

Activation of S1P₂ receptor, a possible mechanism of inhibition of adipogenic differentiation by sphingosine 1-phosphate

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Received March 19, 2014; Accepted August 29, 2014

DOI: 10.3892/mmr.2014.2810

Abstract. Sphingosine 1-phosphate (S1P) belongs to a significant group of signaling sphingolipids and exerts most of its activity as a ligand of G-protein-coupled receptors. In our previous study, S1P demonstrated a novel biological activity with the anti-adipogenesis of 3T3-L1 preadipocytes. In the present study, we identified a possible mechanism of S1P-mediated anti-adipogenic effects, particularly in target pathways of the S1P receptors, including S1P₁ and S1P₂. The mRNA levels of S1P₁ and S1P₂ receptors were increased by MDI media treatment, whereas S1P treatment highly induced S1P₂ but not S1P₁ receptor protein in adipocytes. Triglyceride accumulation assay using an agonist and antagonist of S1P receptors revealed that S1P₂ receptor was only involved in S1P-mediated anti-adipogenic effects. Furthermore, pharmacological inhibition of S1P₂ signals completely retrieved S1P-mediated downregulation of the transcriptional levels of peroxisome proliferator-activated receptor γ , CCAAT/enhancer binding protein α and adiponectin, which are markers of adipogenic differentiation. This study demonstrated that S1P₂ receptor signals may regulate the S1P-mediated anti-adipogenic differentiation and also identifies the S1P₂ receptor as a possible mechanism of anti-adipogenic differentiation.

Introduction

Sphingolipids are ubiquitous components of eukaryotic cell membranes, which can be metabolized to ceramide, sphingosine and their phosphorylated forms, including ceramide 1-phosphate (C1P) and sphingosine 1-phosphate (S1P), which possess bioactivity and vital biological functions in cell growth and survival (1). Experimental studies have denoted S1P as one of the most significant sphingolipid metabolites (2). S1P plays various roles in physiological processes, promotes cellular proliferation, stimulates cell survival and protects cells against apoptosis through G-protein-coupled receptors (GPCRs) or an intracellular receptor-independent mechanism (3,4).

Obesity represents a major risk factor of cardiovascular and endocrine-related diseases, and is defined as an excessive deposit of adipose tissue and cholesterol. Adipogenesis is the process whereby preadipocytes undergo differentiation into mature adipocytes. Adipocytes are derived from mesenchymal stem cells, which have the potential to differentiate into myoblasts, chondroblasts, osteoblasts or adipocytes (5). Adipocyte differentiation involves an elaborate network of transcription factors that regulate the expression of numerous genes responsible for the phenotype of mature adipocytes (6). The main transcription factor that mediates preadipocyte differentiation is peroxisome proliferator-activated receptor γ (PPAR γ), which is considered the 'master regulator of adipogenesis'. Other adipogenic transcription factors include the CCAAT/enhancer binding proteins (C/EBP α , C/EBP β and C/EBP γ) (6-8).

The ATP-dependent phosphorylated form of sphingosine, S1P, may only be produced from sphingosine by sphingosine kinases, and S1P phosphatase dephosphorylates S1P to sphingosine (9,10). Acylation of sphingosine and other long-chain base sphingolipids leads to the formation of ceramide in the turnover pathway (11). S1P has been identified in numerous mammalian cells and other organisms, and harbors distinctive biological functions (13).

Sphingolipids have emerged as multifunctional intra- and intercellular signal-transducing molecules, in addition to performing their well-established roles as structural components of cellular lipid membrane bilayers. S1P belongs to a significant group of signaling sphingolipids recognized to play a role in a diverse array of cellular processes, including

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Abbreviations: S1P, sphingosine 1-phosphate; PPAR γ , peroxisome proliferator-activated receptor γ ; C/EBP α , CCAAT/enhancer binding protein α

Key words: sphingosine 1-phosphate, S1P₂ receptor, 3T3-L1, adipocyte, differentiation

apoptosis, cell motility, differentiation and proliferation in a variety of cell types, including endothelial cells, smooth muscle cells and macrophages (14,15).

