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Involvement of p38 MAP kinase-mediated cytochrome *c* release on sphingosine-1-phosphate (S1P)- and *N*-monomethyl-S1P-induced cell death of PC12 cells

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Abstract

D-*erythro*-Sphingosine-1-phosphate (S1P), a sphingolipid metabolite, affects various neuronal functions including cell fate. S1P appears to have contradictory effects in PC12 cells, a neuronal model cell line; neurite retraction and cell survival/differentiation. In the present study, we examined whether S1P induces cell death in undifferentiated PC12 cells. Culture with S1P at 20 μM for 4 h caused lactate dehydrogenase leakage 24 h later. The response was reduced by an inhibitor of caspases and accompanied by the release of cytochrome *c* and DNA fragmentation. S1P caused the phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) within 10 min. An inhibitor of p38 MAPK (10 μM SB203580) inhibited both the release of cytochrome *c* and DNA fragmentation induced by S1P. Treatment with nerve growth factor or pertussis toxin (PTX) decreased S1P-induced phosphorylation of p38 MAPK and cell death. These findings suggest that S1P-activated p38 MAPK acts as a death signal upstream of the release of cytochrome *c*. *N*-Monomethyl-S1P (MM-S1P), a weak agonist in cells expressing $S1P_1$ receptors, had marked effects (phosphorylation of p38 MAPK, release of cytochrome *c* and DNA fragmentation) at lower concentrations than S1P and in a PTX-sensitive manner. These findings show that the activation of S1P receptors by S1P and MM-S1P causes cell death accompanied by DNA fragmentation via the p38 MAPK pathway-mediated release of cytochrome *c* in PC12 cells. The potential of S1P and MM-S1P to act as agonists of S1P receptors and as intracellular messengers is discussed.

Keywords: Sphingosine-1-phosphate (S1P); *N*-Monomethyl-S1P; p38 MAPK; Cytochrome *c*; Apoptosis; PC12 cells

1. Introduction

The sphingomyelin cycle has been studied extensively as a general signal transduction pathway regulating cell growth, differentiation and cell survival. Sphingolipid metabolites including ceramide and sphingosine are proposed as a new class of lipid second messengers. D-*erythro*-Sphingosine-1-phosphate (S1P), a sphingolipid metabolite, affects a variety of cellular processes including differentiation and cell death [1–3]. There is evidence in abundance that S1P acts as both an intracellular messenger and an extracellular ligand for a family of five specific G protein-coupled S1P receptors [1–4]. It is widely accepted that many of the actions of S1P are mediated through the EDG family receptors, which include $S1P_1$/EDG1, $S1P_2$/EDG5/AGR16, $S1P_3$/EDG3, $S1P_4$/EDG6 and $S1P_5$/EDG8 [3,5]. The activation of S1P receptors is coupled to multiple effector pathways including the mobilization of Ca^{2+} and stimulation of the mitogen-activated protein kinase (MAPK) family member's extracellular regulated protein kinase (ERK1/2) and p38 MAPK [3,5]. These signaling

Abbreviations: S1P, D-*erythro*-Sphingosine-1-phosphate; MM-S1P, D-*erythro*-*N*-Monomethyl-S1P; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular regulated protein kinase 1/2; NGF, nerve growth factor; PTX, pertussis toxin; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole; LDH; lactate dehydrogenase; ZVAD-fmk, z-Val-Ala-Asp(OMe)-fluoromethylketone; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; TUNEL, TdT-mediated dUTP nick end labeling

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pathways are shown to modulate cell growth, survival and apoptosis in various cell types including neurons [4,6]. However, the signaling pathway(s) regulating apoptosis and/or cell death induced by the activation of S1P receptors has not been elucidated.

PC12 rat pheochromocytoma cells differentiate into sympathetic neurons in response to nerve growth factor (NGF). PC12 cells express $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$ receptors [7–11]. S1P appears to have contradictory effects on apoptosis and/or cell death in PC12 cells. Exogenously added S1P promoted the survival of PC12 cells [12,13]. The activation of sphingosine kinase leading to an increase in intracellular S1P may contribute to the NGF-induced neurite outgrowth [14] and effect on survival [12]. Microinjection of S1P into cells [13] and enforced expression of sphingosine kinase which leads to an increase in intracellular S1P [15] suppressed apoptosis in PC12 cells. By contrast, S1P induced neurite retraction, which is one of the first steps in the apoptotic process of neuronal cells, in PC12 cells [16]. Activation of $S1P_2$ and $S1P_5$ receptors in PC12 cells caused cell rounding, inhibition of neurite extension and neurite retraction [11,17]. It is still unclear whether S1P causes cell death including apoptosis in PC12 cells. NGF withdrawal led to apoptosis via activation of p38 MAPK in differentiated PC12 cells [18,19], but NGF-induced differentiation of the cells was also mediated by the p38 MAPK pathway [20,21]. The role of MAPKs, specifically p38 MAPK, in apoptosis and/or survival has not been well elucidated in neuronal cells including PC12 cells.

