 20 μM L-*t*-DMBn-S1P did not show significant cross-reactivity.

2.4. Measurement of PLA_2 activity in vitro in soluble fractions from human embryonic kidney (HEK) 293T cells expressing $cPLA_{2\alpha}$

HEK293T cells were transfected with pcDNA4/HisMax A-human $cPLA_{2\alpha}$ by LipofectAMINE (Invitrogen, Carlsbad, CA, USA) according to the manufacture's protocols. The expression of $cPLA_{2\alpha}$ was confirmed by immunoblotting using anti-$cPLA_2$ antibody (Santa Cruz, N-216, Santa Cruz, CA, USA). $cPLA_2$ activity was measured using 1-palmitoyl-2-[^{14}C]-arachidonyl phosphatidylcholine as a substrate as previously described [20]. Briefly, radiolabeled phospholipid was mixed with distilled water and 0.1% Triton X-100 at 10 μM, and then the mixture was sonicated. To 25 μl of the cell lysate fractions (12.5 μg of protein per tube) were added 175 μl of reaction buffer (50 mM HEPES, 1 mg/ml of bovine serum albumin, 4 mM $CaCl_2$, 10 mM dithiothreitol, pH 7.4) and 50 μl of the lipid vesicle (~60,000 dpm per tube). The reaction mixture (total 250 μl) was incubated at 37 °C for 30 min. The reaction was stopped by adding 1.25 ml of Dole's reagent

(1N H_2SO_4/*n*-heptane/isopropanol = 2/20/78 ratio). Then, 0.75 ml of *n*-heptane and 0.5 ml of water were added to the respective tubes and centrifuged (5000 × *g*; 5 min). To 0.75 ml of the supernatant was added 0.75 ml of *n*-heptane and 100 mg of silica. After centrifugation, the radioactivity of the *n*-heptane phase containing fatty acids was quantified.

2.5. Plasmid construction, transfection and confocal microscopy

The plasmid for a chimeric protein containing green fluorescent protein (GFP) at the amino terminus of $cPLA_{2\alpha}$ was prepared as previously described [26]. For $cPLA_{2\alpha}$-GFP expression, HEK293T cells (60-mm dishes) were transiently transfected with 2 μg of the expression vector with LipofectAMINE PLUS according to the manufacturer's protocol and used for experiment between 48 and 72 h after transfection. Confocal microscopy was performed using an inverted confocal laser scanning microscope (Olympus, Tokyo, Japan).

2.6. Measurement of lactate dehydrogenase (LDH) leakage and noradrenaline release from cells

Cells were cultured in DMEM without FBS in the presence of the indicated concentrations of S1P analogs for 30 min or 24 h. In some experiments, cells were cultured with the indicated S1P analogs for 4 h, and the washed cells were further cultured with S1P analog-free medium for 20 h. Cell viability was estimated by the leakage of LDH as previously described [13,27]. Leakage (%) was defined as the ratio of LDH activity in the culture medium to total activity [% = (extracellular activity)/(extracellular activity and remaining cellular activity) × 100] per well. Although treatment with the vehicle (2.5% DMSO) for 30 min did not stimulate LDH leakage (under 5%), treatment for 24 h increased LDH leakage by 20% in the absence of FBS. The measurement of [^{3}H]noradrenaline release for 10 min from prelabeled PC12 cells was determined as previously reported [28]. The release was presented the percentage of total incorporated [^{3}H]noradrenaline (∼15,000 dpm per tube).

2.7. Statistics

Values are the means ± S.E.M. for 3–4 independent experiments performed in triplicate. In some cases, data are shown as the means ± S.D. of two or three observations in a typical representative experiment. In the case of multiple comparisons, the significance of differences was determined using one-way analysis of variance by Dunnett's or Tukey's test. For pairwise comparisons, the Student's two-tailed *t*-test was used. P values < 0.05 were considered to be significant.

3. Results

3.1. Effects of tested D-e-S1P and L-t-S1P analogs on AA release from PC12 cells

We examined the effects of the indicated synthetic S1P analogs at 100 μM on [^{3}H]AA release for 30 min from labeled PC12 cells (Table 1). Also, we reported log P values calculated by CLOG P program (Biobyte Corp., ver. 4.0, Claremont, CA, USA) as the lipophilicity parameters. The tested analogs except for D-*e*-C13-S1P and D-*e*-C8-S1P are lipophilic compounds with log P values ranging 4.89–7.51. As previously described [20], the addition of D-*e*-S1P did not stimulate AA release, and D-*e*-C13-S1P or D-*e*-C8-S1P did not stimulate release as well. The

Table 1
Effects of the tested S1P analogs on [^{3}H]AA release and LDH leakage in PC12 cells