SIP is generated by the phosphorylation of the sphingosine mediated by sphingosine kinases 1 (Sphk-1) and 2 (Sphk-2) (16). These two isoforms have been identified in mammals. Sphk enzyme activity is expressed in various cell types, and is dynamically regulated by various extracellular stimuli. SIP exerts most of its activity as a ligand of GPCRs (17). Chemotherapy induces downregulation of SIP by inhibiting Sphk. Lowering of the circulating SIP by chemotherapy may switch SIP-mediated adipose cell stasis to adipogenesis. However, the signal pathways of SIP and the SIP receptors in the differentiation of preadipocytes in the context of cellular physiology remain largely unknown.

In the present study, we examined the hypothesis that SIP regulates adipogenic differentiation and modulates its functions via SIP receptor 2 (SIP₂). We provide evidence that the adipogenesis of cultured mouse 3T3-L1 preadipocytes is correlated with the SIP₂ receptor, and further demonstrate that pharmacological inhibition of SIP₂ signals completely retrieved SIP-mediated downregulation of the transcriptional levels of PPAR γ , C/EBP α and adiponectin, which are markers of adipogenic differentiation.

Materials and methods

Reagents. SIP was purchased from Cayman Chemical (Ann Arbor, MI, USA) and Sigma-Aldrich (St. Louis, MO, USA). SIP was prepared as a 2-mM solution in 0.3 M NaOH and then further diluted in cell culture medium. SEW2871, a SIP₁ agonist, and W146, a SIP₁ antagonist, were purchased from Cayman Chemical. The SIP₂ JTE-013 antagonist (Cayman Chemical) was prepared as a 2-mM solution in ethanol and then further diluted in cell culture medium. Anti-SIP2 goat polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture and differentiation. The 3T3-L1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum and antibiotics (100 $\mu\text{g}\cdot\text{ml}^{-1}$ gentamycin and 100 $\mu\text{g}\cdot\text{ml}^{-1}$ penicillin-streptomycin). To induce differentiation, 2-day postconfluent 3T3-L1 cells were incubated in MDI induction medium (DMEM containing 10% fetal bovine serum, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone and 1 $\mu\text{g}/\text{ml}$ insulin) for 2 days. In certain experiments, SIP (10 μM), SEW2871 (from 0.1 to 50 μM), W146 (from 0.01 to 10 μM) and JTE-013 (from 0.02 to 2 μM) were added at the time of the induction of differentiation. Two days after MDI media treatment (day 2), the cell number was determined by a trypan blue exclusion test (Life Technologies, Grand Island, NY, USA) and the medium was changed to insulin medium. The AdipoRed assay and detection of glycerol release contents were performed on day 7.

Quantification of lipid content. Lipid content was quantified using the commercially available AdipoRed assay reagent (Lonza, Verviers, Belgium) according to the manufacturer's

instructions. In brief, preadipocytes grown in 24-well plates were incubated with MDI medium or test compounds, including SIP, SEW2871, W146 and JTE-013, during the adipogenic phase and, on day 7, culture supernatant was removed and the cells were carefully washed with 500 μl phosphate-buffered saline (PBS). The wells were then filled with 300 μl PBS, and 30 μl AdipoRed reagent was added and incubated for 10 min at 37°C. Fluorescence was measured with an excitation of 485 nm and emission of 572 nm.