Previously, we examined the effects of some synthetic S1P analogs on the release of arachidonic acid and cell death in PC12 cells [22]. Treatment for 4 h with S1P, which did not stimulate arachidonic acid release, caused cell death 24 h later. D-*erythro*-*N*-Monomethyl-S1P (MM-S1P) had a marginal effect on arachidonic acid release but was markedly more toxic than S1P. In the present study, we investigated the signaling mechanism(s) of cell death induced by S1P and MM-S1P in native, undifferentiated PC12 cells. We found that (1) activation of S1P receptors is coupled with the activation of p38 MAPK via pertussis toxin (PTX)-sensitive G-proteins, and causes the release of cytochrome *c*, DNA fragmentation and cell death, and (2) MM-S1P stimulates, probably via S1P receptors, the p38 MAPK pathway and subsequent cell death in PC12 cells.

2. Materials and methods

2.1. Materials

S1P and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole (SB203580) were obtained from Sigma (St. Louis, MO, USA). MM-S1P was prepared by standard methods in our laboratories. The cytotoxicity detection kit for the measurement of lactate dehydrogenase (LDH) activity was from Roche (Mannheim, Germany). z-Val-Ala-Asp(OMe)-fluoromethylketone (ZVAD-fmk) was purchased from Biomol (Plymouth Meeting, PA, USA). NGF, PTX and H_2O_2 were from Wako (Osaka, Japan). DMSO was used as a solvent to dissolve S1P, MM-S1P and SB203580. The concentrations of ZVAD-fmk, NGF, PTX and H_2O_2 were selected based on previous studies [22–24].

2.2. Culture of PC12 cells and LDH leakage assay

PC12 cells were grown in collagen-coated dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS, Thermo-Trace, Melbourne, Australia). Sub-confluent PC12 cells were used at 24 h after the final change of the medium. For experiments, cells were cultured with DMEM containing 0.1% FBS, since serum-deprivation alone resulted in marked cytotoxicity [23,25]. PC12 cells were cultured with S1P or MM-S1P for 4 h, and followed by the S1P-free medium for an additional 20 h. In some cases, PC12 cells were cultured with 50 or 100 ng/mL NGF for 15 h, 20 μM ZVAD-fmk (an inhibitor of caspases) for 2 h or 200 ng/mL PTX for 16 h before the addition of S1P analogs. The S1P analogs were dissolved in DMSO, and the final concentration of DMSO in the medium was under 2%. Although treatment with the vehicle containing DMSO for 24 h was slightly toxic, treatment with the vehicle alone for 4 h had no effect [22]. Cell death was estimated by the LDH leakage method as described previously [23]. LDH leakage (%) was defined as the LDH activity in the culture medium divided by total activity times 100 [(extracellular activity)/(extracellular activity and remaining cellular activity) × 100%].

2.3. Measurement of nuclear DNA fragmentation

For fluorescence microscopy, cells were stained with the chromatin dye Hoechst 33258 (10 μM, Wako). Nuclei of apoptotic cells were observed as fragmented as previously reported [23]. Quantitative analyses for DNA fragmentation were conducted with an apoptosis screening kit (Wako, Osaka, Japan) using TdT-mediated dUTP nick end labeling (TUNEL). Since the absolute values of OD_{492} in the control cells changed depending on the individual experiment (0.39–0.74, $n = 4$), the data were presented as percentages of the control value obtained with vehicle.

2.4. Western blotting of p38 MAPK and ERK1/2

For the detection of p38 MAPK and its phosphorylated form, PC12 cell lysates were fractionated by SDS-PAGE gel, and transferred to polyvinyldifluoride membranes. The blocked membranes were then incubated with the respective antibody. Anti-phospho-p38 MAPK (Thr^{180}/Tyr^{182}) antibody (no. 9211) and anti-p38 MAPK antibody (no.

9212) were obtained from Cell Signaling Tech. (Beverly, MA, USA). Anti-phospho-ERK1/2 antibody (no. V803A) and anti-ERK1/2 antibody (no. V114A) were obtained from Promega (Madison, WI, USA). The immunoreactive bands were visualized using chemiluminescent reagent, and the signals of the bands were quantitated using a computer imaging analysis (NIH image 1.57).

2.5. Detection of cytochrome c levels in cytosolic fractions

PC12 cells were cultured with the appropriate reagents for 3 h. Then, the collected cells were suspended with ice-cold buffer (250 mM sucrose, 10 mM HEPES, 1 mM EDTA, and protease inhibitors, pH 7.4) and homogenized using a glass-Teflon homogenizer at 4 °C. The homogenates were centrifuged at 18,000 × *g* for 20 min. Equal amounts of protein were subjected to Western blotting using anti-cytochrome *c* antibody (PharMingen Int., cat. no. 556433, San Francisco, CA, USA, diluted to 1:500).