Compounds	[^{3}H]AA release (% of total)	LDH leakage (%) (treatment for 4 h)	LDH leakage (%) (treatment for 24 h)	Lipophilicity (CLOG P)
Vehicle (DMSO)	0.88 ± 0.13	5.3 ± 0.6	20.5 ± 4.2	
D-*e*-S1P; $C_{18}H_{38}NO_5P$	0.48 ± 0.17	27.6 ± 2.6[a]	43.7 ± 4.1	4.89
D-*e*-C13-S1P; $C_{13}H_{28}NO_5P$	0.62 ± 0.20	Not determined	15.2 ± 1.9	2.24
D-*e*-C8-S1P; $C_8H_{18}NO_5P$	0.78 ± 0.21	Not determined	17.6 ± 0.5	−0.40
D-*e*-MM-S1P; $C_{19}H_{40}NO_5P$	1.52 ± 1.02	59.8 ± 3.9[a]	84.9 ± 11.0	5.35
D-*e*-TM-S1P; $C_{21}H_{44}NO_5P$	4.34 ± 0.42[a]	17.3 ± 3.9	32.7 ± 10.3	5.38
L-*t*-S1P; $C_{18}H_{38}NO_5P$	0.97 ± 0.22	Not determined	40.4 ± 4.4	4.89
L-*t*-DM-S1P; $C_{20}H_{42}NO_5P$	3.24 ± 0.29[a]	Not determined	18.6 ± 0.9	4.92
L-*t*-Bn-S1P; $C_{25}H_{44}NO_5P$	1.39 ± 0.23	Not determined	10.5 ± 0.6	7.48
L-*t*-DMBn-S1P; $C_{27}H_{48}NO_5P$	9.80 ± 1.23[a]	26.5 ± 3.1[a]	32.8 ± 1.0	7.51

For the measurement of [^{3}H]AA release, labeled PC12 cells were incubated with vehicle or the indicated S1P analogs (100 μM) for 30 min in the presence of 2 mM $CaCl_2$. Data are presented as percentages of the total incorporated [^{3}H]AA, and are the mean ± S.E.M. for three independent experiments done in triplicate. [a] $P < 0.05$, significantly different from the control value. For the measurement of LDH leakage, PC12 cells were cultured with vehicle, D-*e*-S1P (50 μM) or the indicated S1P analogs (30 μM) for 4 h. The cells were washed with S1P analog-free DMEM and then cultured for an additional 20 h without S1P analogs. Data are the means ± S.E.M. for four to eight independent experiments done in triplicate. [a] $P < 0.05$, significantly different from the control value. In some experiments, PC12 cells were cultured with vehicle or the indicated S1P analogs (50 μM) for 24 h. Data are the means ± S.D. for a typical experiment. The data are representative of three independent experiments. Values of calculated octanol–water partition coefficient (CLOG P) are shown as the lipophilicity parameters.