Real-time polymerase chain reaction (PCR). Total RNA was extracted from 3T3-L1 cells treated with SIP using the Easy-spinTM total RNA extraction kit (Intron Biotechnology, Seoul, Korea). cDNA synthesis was carried out following the instructions of the Takara PrimeScriptTM First Strand cDNA synthesis kit (Takara Bio, Tokyo, Japan). For real-time PCR, 1 μl gene primers with SYBR-Green (Bio-Rad Laboratories, Hercules, CA, USA) in 20 μl reaction volume was applied. The sequences of the primers used for real-time PCR were as follows: SIP₁ (forward, 5'GAAACTACACAACGGGAG CAACAG3' and reverse, 5'AAGCAGGAGCAGAGTGAAGACG3'), SIP₂ (forward, 5'AACAGCAAGTTCCACTCAGCAATG3' and reverse, 5'GGCGGAGAGCGTGATGAAGG3'), PPAR γ (forward, 5'CGGAAGCCCTTTGGTGACTTTATG3' and reverse, 5'GCAGCAGGTTGTCTTGGATGTC3'), C/EBP α (forward, 5'CGGGAACGCAACAACATCGC3' and reverse, 5'TGTCCAGTTCACGGCTCAGC3'), adiponectin (forward, 5'TGACGGCAGCACTGGCAAG3' and reverse, 5'TGATACTGGTTCGTAGGTGAAGAGAAC3'), β -actin (forward, 5'TGAGAGGGAAATCGTGCGTGAC3' and reverse, 5'GCTCGTTGCCAATAGTGATGACC3'). All reactions with iTaq SYBR-Green Supermix were performed on the CFX96 real-time PCR detection system (Bio-Rad Laboratories).

Western blot analysis. The 3T3-L1 cells were lysed in a lysis buffer (25 mM HEPES; pH 7.4, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 0.1 mM dithiothreitol and protease inhibitor mixture). Proteins were electrophoretically resolved on an 8-15% sodium dodecyl sulfate gel, and immunoblotting was performed as previously described (18). Images were captured using the Fusion FX7 acquisition system (Vilbert Lourmat, Eberhardzell, Germany). The antibodies used for immunoblotting were PPAR γ , SIP₁, SIP₂ (Santa Cruz Biotechnology, Inc.) and β -actin (Sigma-Aldrich).

Statistical evaluation. All data are expressed as the mean \pm SEM, and the data were compared using Student's t-test, analysis of variance and Duncan's multiple range test, with the SAS statistical package (SAS Institute, Inc., Cary, NC, USA). P<0.05 and P<0.01 were considered to indicate a statistically significant difference.

Results

SIP₁ and SIP₂ receptor expression in preadipocytes and differentiated adipocytes. When 3T3-L1 cells were differentiated for over 6 days in the presence of SIP, lipid accumulation was reduced dose-dependently (Fig. 1A). The biological action