2.6. Data presentation and statistics

Values are mean ± S.E.M. of more than three independent experiments. Some data show the results of two independent experiments or are from one experiment representative of two or three. In the case of multiple comparisons, the significance of difference was determined using a one-way analysis of variance followed by the Bonferroni test. *P*-values < 0.05 were considered significant.

3. Results

3.1. Death of PC12 cells caused by S1P and MM-S1P

First, we investigated the toxicity of S1P and MM-S1P by assaying the leakage of LDH 24 h after their addition in the presence of 0.1% FBS. S1P at concentrations greater than 10 μM caused LDH leakage (Fig. 1, panel A). The leakage induced by 10 μM MM-S1P was significant compared with that caused by vehicle, and the maximal response to MM-S1P was obtained at 30–50 μM. The ED_{50} value of MM-S1P was 19.8 ± 3.5 μM ($n = 3$). The cytotoxic effect of MM-S1P was much greater than that of S1P at all concentrations tested. Next, we investigated the effects of treatment with NGF and ZVAD-fmk on 50 μM S1P- and 20 μM MM-S1P-induced cell death (panel B). Basal LDH leakage probably because of serum withdrawal (0.1%) was significantly inhibited in the cells cultured with 50 ng/mL NGF for 15 h and in the cells treated with 20 μM ZVAD-fmk for 2 h. Both treatments significantly inhibited, by about half but not completely, the 50 μM S1P- and 20 μM MM-S1P-induced LDH leakage. Treatment with 100 ng/mL NGF for 15 h, 50 ng/mL NGF for 48 h or