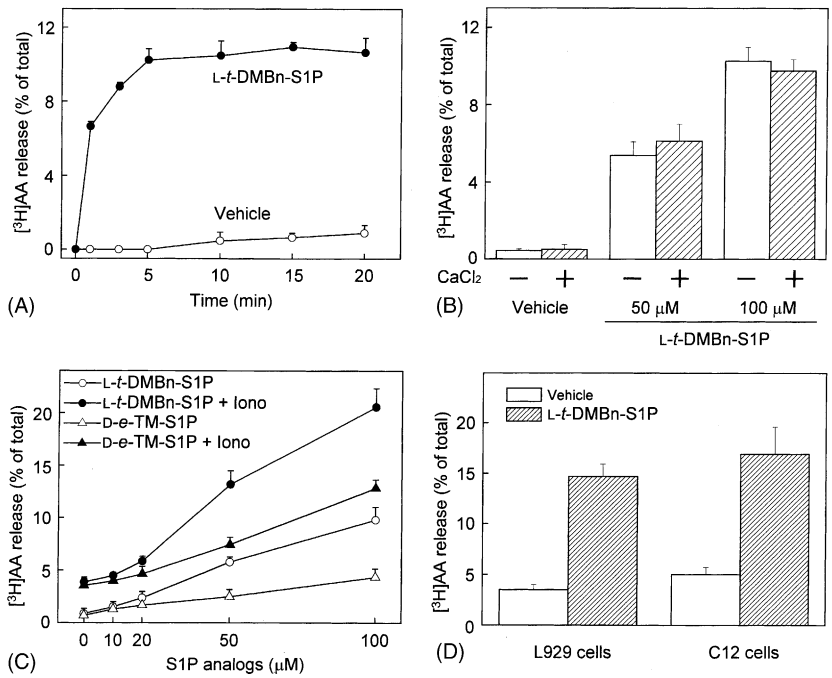

Fig. 2. [^{3}H]AA release induced by L-*t*-DMBn-S1P and D-*e*-TM-S1P in PC12, L929 and C12 cells. In Panel A, PC12 cells labeled with [^{3}H]AA were incubated with vehicle or 100 μM L-*t*-DMBn-S1P for the indicated time in the presence of 2 mM $CaCl_2$. Values are the means ± S.D. for duplicate samples in a typical experiment. In Panel B, PC12 cells were incubated with vehicle or 50 and 100 μM L-*t*-DMBn-S1P for 30 min in the absence and presence of 2 mM $CaCl_2$. The assay mixture was further supplemented with 0.2 mM EGTA. In Panel C, PC12 cells were incubated with the indicated concentrations of L-*t*-DMBn-S1P (○, ●) and D-*e*-TM-S1P (△, ▲) for 30 min in the presence of 2 mM $CaCl_2$. The assay mixture was further supplemented with vehicle (○, △) or 5 μM ionomycin (●, ▲). In Panel D, L929 and C12 cells labeled with [^{3}H]AA were incubated with vehicle or 100 μM L-*t*-DMBn-S1P for 30 min in the presence of 2 mM $CaCl_2$. In Panels B–D, data are the means ± S.E.M. for three independent experiments done in triplicate.

addition of D-*e*-TM-S1P but not D-*e*-MM-S1P significantly stimulated release. L-*t*-S1P and L-*t*-Bn-S1P had no effect, but L-*t*-DM-S1P and L-*t*-DMBn-S1P-stimulated AA release. The AA release response induced by L-*t*-DMBn-S1P was detected after 1 min and became saturated between at 5 and 10 min after addition (Fig. 2A). The effects of L-*t*-DMBn-S1P and D-*e*-TM-S1P were concentration-dependent (Fig. 2C). Although the effects of L-*t*-DMBn-S1P (Fig. 2B) and L-*t*-TM-S1P (data not shown) were independent of extracellular $CaCl_2$, the two analogs enhanced 5 μM ionomycin stimulated AA release in the presence of 2 mM $CaCl_2$ (Fig. 2C). In the following experiments, we investigated the effect of L-*t*-DMBn-S1P, which was the most effective compound for inducing AA release among the S1P analogs. The addition of 20 μM L-*t*-DMBn-S1P-stimulated PGE_2 formation for 20 min, and $PGF_{2\alpha}$ formation for 10 min in the PC12 cell medium (Table 2). Twenty micromolar L-*t*-DMBn-S1P enhanced 5 mM Na_3VO_4/5 μM ionomycin-induced PGE_2 and $PGF_{2\alpha}$ formation in PC12 cells.

The S1P analogs did not stimulate [^{3}H]noradrenaline release after 10 min from prelabeled PC12 cells. For instance, the values in the presence of 2 mM $CaCl_2$ were 4.8 ± 0.4 and 5.3 ± 0.6 (percent of the total incorporated [^{3}H]noradrenaline) in the vehicle- and 100 μM L-*t*-DMBn-S1P-treated cells, respectively (n = 3). The addition of 5 μM ionomycin caused release, 10.9 ± 1.2% (n = 3).

3.2. AA release induced by L-t-DMBn-S1P without the involvement of $cPLA_{2\alpha}$

Next, we investigated the direct effects of S1P analogs on $cPLA_{2\alpha}$ activity in vitro. Since the activity in the soluble fraction of the PC12 cells was low (100–200 dpm per assay tube), we used the soluble fraction of the HEK293T cells transiently expressing $cPLA_{2\alpha}$ for this purpose. PLA_2 activity in vitro in the soluble fraction from control vector-transfected HEK293T cells was low (under 100 dpm per tube). The activity of that from HEK293T cells expressing $cPLA_{2\alpha}$ was high (over 2000–3000 dpm per assay tube) in the presence of 4 mM $CaCl_2$. The addition of L-*t*-

Table 2
PGE_2 and $PGF_{2\alpha}$ formation induced by L-*t*-DMBn-S1P in PC12 cells

Addition	PGE_2 (pg/mL)	$PGF_{2\alpha}$ (pg/mL)
None	14 ± 1	165 ±12
L-*t*-DMBn-S1P	112 ± 9	237±16
Na_3VO_4/ionomycin	188 ± 15	537 ± 29
Na_3VO_4/ionomycin + L-*t*-DMBn-S1P	356 ± 3	1737 ± 128

PC12 cells were incubated with DMEM in the presence of 0.2% fatty acid-free bovine serum albumin for 20 min (PGE_2) or 10 min ($PGF_{2\alpha}$) at 37 °C. The medium was further supplemented with vehicle, 20 μM L-*t*-DMBn-S1P and/or 5 mM Na_3VO_4 plus 5 μM ionomycin. The amounts of PGE_2 and $PGF_{2\alpha}$ in the medium were measured by respective EIA kits. Values are the means ± S.D. for duplicate samples in a typical experiment. The data are representative of two independent experiments.