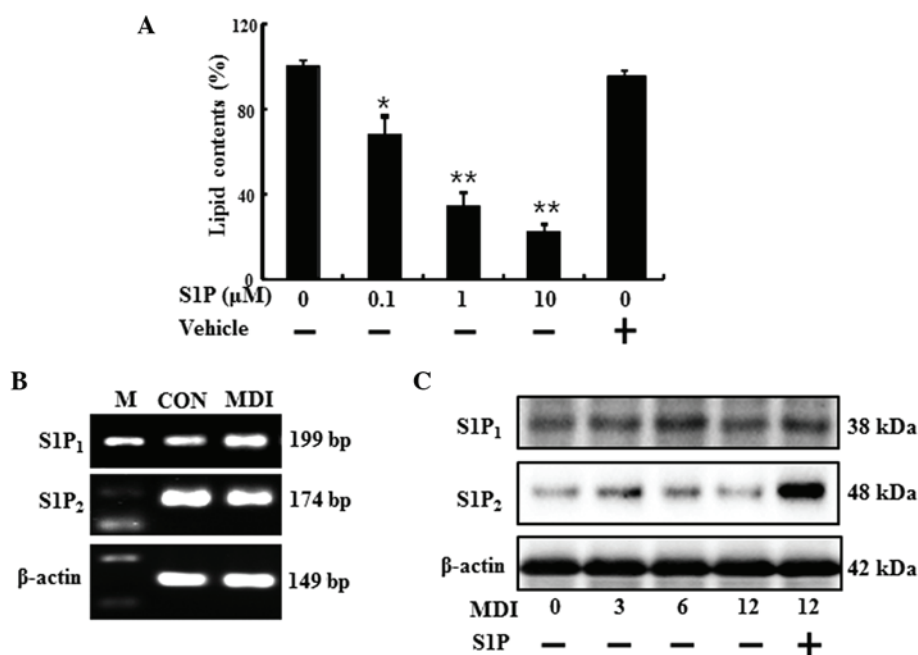


Figure 1. Sphingosine 1-phosphate (SIP) inhibits lipid accumulation and detection of mRNA and protein for S1P₁ and S1P₂ receptors. (A) Preadipocytes were induced to differentiate with increasing concentrations of SIP. *P<0.05 and **P<0.01. (B) Real-time polymerase chain reaction was performed from 1 μg total RNA extracted from 3T3-L1 preadipocytes and differentiated adipocytes. M, marker; CON, control; MDI, MDI induction media treatment. (C) Preadipocytes were induced to differentiate with or without 10 μM SIP harvested at 3, 6 and 12 h during the differentiation period. S1P₁ and S1P₂ receptor were analyzed by immunoblot assay with antibodies specific to S1P₁ and S1P₂ receptor.

of SIP is largely ascribed to ligation to specific S1PRs, which evokes distinct biological responses. To assess which receptor is associated with the SIP anti-adipogenic effect, the expression pattern of the known S1P-specific receptors was studied in the 3T3-L1 cells following the addition of MDI (Fig. 1B and C). As shown in Fig. 1B, the preadipocytes and differentiated adipocytes expressed mRNA for S1P₁ and S1P₂ receptors, as indicated by the presence of 199- and 174-bp bands, respectively. In addition, the treatment of SIP increased the protein expression of S1P₂ receptor (Fig. 1C). These results indicated that preadipocytes and differentiated adipocytes express S1P₁ and S1P₂ receptor, which are known to be associated with cell differentiation, and these receptors may be correlated with the anti-adipogenic function of SIP.

S1P₁ receptor does not affect adipogenic differentiation. To test the involvement of the S1P₁ receptor in anti-adipogenic differentiation, 3T3-L1 preadipocytes were stimulated with SEW2871, a S1P₁ selective agonist, to specifically activate the S1P₁ receptor. As shown in Fig. 2A and B, SEW2871 had no effect on the adipocyte differentiation inhibitory action of SIP. In addition, the inhibitory action of SIP on lipid accumulation was not restored by blocking the S1P₁ receptor using W146, a selective S1P₁ antagonist (Fig. 2C and D). These results demonstrate that the anti-adipogenic action of SIP does not occur through the S1P₁ receptor.

Adipogenic differentiation is regulated via activation of the S1P₂ receptor and blockade of the S1P₂ receptor using S1P₂ antagonist. To determine whether the S1P₂ receptor was involved in the anti-adipogenic activity of SIP, adipocyte differentiation was analyzed in mouse 3T3-L1

preadipocytes. Day 0 postconfluent 3T3-L1 preadipocytes were induced to differentiate into adipocytes using the standard differentiation protocol and co-treatment with S1P₂ antibody, dose-dependently (0.05–0.4 μg), or S1P (10 μM). The effect of S1P₂ antibody was significant at 0.1 μg and was maximal at 0.4 μg, having the same anti-adipogenic effect of 10 μM SIP (Fig. 3A and B). Differentiation of the preadipocytes was induced by MDI treatment and co-treatment with JTE013 as a S1P₂ antagonist at various doses (from 0.02 μM to 2 μM) and/or 10 μM SIP during the adipogenesis period. When JTE013 was added to the cultures, the reduction of lipid accumulation by SIP, compared with the MDI control, was restored as treatment with JTE013 increased (Fig. 3C and D). The effect of JTE013 was significant at 0.2 μM and was maximal at 2 μM. At 0.2 μM JTE013, lipid accumulation returned to the triglyceride level of the MDI control. Moreover, 2 μM of JTE013 increased lipid accumulation compared with MDI induction only. A single treatment of JTE013 alone enhanced adipocyte differentiation (Fig. 3C and D).