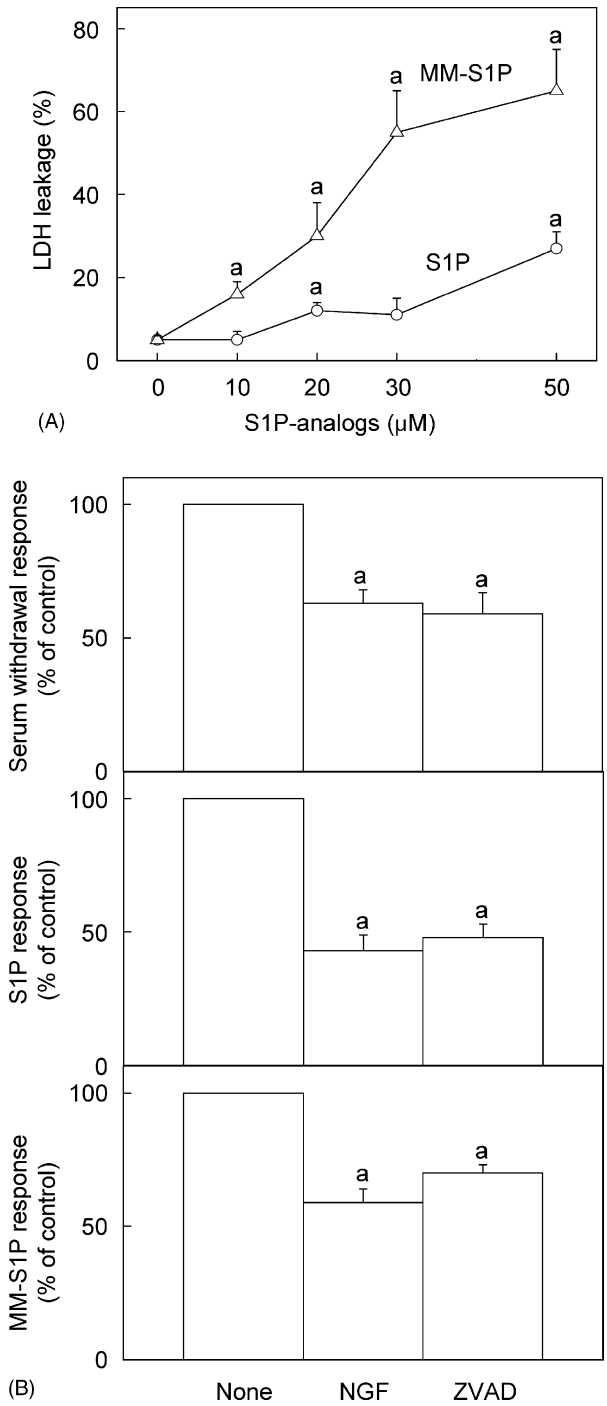

Fig. 1. Inhibition of S1P- and MM-S1P-induced LDH leakage by NGF and ZVAD-fmk. In panel A, PC12 cells were cultured with the indicated concentrations of S1P (○) or MM-S1P (△) for 4 h, and then with fresh medium without the reagents for an additional 20 h. In panel B, cells were cultured with vehicle (none), 50 ng/mL of NGF for 15 h or 20 μM ZVAD-fmk for 2 h, and further cultured with 50 μM S1P and 20 μM MM-S1P for an additional 4 h. Then, the cells were cultured with fresh medium without S1P and MM-S1P in the presence of the same concentrations of NGF and ZVAD-fmk. The LDH leakage (% of control) was normalized as a percentage of LDH leakage induced by vehicle, S1P and MM-S1P, respectively, in the cells not treated with NGF and ZVAD-fmk. The absolute values of LDH leakage (% of total) were 6.2 ± 1.0% in the control (serum withdrawal) cells, 27.8 ± 3.3% in the S1P-treated cells, and 54.5 ± 6.6% in the MM-S1P-treated cells. Data are the mean ± S.E.M. for three independent experiments. [a]$P < 0.05$, significantly different from the value in the control cells.

50 μM ZVAD-fmk for 2 h did not have an additional protective effect, and half inhibited LDH leakage induced by S1P and MM-S1P. PTX treatment (200 ng/mL and 1 μg/mL for 16 h) inhibited partially, not completely, LDH leakage induced by 50 μM S1P and 20 μM MM-S1P (data not shown). Although Edsall et al. [12] reported that culture with 5 μM S1P protected PC12 cells from apoptosis induced by serum withdrawal for 24 h, S1P did not have a protective effect at any concentration under our experimental conditions.

The nuclear staining of PC12 cells with the chromatin dye Hoechst 33258 is shown in Fig. 2, panel A. Addition of 50 μM S1P and 20 μM MM-S1P caused the fragmentation of chromatin 24 h later in some cells. As similar as reported previously [12,26,27], condensation of chromatin in PC12 cells was not obvious under our conditions. The cells showing clear DNA fragmentation accounted for about 10–20% of all cells under our conditions. The treatment with 20 and 50 μM S1P significantly caused DNA fragmentation, as detected by the TUNEL method, 24 h after the addition (panel B). MM-S1P caused DNA fragmentation at much lower concentrations than S1P; the addition of 5 and 10 μM MM-S1P stimulated a response slightly and markedly, respectively. The response induced by 20 μM MM-S1P was almost the same as that evoked by 50 μM S1P. Treatment with 10 μM SB203580 for 5 h, an inhibitor of p38 MAPK, alone caused slight DNA fragmentation 24 h later; the OD_{492} values were larger than those for the control in representative experiments (panel C). DNA fragmentation induced by 50 μM S1P and 20 μM MM-S1P was inhibited in the cells treated with 10 μM SB203580 for 1 h before the stimulation (panel D).