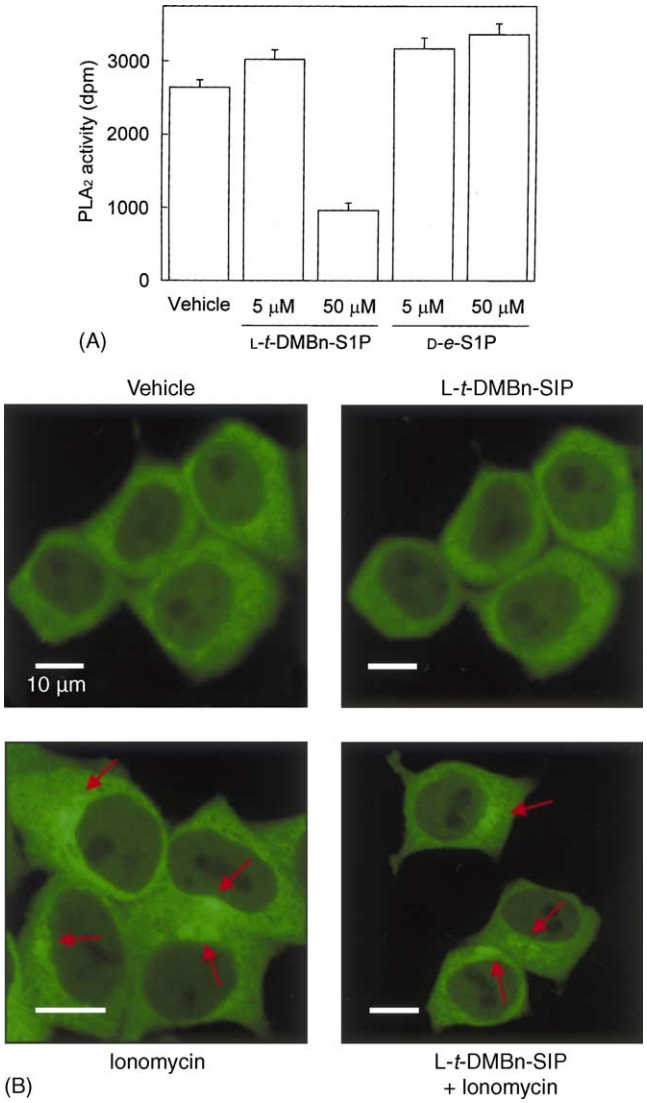

Fig. 3. Non-involvement of $cPLA_{2\alpha}$ activity by L-*t*-DMBn-S1P. In Panel A, the activity of PLA_2 in soluble fractions from HEK293T cells expressing $cPLA_{2\alpha}$ was measured. The assay mixture was further supplemented with the indicated concentrations of L-*t*-DMBn-S1P and D-*e*-S1P. Values are the means ± S.D. for duplicate samples in a typical experiment. In Panel B, translocation of $cPLA_{2\alpha}$-GFP fusion protein by ionomycin was examined. HEK293T cells transiently transfected with the expression vector coding $cPLA_{2\alpha}$-GFP were stimulated with vehicle, 5 μM ionomycin, 20 μM L-*t*-DMBn-S1P and 5 μM ionomycin/20 μM L-*t*-DMBn-S1P. Confocal fluorescence images were recorded at 10 min after the addition. The *scale bars* are equal to 10 μm. The data are typical of three independent experiments.

DMBn-S1P at 5 and 20 μM did not modify the activity, but at 50 μM it was inhibited although D-*e*-S1P had no effect (Fig. 3A). Treatment of intact HEK293T cells expressing $cPLA_{2\alpha}$ with 100 μM L-*t*-DMBn-S1P for 20 min did not change PLA_2 activity; that of the soluble fraction from treated cells was almost the same as that of vehicle-treated cells (data not shown). In the absence of $CaCl_2$ in the assay mixture, the activity in HEK293T cells expressing $cPLA_{2\alpha}$ was low (150–200 dpm), and L-*t*-DMBn-S1P had no effect on the activity (data not shown). We then investigated the effect of L-*t*-DMBn-S1P on the localization of a chimeric protein ($cPLA_{2\alpha}$-GFP) by fusing GFP to the amino terminus of $cPLA_{2\alpha}$. Stimulation of the cells with 5 μM ionomycin triggered the translocation of $cPLA_{2\alpha}$-GFP to the perinuclear region within 1–2 min (Fig. 3B). Stimulation with 10 (data not shown) and 20 μM L-*t*-DMBn-S1P did not cause translocation and did not modify translocation induced by ionomycin.