SIP induces downregulation of PPARγ, C/EBPα and adiponectin expression. Next, the mRNA levels of the key genes of differentiation were examined to evaluate the effects of SIP on the regulation of the main transcriptional factors in adipogenesis via the S1P₂ receptor. The 3T3-L1 cells underwent differentiation with MDI treatment and were co-treated with 10 μM SIP and/or 0.2 μM JTE013. After two days, the cells were collected for real-time PCR. A reduction in the mRNA levels of PPARγ, C/EBPα and adiponectin was observed with the addition of SIP; however, the S1P₂ antagonist effectively restored the mRNA levels of PPARγ, C/EBPα and adiponectin

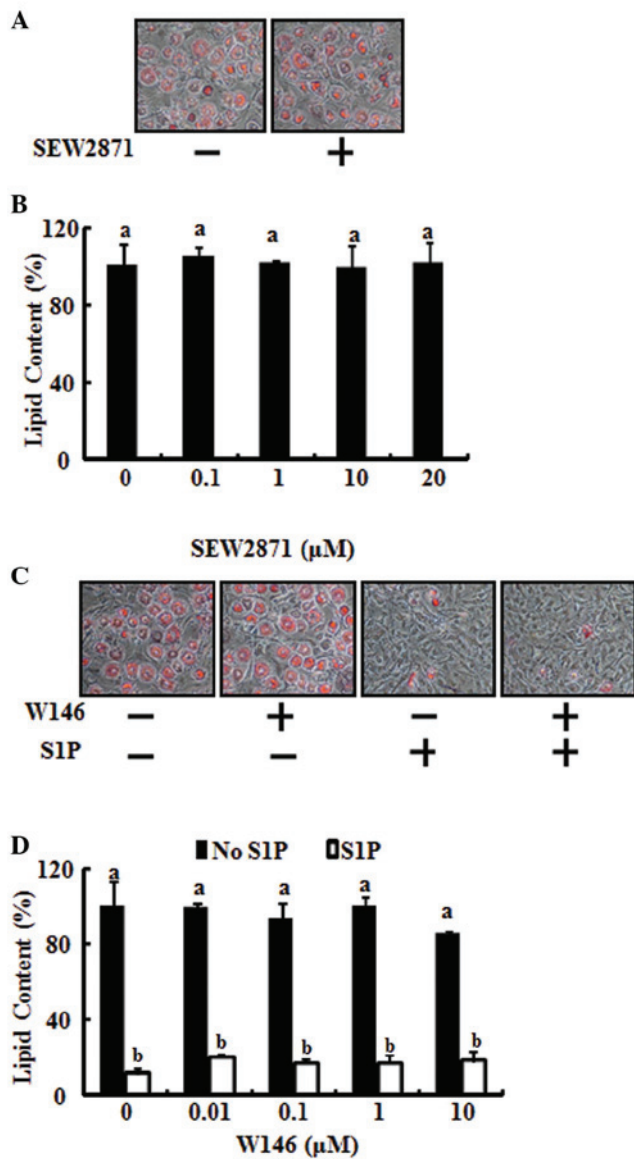


Figure 2. Sphingosine 1-phosphate receptor 1 (SIP₁) is not correlated with the anti-adipogenic effects of SIP. (A-D) Preadipocytes were induced to differentiate with SEW2871 (A and B), known as SIP₁ receptor selective agonist, or W146 (C and D), known as SIP₁ receptor antagonist, at increasing concentrations for 6 days. (A and C) The AdipoRed assays (Lonza, Verviers, Belgium) were performed on day 6 and captured with a light microscope (magnification, x200). (B and D) Fluorescence was measured with excitation of 485 nm and emission of 572 nm. The bar graphs indicate the mean ± SEM (*n*=3). The data were analyzed using analysis of variance and Duncan's multiple range test (*P*<0.05). Means sharing a common alphabetical symbol did not significantly differ.

to the level of the MDI control (Fig. 4A, D and E). In addition, the protein expression of PPAR γ showed the same results as the mRNA levels (Fig. 4B). In addition, the anti-mitotic activity of C/EBP α induces 3T3-L1 cell arrest (19). Following the initial phase of DNA replication, 3T3-L1 cells are arrested in the G1 phase of the cell cycle. The arrest coincides with the induction of C/EBP α expression (20). These previous studies supported our results that SIP treatment increases the number of cells 48 h after MDI induction, which coincides with a lowering of C/EBP α expression. However, in the presence of JTE013,