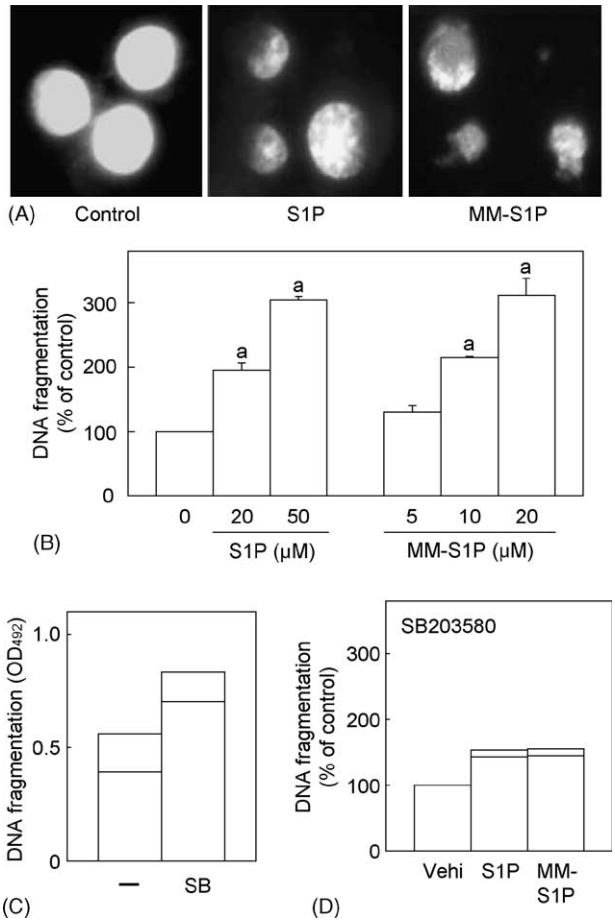

Fig. 2. Nuclear DNA fragmentation induced by S1P and MM-S1P. PC12 cells were cultured with 50 μM S1P and 20 μM MM-S1P for 4 h, and then with fresh medium without the reagents for an additional 20 h. In panel A, fluorescence micrographs were taken of cells stained with Hoechst 33258. Data are representative of three independent experiments. In panels B–D, DNA fragmentation was determined by the TUNEL method 24 h after the addition of S1P and MM-S1P. In panel B, cells were cultured with the indicated concentrations of S1P and MM-S1P for 4 h. The data (% of control) were normalized as percentages of the OD_{492} value obtained with by vehicle. The OD_{492} values were from 0.393 to 0.742, depending on each experiment (0.58 ± 0.15 (S.D.), $n = 4$). $^{a}P < 0.05$, significantly different from the value in the control cells. In panel C, cells were treated with vehicle or 10 μM SB203580 (SB) for 5 h and then cultured with fresh medium without the reagents for an additional 20 h. In panel D, cells were treated with vehicle or 10 μM SB203580 for 1 h, and cultured with vehicle (Vehi), 50 μM S1P and 20 μM MM-S1P for an additional 4 h in the presence of SB203580. Then the cells were cultured with fresh medium without the reagents for an additional 20 h. The data (% of control) were normalized as percentages of OD values in the SB203580-treated cells. In panels C and D, two lines in the bars show results of two independent experiments. The S.D. values of two determinations in a typical experiment were within 10%.

3.2. Phosphorylation of p38 MAPK induced by S1P or MM-S1P and its inhibition by PTX and NGF

Next, we investigated the effects of S1P and MM-S1P on phosphorylation of p38 MAPK in PC12 cells (Figs. 3 and 4). Addition of S1P caused the phosphorylation of p38 MAPK (Fig. 3A). Although the phosphorylation of p38 MAPK induced by 10 μM S1P was not marked at 5 min, it was maximal at 10–20 min in representative experiments. Addition of MM-S1P at 5 μM caused marked phosphorylation of p38 MAPK after 10 min (lanes 8–11). The response induced by S1P or MM-S1P at all concentrations decreased at 60 min after the addition (data not shown). Addition of H_2O_2 caused phosphorylation of p38 MAPK in PC12 cells, as reported previously [21,24,28]. The phosphorylation of p38 MAPK induced by 20 μM S1P and 10 μM MM-S1P, but not by 0.2 mM H_2O_2, was inhibited in the PTX-treated PC12 cells (panel B). Similarly, the responses induced by S1P and MM-S1P were inhibited in the NGF-treated cells (panel C). NGF treatment inhibited H_2O_2-induced phosphorylation of p38 MAPK. Results of quantitative analyses of the data from three independent experiments are shown in Fig. 4. The phosphorylation of p38 MAPK induced by S1P and MM-S1P were significantly decreased by treatment with PTX and NGF. However, the H_2O_2-induced phosphorylation of p38 MAPK was significantly inhibited by NGF treatment, but not by PTX treatment. Co-addition of 20 μM S1P and 10 μM MM-S1P did not have an additive effect and the responses induced by 50 μM S1P and 20 μM MM-S1P were also abolished in the PTX- and NGF-treated cells (data not shown). Treatment of PC12 cells with 10 and 20 μM