The L929 cell line expresses $cPLA_2$ (probably $cPLA_{2\alpha}$) and causes AA release in response to TNFα, but its variant C12 cells lack $cPLA_2$ and the resulting TNFα response [25,29,30]. We confirmed that TNFα could not stimulate AA release from C12 cells; basal- and 10 nM TNFα-induced AA release for 6 h were 1.3 ± 0.1 and 3.5 ± 0.1 (percent of total) for L929 cells, and were 1.6 ± 0.1% and 1.8 ± 0.1% for C12 cells, respectively (n = 3). The addition of 50 (data not shown) and 100 μM L-*t*-DMBn-S1P stimulated AA release from L929 and C12 cells for 30 min to a similar degree (Fig. 2D). As with PC12 cells, D-*e*-S1P did not stimulate AA release and the effects of D-*t*-DMBn-S1P on AA release were observed in the absence of extracellular $CaCl_2$ for both cell types (data not shown).

3.3. Inhibition of L-*t*-DMBn-S1P-induced AA release by D-*e*-sphingosine in PC12 and L929 cells

We investigated the effects of several inhibitors of PLA_2 and/or AA release on L-*t*-DMBn-S1P-induced AA release. Treatment with the non-specific PLA_2 inhibitors (50 μM *p*-bromophenacyl bromide and 100 μM mepacrine, Ref. [31]) slightly inhibited the L-*t*-DMBn-S1P response; the values were 14.6 ± 0.8 for the control cells and 11.6 ± 0.5 and 12.8 ± 0.6% for the respective inhibitor-treated cells (means ± S.D. for a typical experiment). Treatment with relatively selective inhibitors of Ca^{2+}-independent PLA_2 (50 μM bromoenol lactone) and $cPLA_2$ (20 μM arachidonyl trifluoromethyl ketone, Ref. [31]) showed no and/or marginal effects. Treatment with inhibitors (100 μM RHC80275 for diacylglycerol kinase, 20 μM U0126 for extracellular signal-regulated kinase pathway, 10 μM SB203580 for p38 kinase, 10 μM Gö6976 for conventional protein kinase Cs), pertussis toxin (1 mg/mL, 24 h, a toxin for the G proteins) or 5 mM dithiothreitol (an inhibitory reagent for secretory PLA_2s) did not inhibit the L-*t*-DMBn-S1P response (data not shown).

Previously, we reported that D-*e*-sphingosine and D-*e*-dihydrosphingosine act as inhibitors of AA release from PC12 cells and $cPLA_{2\alpha}$ activity in vitro [20]. The co-addition of D-*e*-sphingosine and D-*e*-dihydrosphingosine but not DL-*t*-dihydrosphingosine inhibited 100 μM L-*t*-DMBn-S1P-induced AA release in a concentration dependent manner (Fig. 4). The inhibitory effects induced by these sphingosine analogs were observed from 50 μM onward. D-*e*-*N*,*N*-Dimethylsphingosine also inhibited the L-*t*-DMBn-S1P response, but D-*e*-S1P even at 100 μM did not inhibit it. One hundred micromolar L-*t*-DMBn-S1P-induced AA release from L929 cells was inhibited by

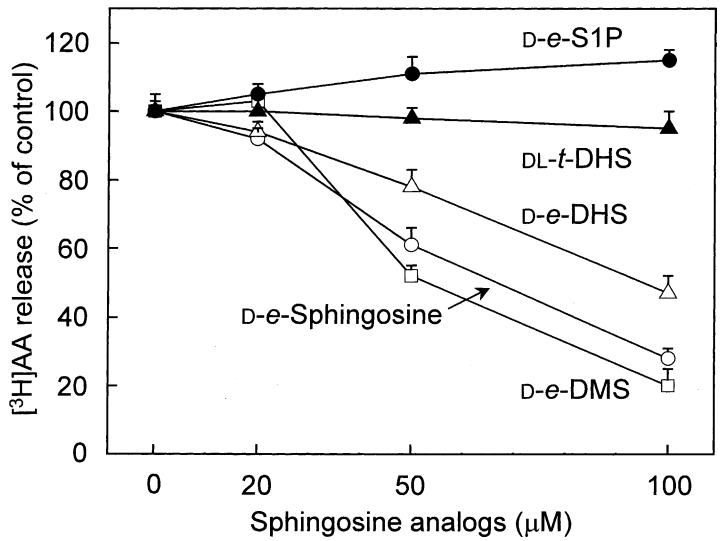

Fig. 4. Inhibitory effects of D-*e*-sphingosine analogs on L-*t*-DMBn-S1P stimulated [^{3}H]AA release in PC12 cells. Labeled PC12 cells were incubated with 100 μM L-*t*-DMBn-S1P in the presence of the indicated concentrations of D-*e*-sphingosine, D-*e*-dihydrosphingosine (D-*e*-DHS), D-*e*-*N*,*N*-dimethylsphingosine (D-*e*-DMS), DL-*t*-dihydrosphingosine (DL-*t*-DHS) and D-*e*-S1P. The data are normalized as the percentage of [^{3}H]AA release induced by 100 μM L-*t*-DMBn-S1P alone. The absolute value of [^{3}H]AA release by L-*t*-DMBn-S1P is shown in Fig. 2. Values are the means ± S.E.M. for three independent experiments done in triplicate.