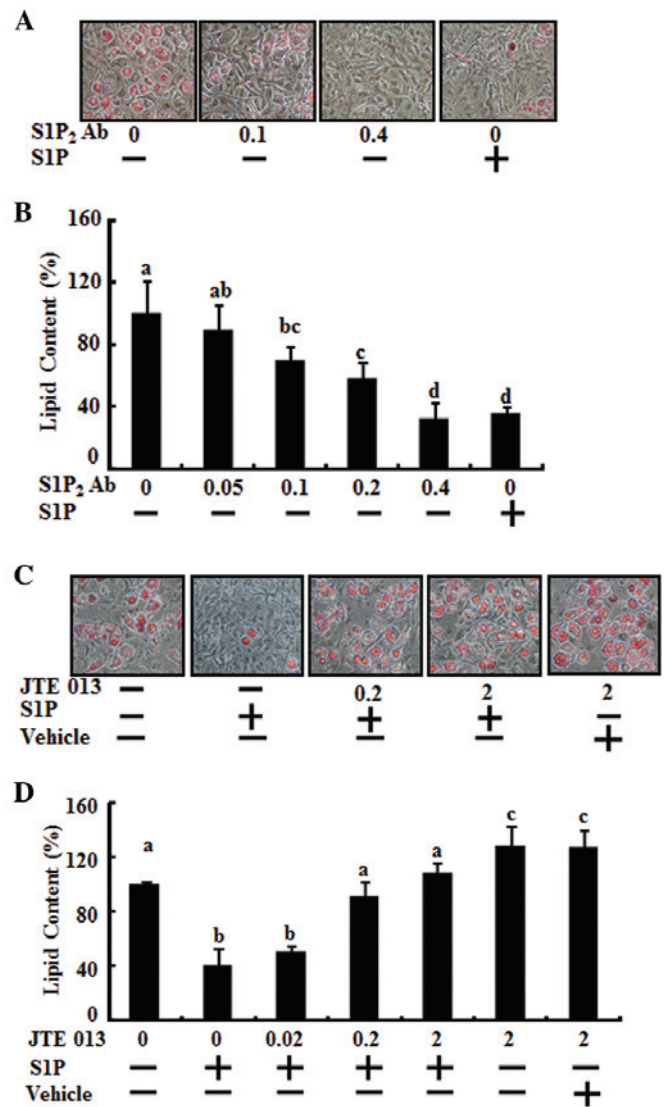


Figure 3. Treatment of sphingosine 1-phosphate receptor 2 (SIP₂) antibody inhibits the triglyceride accumulation of SIP whereas the blockade of SIP₂ receptor reverses the inhibition of triglyceride accumulation by SIP. (A and B) AdipoRed assays (Lonza, Verviers, Belgium) for triglycerides in 3T3-L1 cells differentiated for 6 days with 3-isobutyl-1-methylxanthine (IBMX), dexamethasone and insulin plus SIP₂ antibody (dose-dependently; 0.05-0.4 μ g), normal goat IgG (0.4 μ g) or SIP 10 μ M. On day 7, cells stained by AdipoRed assays were captured with a light microscope (magnification, x200). Fluorescence was measured with excitation of 485 nm and emission of 572 nm. The bar graphs indicate the mean ± SEM (*n*=3). (C) AdipoRed assays for triglycerides in 3T3-L1 cells differentiated for 6 days with IBMX, dexamethasone and insulin plus 10 μ M SIP or vehicle control (0.3N NaOH) and/or JTE013, known as SIP₂ antagonist, dose-dependently. AdipoRed assays were performed as described in Fig. 3A. (B and D) Fluorescence was measured as described in Fig. 3B. The bar graphs indicate the mean ± SEM (*n*=3). The data were analyzed using analysis of variance and Duncan's multiple range test (*P*<0.05). Means sharing a common alphabetical symbol did not significantly differ.