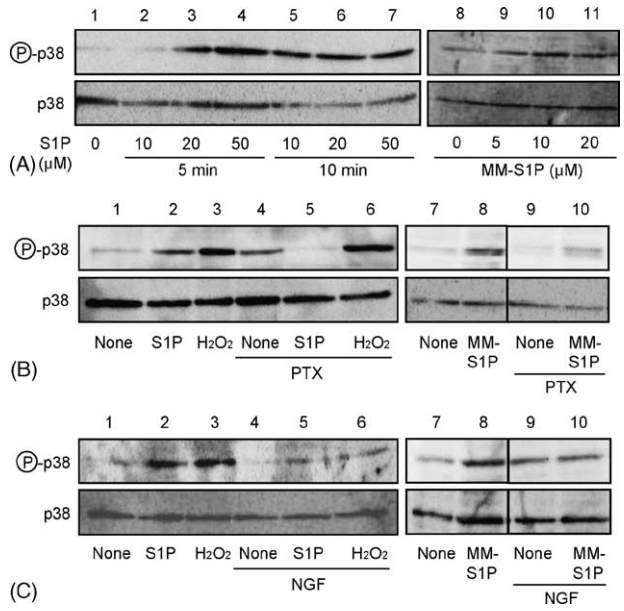

Fig. 3. S1P- and MM-S1P-induced phosphorylation of p38 MAPK and its inhibition by treatment with PTX and NGF. In panel A, PC12 cells were cultured with vehicle (lanes 1 and 8) or the indicated concentrations of S1P (lanes 2–7) and MM-S1P (lanes 9–11) for 5 min (lanes 1–4) and 10 min (lanes 5–11). In panel B, cells were cultured with vehicle (lanes 1–3, 7 and 8) or PTX (200 ng/mL, lanes 4–6, 9 and 10) for 16 h, and further cultured with the vehicle (lanes 1, 4, 7 and 9), 20 μM S1P (lanes 2 and 5), 0.2 mM H_2O_2 (lanes 3 and 6) and 10 μM MM-S1P (lanes 8 and 10) for 10 min. In panel C, cells were cultured with vehicle (lanes 1–3, 7 and 8) and 50 ng/mL of NGF (lanes 4–6, 9 and 10) for 16 h, and further cultured with the vehicle (lanes 1, 4, 7 and 9), 20 μM S1P (lanes 2 and 5), 0.2 mM H_2O_2 (lanes 3 and 6) and 10 μM MM-S1P (lanes 8 and 10) for 10 min. The cell lysates were analyzed by Western blotting with anti-phospho-p38 MAPK (p-p38) and anti-p38 MAPK antibody. Data are representative of three independent experiments. The results of the quantitative analyses are shown in Fig. 4.

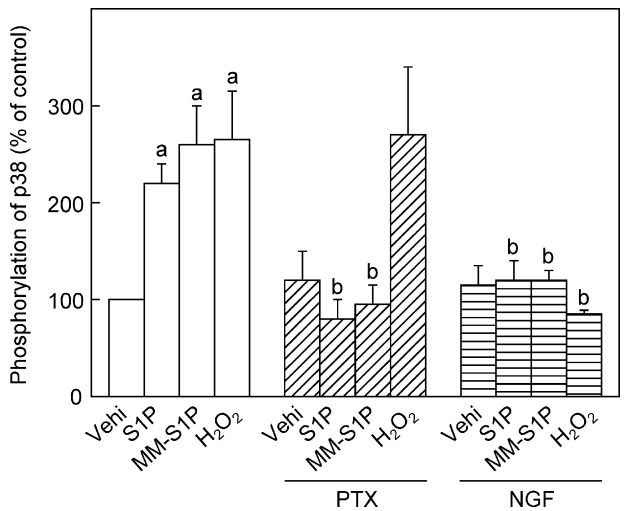

Fig. 4. S1P- and MM-S1P-induced phosphorylation of p38 MAPK and its inhibition by treatment with PTX and NGF. The values were calculated as percentages of the intensity of the band in the control cells. $^{a}P < 0.05$, significantly different from the vehicle in the control cells. $^{b}P < 0.05$, significantly different from the control cells without PTX and NGF treatment.

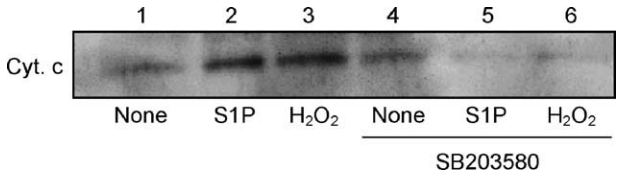

Fig. 5. Increase of cytochrome *c* levels in the cytosolic fractions in PC12 cells treated with S1P and H_2O_2. In panel A, cells were cultured with vehicle (lanes 1–3) or 10 μM SB203580 (lanes 4–6) for 1 h. Then, PC12 cells were cultured with vehicle (lanes 1 and 4), 50 μM S1P (lanes 2 and 5) and 0.2 mM H_2O_2 (lanes 3 and 6) for 3 h in the presence of 0.1% FBS. The levels of cytochrome *c* in the cytosolic fractions were analyzed by Western blotting with anti-cytochrome *c* antibody (Cyt. *c*). Data are representative of two independent experiments.

SB203580 for 1 h did not inhibit the phosphorylation of p38 MAPK induced by stimulants such as S1P and H_2O_2 in PC12 cells (data not shown), as described previously [29,30]. Addition of 20 μM S1P and 10 μM MM-S1P caused phosphorylation of ERK1/2 within 10 min in PC12 cells (data not shown).

3.3. SB203580-sensitive cytochrome c release induced by S1P and MM-S1P

It is established that H_2O_2 caused apoptosis via the release of cytochrome *c* from mitochondria to the cytosol in PC12 cells [28]. Next, we investigated whether S1P causes the release of cytochrome *c* in PC12 cells (Fig. 5). Like 0.2 mM H_2O_2, 50 μM S1P caused the release of cytochrome *c* into the cytosolic fraction 3 h after its addition. The release of cytochrome *c* induced by S1P and H_2O_2 was inhibited in the cells treated with 10 μM SB203580. Treatment with 20 μM MM-S1P for 3 h caused the release of cytochrome *c* in a SB203580-sensitive manner (data not shown). Under our conditions, treatment with 20 μM S1P for longer periods (6 and 12 h) resulted in the release of cytochrome *c* in some experiments but not all.