50 μM D-*e*-sphingosine; the values were 2.8 ± 0.2% for control, 1.8 ± 0.7% for D-*e*-sphingosine-, 15.7 ± 1.8% for L-*t*-DMBn-S1P-, and 6.1 ± 1.1% for D-*e*-sphingosine/L-*t*-DMBn-S1P-treated cells (means ± S.D. for a typical experiment, n = 2).

3.4. Effects of tested S1P analogs on cell death

Treatment with tested S1P analogs at 100 μM for 30 min did not stimulate LDH leakage from PC12 cells; the values were under 5% for the control- (without DMSO), vehicle- (containing 2.5% DMSO) and S1P analog-treated cells. In PC12 cells, serum-deprivation for 24 h alone caused cell toxicity [27]. Similarly, basal (vehicle-induced) LDH leakage over a period of 24 h was 20% in serum-free DMEM in the present study. The vehicle containing 2.5% DMSO alone showed toxicity over 24 h after addition depending on the experiment. Several compounds of the S1P analogs stimulated LDH leakage 24 h after addition. Treatment with D-*e*-S1P and D-*e*-MM-S1P at 50 μM for 24 h stimulated LDH leakage, although D-*e*-C13-S1P, D-*e*-C8-S1P and D-*e*-TM-S1P showed little and/or marginal effect (Table 1). In representative experiments, the ED_{50} of D-*e*-S1P and D-*e*-MM-S1P were 56 ± 7 and 22 ± 4 μM (means ± S.D. for a typical experiment), respectively. Treatment with L-*t*-S1P and L-*t*-DMBn-S1P slightly stimulated LDH leakage, but L-*t*-DM-S1P and L-*t*-Bn-S1P had no effect. The lipophilicity cannot account alone for variations in cell toxicity of S1P analogs, since the effects of lipophilic analogs (L-*t*-Bn-S1P and L-*t*-DMBn-S1P) were limited. Next, we examined the effect of treatment with the S1P analogs for 4 h. In those experiments, PC12 cells were first cultured with the indicated S1P analogs for 4 h, washed with S1P analog-free DMEM and then cultured without S1P analogs/DMSO for an additional 20 h. The values of LDH leakage were 5.3 ± 0.6% for vehicle-treated cells, and 10% (n = 2) and 27.6 ± 2.6% (n = 8) for 30 μM and 50 μM D-*e*-S1P-treated cells, respectively. The effect of 30 μM D-*e*-MM-S1P was significantly greater than that of 50 μM D-*e*-S1P. Treatment with 30 μM D-*e*-TM-S1P stimulated LDH leakage in some experiments, but the effect was not significant because of a wide variation. Treatment with 30 μM L-*t*-DMBn-S1P for 4 h caused significant LDH leakage. Treatment with nerve growth factor (100 ng/mL) and a pan-caspase inhibitor (20 μM) before the addition of S1P analogs decreased LDH leakage induced by D-*e*-S1P and D-*e*-MM-S1P (manuscript in preparation).

In L929 and C12 cells, treatment with 50 μM D-*e*-MM-S1P but not L-*t*-Bn-S1P caused LDH leakage over 24 h after the addition; the values for D-*e*-MM-S1P treatment were 82 ± 10% (mean ± S.D. for two determinations in a typical experiment) and 78 ± 8% in L929 and C12 cells, respectively, and the values for L-*t*-Bn-S1P-treatment were 10 ± 4% and 15 ± 5% in L929 and C12 cells, respectively. The value induced by the vehicle was 10% for both types of cells.

4. Discussion

4.1. Effects of synthetic S1P analogs on AA release

In this study, we examined the effects of several synthetic D-*e*-S1P and L-*t*-S1P analogs on AA release and cell death. Among the tested compounds, D-*e*-TM-S1P, L-*t*-DM-S1P and L-*t*-DMBn-S1P stimulated AA release at pharmacological concentrations (50 and 100 μM) in PC12 cells. In addition, L-*t*-DMBn-S1P at 20 μM stimu-

lated prostanoid production in PC12 cells. L-*t*-DMBn-S1P at these concentrations did not show cell toxicity (LDH leakage) or induce noradrenaline release, and L-*t*-DMBn-S1P-induced AA release was inhibited by D-*e*-sphingosine. Thus, L-*t*-DMBn-S1P seems to be selectively coupled with AA metabolism for at least 30 min.