the numbers of cells were lowered and the C/EBP α expression was reversed similar to MDI induction in the controls (Fig. 4C and 4D). It was observed that cell numbers of the MDI induction were doubled in the control and those of the SIP treatment were similar to the control. There were no significant quantitative differences between the control and MDI induction. MDI induction and SIP co-treatment increased cell numbers effectively compared with MDI treatment alone (Fig. 4C). These

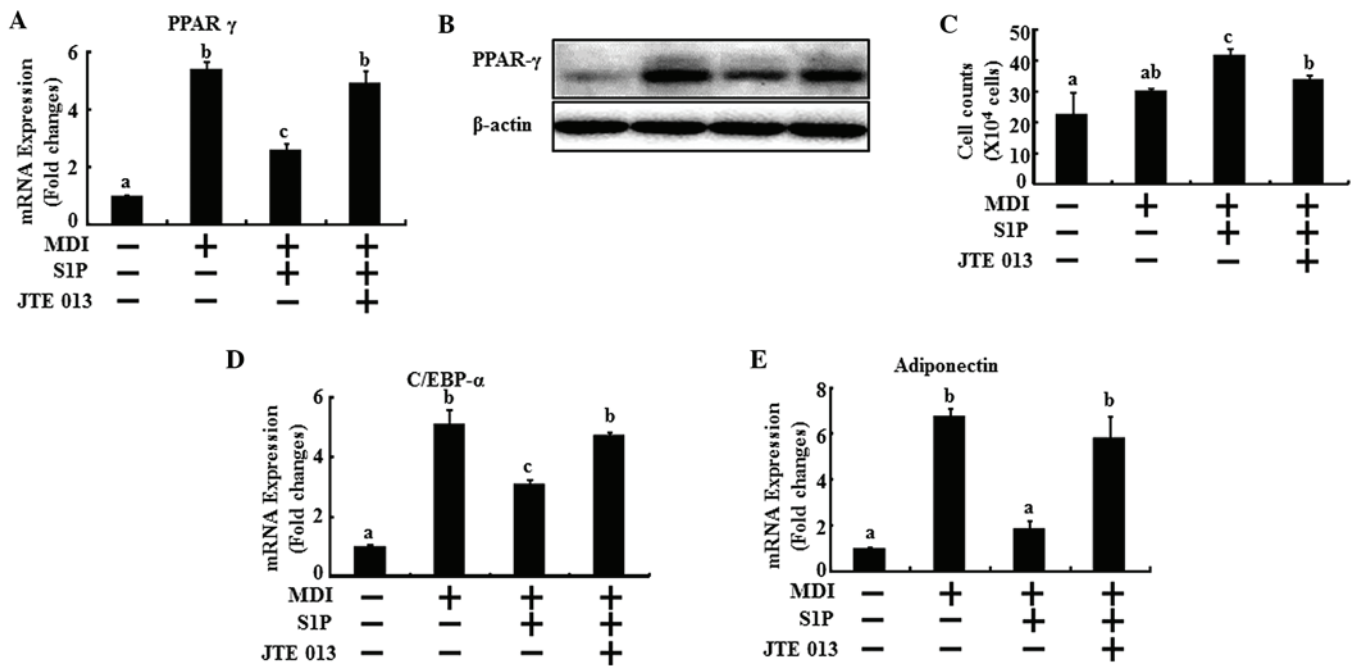


Figure 4. Spingosine 1-phosphate receptor 2 (SIP_2) is correlated with downregulation of peroxisome proliferator-activated receptor γ ($PPAR\gamma$), CCAAT/enhancer binding protein α ($C/EBP\alpha$) and adiponectin expression induced by SIP. (A) Preadipocytes were induced to differentiate with $10 \mu\text{M}$ SIP or/and $0.2 \mu\text{M}$ JTE013 and harvested on day 2 during the differentiation period. The mRNA expression of $PPAR\gamma$ (A), $C/EBP\alpha$ (D) and adiponectin (E) was analyzed by real-time polymerase chain reaction. Values are the mean \pm SD of data from three separate experiments; each experiment was performed in triplicate. (B) Preadipocytes were treated as described in Fig. 4A. On day 2, the protein levels of $PPAR\gamma$ were analyzed by western blot analysis using antibodies for $PPAR\gamma$. (C) Day 0 postconfluent 3T3-L1 preadipocytes were treated as described in Fig. 4A. On day 2, the cell number was determined by trypan blue exclusion test, and the absolute cell number was graphed. The experiments were repeatedly performed to confirm the results. The data were analyzed using analysis of variance and Duncan's multiple range test ($P < 0.05$). Means sharing a common alphabetical symbol did not significantly differ.