4. Discussion

4.1. S1P receptor-mediated cell death accompanied by DNA fragmentation in PC12 cells

S1P receptors activate both pro-apoptotic and anti-apoptotic pathways in PC12 cells, as described in Section 1. Under our conditions, S1P did not have anti-apoptotic effect and/or influence survival in undifferentiated PC12 cells. Activation of S1P receptors caused neurite retraction and cell rounding, which are characteristic phenomena in the apoptotic process of neuronal cells, in PC12 cells [11,16,17]. In the present study, we showed that treatment with S1P at concentrations greater than 20 μM for 4 h caused cell death accompanied by the release of cytochrome *c* and fragmentation of DNA at 24 h after the

treatment in undifferentiated PC12 cells. It has been established that activation of p38 MAPK positively regulates an apoptotic pathway in neuronal cells including PC12 cells [18,28,31]. We showed that S1P induced phosphorylation of p38 MAPK and an inhibitor of p38 MAPK abolished the release of cytochrome *c* and DNA fragmentation induced by S1P in PC12 cells. The S1P-induced phosphorylation of p38 MAPK was inhibited in PTX-treated cells, like in endothelial cells expressing $S1P_1$ and $S1P_3$ receptors [32,33]. PTX treatment did not change the S1P-induced activation of p38 MAPK in CHO cells expressing $S1P_2$ (EDG5) receptors [34]. In CHO cells expressing $S1P_5$ receptors, S1P activated the ERK1/2 pathway in a PTX-sensitive manner, but this was not coupled with the activation of the p38 MAPK pathway [9]. Thus, S1P appears to activate S1P receptors (probably $S1P_1$ and/or $S1P_3$ receptors) and subsequently p38 MAPK via PTX-sensitive G proteins in PC12 cells. In addition to the inhibition of p38 MAPK, SB203580 is shown to have other biological effects [30]. Treatment with SB203580 alone induced DNA fragmentation in this study. Thus, the role of p38 MAPK on cell death needs to be confirmed by using the molecular and genetic approach.

The antagonism concerning the response to S1P and cell death may be due to the balance between apoptotic and anti-apoptotic signaling pathways in cells. Like S1P, prostaglandin promoting neurite outgrowth showed both anti- and pro-apoptotic effects accompanied by a change of MAPK pathways in PC12 cells [35]. Stimulation of $S1P_{1-4}$ receptors activates the ERK1/2 pathway which contributes to cell survival in PC12 cells [5,15]. Co-treatment with NGF and peroxynitrite resulted in a facilitation of PC12 cell death, although pre-treatment with NGF protected against cell death [36]. Undifferentiated PC12 cells exhibited greater sensitivity to the induction of apoptosis by microinjected cytochrome *c* than differentiated cells [37]. The diversity of intracellular signaling, its balance or unidentified factors up- and down-stream of mitochondria may change depending on the culture conditions, cell types tested and levels of differentiation in PC12 cells.

4.2. p38 MAPK-mediated release of cytochrome c induced by S1P

Activation of p38 MAPK appears to regulate the apoptosis of neuronal cells via two mechanisms; regulation of gene transcription and post-translational modifications such as protein phosphorylation and translocational changes. In the present study, we showed that the activation of S1P receptors stimulates the release of cytochrome *c* within 3 h of the addition of S1P, and treatment with SB203580 inhibited the S1P-induced release and DNA fragmentation in PC12 cells. The activation of p38 MAPK can cause the translocation of Bax protein from the cytosol to mitochondria and the resulting release of cytochrome *c* and activation of caspases in neuronal cells undergoing apoptosis induced by nitric oxide and cyanide [38,39]. Sarker et al. [31] reported that the p38 MAPK pathway may lie upstream of Bax translocation during anandamide-induced PC12 cell apoptosis, but the response was not mediated by specific receptors such as cannabinoid CB and vanilloid receptors. Our results suggest that stimulation of S1P receptors activates the p38 MAPK pathway and then cell death via the release of cytochrome *c* and DNA fragmentation in PC12 cells, in addition to stimulation with chemicals that react with cells in a non-receptor-mediated manner. Activation of p38 MAPK regulates the transcription factors and/or pro-apoptotic gene expression resulting in cell death in various cells including PC12 cells [6,40]. The mechanism of S1P receptor-mediated apoptosis and/or cell death remains to be elucidated.

As reported in the case of the c-Jun N-terminal kinase pathway [18], NGF treatment blocked S1P-induced cell death upstream of p38 MAPK activation in PC12 cells. NGF treatment for 24 h down-regulated the expression of $S1P_2$, $S1P_3$ and $S1P_5$ receptors but not $S1P_1$ receptors in PC12 cells [7,8,11]. This may explain the protective effect of NGF against S1P-induced cell death. However, phosphorylation of p38 MAPK induced by H_2O_2 was also inhibited by NGF treatment in the present study, and NGF treatment inhibited apoptosis and/or cell death induced by various kinds of stimulation in PC12 cells [23,25,41]. The mechanism by which NGF protects against S1P-induced cell death remained to be clarified.