D-*e*-S1P is the natural ligand for specific G protein-coupled receptors, referred to as EDGs and/or S1P receptors. To date, EDG-1/$S1P_1$, EDG-5/$S1P_2$, EDG-3/$S1P_3$, EDG-6/$S1P_4$ and EDG-8/$S1P_5$ receptors have been identified [1,2]. These receptors are highly specific, binding D-*e*-S1P and D-*e*-dihydro-S1P and not L-*t*-S1P. EDG-5 and EDG-8 are expressed in PC12 cells, and addition of D-*e*-S1P causes responses via cell surface receptors, probably via EDG-5, in PC12 cells [32–35]. However, the effects of L-*t*-DMBn-S1P are not mediated by EDG receptors. The reasoning for this is as follows: (1) D-*e*-S1P between 1 and 100 μM did not stimulate AA release. (2) L-*t*-DMBn-S1P-induced AA release was not inhibited by treatment with pertussis toxin, which decreased S1P responses mediated by EDG receptors in PC12 cells [32–35]. (3) Three ion-pairing interactions are critical for the recognition of D-*e*-S1P by EDG-1; two cationic amino acids, Arg^{120} and Arg^{292}, the anionic phosphate of S1P, as well as an anionic amino acid Glu^{121} and the protonated amino group of S1P [36]. S1P-specific receptors such as EDG-1, -5 and -8, all share corresponding amino acid residues [36]. D-*e*-*O*,*O*-Dimethyl-S1P is inactive upon binding to the cell expressing EDG-1, -3 and -5 [37]. These reports suggest that modifications such as methylation of the C1 phosphate group and the C2 ammonium moiety of D-*e*-S1P eliminate their ability to interact with EDG receptors. In addition, the C3 hydroxy group of D-*e*-S1P is critical for binding to EDG-1, -3 and -5 [37]. In our experiments, *O*,*O*-dimethyl analogs of S1P (D-*e*-TM-S1P, L-*t*-DM-S1P and L-*t*-DMBn-S1P) did not show an agonistic effect on Ca^{2+} mobilization in HeLa cells expressing EDG family receptors (data not shown). However, effective analogs for AA release in the present study were all *O*-methyl analogs. In addition, the *N*-methylated analog (D-*e*-TM-S1P) and the C3 hydroxy group-modified analog (L-*t*-DMBn-S1P) stimulated AA release. (4) The most active analog (L-*t*-DMBn-S1P) was an L-*threo*-form and not D-*erythro*-form. Lim et al. [37] reported that the D-*erythro* configuration of S1P is important for high affinity binding to EDG-1, -3 and -5, and that the L-*erythro*-, D- and L-*threo*-forms of S1P were less effective. Thus, L-*t*-DMBn-S1P does not act as an agonist for reported cell surface EDG receptors, although the possible involvement of unknown receptors cannot be excluded.

D-*e*-S1P also functions as an intracellular messenger in the regulation of responses such as Ca^{2+} homeostasis [2,4,7,8]. S1P is taken up into cells via a member of the ATP binding cassette family of proteins, and uptake influences the availability of S1P to modulate biological effects induced by EDG receptors [9]. In the present study, high concentrations of L-*t*-DMBn-S1P were needed to induce a response (20 μM for PG production and concentrations greater than 50 μM for AA release). S1P analogs in the upper (log $P > 4.92$) lipophilicity range were effective on AA release. Lipophilic analogs of S1P may cross the plasma membrane and then act intracellularly. However, the *3O*-benzyl analog (L-*t*-Bn-S1P) and the *N*-monomethyl analog (D-*e*-MM-S1P), which are lipophilic compounds with larger log *P* values, showed marginal effects. Thus, the lipophilicity cannot account alone for variations in the activities of S1P analogs on AA release. S1P analogs may be taken up via putative S1P transporters into the cells.

4.2. Non-involvement of $cPLA_{2\alpha}$ in L-*t*-DMBn-S1P-stimulated AA release

Ceramide stimulates AA release from cells [16,19,20] and $cPLA_2$ activity (probably α type) in vitro by binding to its Ca^{2+}-lipid binding domain [19]. Kitatani et al. [17] reported that ceramide enhanced Ca^{2+}-dependent translocation of $cPLA_2$ into membrane fractions. These findings suggest an interaction between sphingolipid metabolites and $cPLA_{2\alpha}$. Since L-*t*-DMBn-S1P-induced AA release was inhibited by D-*e*-sphingosine, which inhibited $cPLA_{2\alpha}$ activity in vitro [20], the effect of L-*t*-DMBn-S1P may be mediated by $cPLA_{2\alpha}$ activation. In the present study, however, L-*t*-DMBn-S1P did not modify the activity of $cPLA_{2\alpha}$ in vitro and the translocation of $cPLA_{2\alpha}$-GFP protein in cells. In addition, L-*t*-DMBn-S1P stimulated AA release from C12 cells lacking $cPLA_2$. Thus, pathways other than the $cPLA_{2\alpha}$-dependent system likely mediate L-*t*-DMBn-S1P-induced AA release.