results demonstrate that the SIP_2 receptor plays a dominant role in the anti-differentiating action of SIP.

Discussion

We have shown that treatment with exogenous SIP effectively inhibits adipogenesis by downregulating the expression of adipocyte-specific differentiation markers. Neither the SIP_1 selective agonist (SEW2871) nor the antagonist (W146) affected the adipocyte differentiation function of SIP. By contrast, abrogation of the SIP_2 receptor recovered the impaired differentiation caused by SIP as well as enhancing differentiation. These anti-adipogenic effects of SIP via the SIP_2 receptor occurred through the regulation of MAPK pathways and the master of adipogenic transcriptional factors.

There is a previous relevant study concerning Sphk-1 and adipogenesis, which reveals that Sphk-1 is induced and SIP content increased in adipogenesis, and that its downregulation (using Sphk-1 inhibitors and siRNA) blocks differentiation (21). In addition, SIP content increased in adipogenesis. However, there was no information as to whether direct treatment of SIP has a pro-adipogenic effect. In addition, there is no information on the adipogenic effect of activating Sphk-1 and whether or not the activation of Sphk-1 promotes adipogenesis or increases SIP content. Therefore, we examined the direct effect of SIP and our results revealed that the direct effect of SIP exerted an anti-adipogenic effect via SIP_2 .

Numerous signaling pathways that are activated in response to the stimulation of cells by SIP are initiated by activation of

SIP -specific receptors. The results of this study revealed that the anti-adipogenic action of SIP was not associated with SIP_1 (Fig. 2). Neither the SIP_1 selective agonist (SEW2871) nor the SIP_1 antagonist (W146) affected the inhibition of adipocyte differentiation by SIP. The anti-adipogenic effect of SIP occurred via SIP_2 . In addition, the SIP_2 selective antagonist significantly reversed the inhibition of SIP during adipocyte differentiation (Fig. 3). A single treatment of JTE013 alone enhanced adipocyte differentiation. The adipogenesis process is tightly controlled by $PPAR\gamma$ and $C/EBP\alpha$. $PPAR\gamma$ is the master regulator of adipogenesis. $C/EBP\alpha$ induces numerous adipocyte genes directly (22). As a result, as noted in Fig. 4, SIP lowered $PPAR\gamma$ and $C/EBP\alpha$ expression; however, SIP lost its ability to impair $PPAR\gamma$ and $C/EBP\alpha$ expression in the abrogation of SIP_2 using JTE013 treatment (Fig. 4). These previous studies supported our results that SIP treatment increases the number of cells 48 h after MDI induction, which coincides with a lowering of $C/EBP\alpha$ expression. However, in the presence of JTE013, the numbers of cells were lowered and $C/EBP\alpha$ expression was reversed similar to MDI induction in the controls (Fig. 4).

In conclusion, the results of this study demonstrated that exposure of preadipocytes to SIP inhibited their differentiation into adipocytes, as confirmed by a reduction in triglyceride accumulation and a reduction in the expression of adipocyte-specific genes. Therefore, SIP functioned as an anti-adipogenic compound and SIP_2 was shown to be responsible for the anti-adipogenic activity of SIP. This study identifies the activation of SIP_2 receptor as a possible

mechanism of the anti-adipogenic differentiating action of SIP and suggests that SIP₂ activation may be a therapeutic target for anti-obesity.

Acknowledgements

This study was supported by a grant from the National Research Foundation of Korea (NRF), funded by the Korean government (2012R1A1B3000463).

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