4.3. MM-S1P as an agonist for S1P receptors

Like S1P, MM-S1P caused phosphorylation of p38 MAPK in a PTX-sensitive manner and cell death (DNA fragmentation and LDH leakage) in PC12 cells. ERK1/2 was phosphorylated 10 min after MM-S1P was added (data not shown). Addition of 10 μM MM-S1P caused a rapid increase, within 30 s, in the intracellular Ca^{2+} concentration in HeLa cells expressing $S1P_1$ receptors, although the effect was half of that induced by 10 μM S1P (manuscript in preparation). Thus, MM-S1P appears to be a ligand for some membrane receptors, probably S1P receptors. *N*-Methyl-D-*erythro*-sphingosine is an endogenous compound with some biological effects [42,43]. It should be determined whether MM-S1P is synthesized endogenously by enzymes including sphingosine kinases. Parrill et al. [44] reported that the basic amino acids Arg^{120} and Arg^{292} of the $S1P_1$ receptor ion-pair with the phosphate of S1P and the acidic Glu^{121} residue ion-pairs with the amino moiety of S1P, and the requirement of these interactions for specific ligand recognition has been confirmed. Sanna et al. [45] concluded that full agonism for S1P receptors does not require phosphate-head group interactions, by using a ligand (SEW2871) structurally unrelated to S1P. However, the phosphate moiety of S1P appears to be necessary for the signaling for PC12 cell death, since D-

erythro-N,O,O-trimethyl-S1P did not cause cell death [22] or phosphorylation of p38 MAPK (data not shown). Our findings may suggest that *N*-mono-methylation of S1P is not critical for the death signaling. Forrest et al. [46] reported that S1P analogs having the phosphate-head group and a modified amino moiety still have agonistic activity toward some S1P receptors. Pharmacological analyses using D-*erythro-N,N*-dimethyl-S1P and other analogs are in progress in our laboratory, in order to elucidate the role of the phosphate and amino moieties of S1P. S1P receptors such as $S1P_1$ and $S1P_3$ couple with multiple signaling pathways in the presence and absence of G proteins [3,5]. MM-S1P preferentially stimulates S1P receptors coupled with PTX-sensitive G proteins and p38 MAPK activation, rather than S1P receptors coupled with the Ca^{2+} signaling pathway.

4.4. Metabolism and possible intracellular action of S1P and MM-S1P

Extra and intra-cellular levels of sphingolipids including S1P are regulated by various pathways and enzymes [1,4]. FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol) is a sphingosine analogue that, when phosphorylated, functions as an agonist for four of the five S1P receptors ($S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ but not $S1P_2$) and as an immunosuppressant [2,47]. FTY720-phosphate was more potent in inducing lymphopenia than S1P when administered to rats, perhaps due to reduced degradation by endogenous enzymes such as the non- and S1P-selective phosphohydrolases and S1P lyases [2,47]. MM-S1P may be more resistant to enzymatic degradation and so trigger cell death at lower concentrations than S1P.

Addition of 10 μM S1P markedly stimulated phosphorylation of p38 MAPK within 10 min but had caused little cell death 24 h later. S1P and MM-S1P are lipophilic compounds [22], and S1P can be taken up into cells [2,4]. FTY720 stimulated the release of cytochrome *c* in isolated Bcl-2-poor mitochondria from Jurkat cells [48]. These findings and reports raise the possibility that S1P and MM-S1P are incorporated into cells and show intracellular effects leading to cell death, in addition to activation of p38 MAPK via S1P receptors on cell membranes. Another possibility is the conversion of S1P to sphingosine or ceramide in cells [1,4]. It is difficult to exclude the possibility that the pro-apoptotic effects of S1P in present study are caused by a conversion of S1P to sphingosine or ceramide. A change in sphingolipid metabolites including ceramide induces both apoptosis and necrotic cell death in PC12 cells [41,49,50]. These possibilities may explain that a greater concentration of S1P than 20 μM was needed for cell death. The intracellular actions and/or influence of intracellular sphingolipid metabolism induced by S1P and MM-S1P remain to be elucidated.

Interestingly, the protective effects induced by NGF, PTX and ZVAD-fmk treatment on S1P- and MM-S1P-induced LDH leakage were only 50% complete. The number of cells having clear DNA fragmentation was limited as described in Section 3, and some portion of cells showed a swollen or burst morphology (data not shown). It has been shown that various stimuli including cytokines and ischemia can cause both apoptosis and necrosis in the same cell population [51,52]. In some cases, necrosis is implicated to be involved in programmed cell death under normal physiological conditions [51,52]. Further studies are needed to clarify the role of S1P and sphingolipids on apoptosis and necrosis in neuronal cells.

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