Ca^{2+}-independent and secretory PLA_2s are ubiquitous enzymes, and are expressed in various cell types. Although L-*t*-DMBn-S1P-induced AA release was independent of extracellular $CaCl_2$, the response was enhanced by ionomycin. Treatment with an inhibitor of Ca^{2+}-independent PLA_2 had no effect on the L-*t*-DMBn-S1P response. PLA_2 activity in vitro in the absence of $CaCl_2$, which reflects the enzyme activity of Ca^{2+}-independent PLA_2, was not enhanced by L-*t*-DMBn-S1P. Thus, Ca^{2+}-independent PLA_2 does not appear to be activated by L-*t*-DMBn-S1P. Secretory types of PLA_2 require Ca^{2+} at the millimolar level and disulfide linkages (the S–S bridges) for their activation [38,39]. Treatment with dithiothreitol, which inhibited AA release induced by phenylarsine oxide (a sulfhydryl reagent and thus an activator of secretory PLA_2s) in PC12 cells [40], did not modify the L-*t*-DMBn-S1P response. These findings suggest the involvement of other types of PLA_2 such as γ type $cPLA_2$ [41] and/or other pathway(s) in AA release induced by L-*t*-DMBn-S1P. Evidence suggests that AA is also produced through the hydrolyzation of 2-arachidonoylglycerol and anandamide induced by monoacylglycerol lipase and fatty acid amide hydrolase, respectively [42,43]. Identification of the molecular target(s) and pathway(s) of L-*t*-DMBn-S1P for AA release remained to be solved.

4.3. Interaction between AA release and cell death

The stimulation of PLA_2 causing AA release is essential for cytotoxic action and/or apoptosis by stimulants and stress in various cell types [12,13,44]. Treatment with AA at concentrations ranging 1–100 μM for 24 h caused LDH leakage from PC12 cells [45]. TNFα showed cytotoxic action via the activation of $cPLA_2$ (probably the α type) in L929 cells but not in TNFα-resistant C12 cells lacking $cPLA_2$ [25,29]. In our experiments, however, cell death induced by L-*t*-DMBn-S1P or D-*e*-TM-S1P (potent stimulators of AA release) was less than that induced by D-*e*-MM-S1P or D-*e*-S1P (a week- or non-stimulator for AA release) in PC12 cells. In addition, D-*e*-MM-S1P caused LDH leakage from C12 cells lacking $cPLA_2$, similarly from L929 cells. These findings suggest that D-*e*-S1P and D-*e*-MM-S1P cause cell death without AA release, at least in the tested cell lines.

S1P elicits anti-apoptotic effects in cells in general [1,2]. In PC12 cells, the addition of S1P and expression of sphingosine kinase resulted in the suppression of apoptosis induced by trophic factor withdrawal or ceramides [46]. However, the apoptotic effect of S1P has occasionally been described for various cells including neuronal and PC12 cells [10,34,47]. These reports suggest that the balance between survival and apoptotic (death) signaling pathways and/or the cellular levels of sphingolipid metabolites may be critical for determining the final response to S1P. In the present study, treatment with D-*e*-S1P and D-*e*-MM-S1P at concentrations greater than 20 μM caused cell death in the three cell lines. Cell death induced by D-*e*-S1P or D-*e*-MM-S1P was inhibited by treatment with nerve growth factor and an inhibitor of pan-caspases, as described in Section 4. In a preliminary experiment, D-*e*-MM-S1P acted as a week agonist in HeLa cells expressing EDG family receptors. Like D-*e*-S1P, D-*e*-MM-S1P activated p38 kinase about 5–15 min after addition to PC12 cells (manuscript in preparation). Since activation of p38 kinase is coupled with apoptosis in neuronal cells including PC12 cells [48,49], D-*e*-MM-S1P may activate the signaling pathway causing apoptosis independently mediated by EDG receptors during AA metabolism. Interestingly, cell death induced by D-*e*-MM-S1P was observed at lower concentrations compared with that induced by D-*e*-S1P in PC12 cells. D-*e*-MM-S1P in cells may be more stable than D-*e*-S1P, which is metabolized by enzymes such as S1P phosphatases and lyases [2]. Synthetic S1P analogs are useful tools for studying AA metabolism and cell death induced by sphingolipid metabolites in cells.

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