The Role of Sphingosine 1-phosphate in adipogenesis of Graves' ophthalmopathy

Sung Eun Kim

Department of Medicine

The Graduate School, Yonsei University

The Role of Sphingosine 1-phosphate in adipogenesis of Graves' ophthalmopathy

Directed by Professor Jin Sook Yoon

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Sung Eun Kim

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This certifies that the Doctoral Dissertation
of Sung Eun Kim is approved.

Thesis Supervisor : Jin Sook Yoon

Thesis Committee Member#1 : Eun Jig Lee

Thesis Committee Member#2 : Joon H. Lee

Thesis Committee Member#3: Suk Woo Yang

Thesis Committee Member#4: Kee Hyun Nam

The Graduate School
Yonsei University

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Sung Eun Kim

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ABSTRACT

The Role of Sphingosine 1-phosphate in adipogenesis of Graves' ophthalmopathy

Sung Eun Kim

Department of Medicine
The Graduate School, Yonsei University

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Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid that mediates diverse cellular responses including proliferation, immune cell traficking and differentiation. We investigated the expression and action of S1P in orbital fibroblasts and adipocyte differentiation and its putative role in the pathogenesis of Graves' ophthalmopathy.

Primary cultures of preadipocyte orbital fibroblasts were stimulated for adipogenesis in enriched culture medium. Semiquantitive RT-PCR was performed to evaluate the expression of S1P receptor mRNA.

To evaluate the effect of S1P and S1P receptor blockers (W146, JTE013, and FTY720) on adipocyte differentiation, we exposed cultures to them for the first 4 days of differentiation period. Differentiated cells were stained with oil red O, and the expression of peroxisome proliferator activator gamma (PPARγ), CCAAT-enhancer-binding proteins (C/EBP) α and β were determined by western blot.

S1P receptor 1, 2 and 3 mRNA expression levels were all significantly higher in GO tissues samples. S1P receptor 1-5 mRNA expression was significantly increased during 10 days of adipogenesis. S1P treatment increased the size and number of adipocytes and increased accumulation of lipid droplets

and also increased the expression of adipogenic transcriptional regulators. Treatment of S1P receptor 1 inhibitor (W146) and S1P2 blocker (JTE013) for 4 days after induction of adipogenesis attenuated adipocyte differentiation. Additionally, we examined whether S1P receptor blockers may decrease reactive oxygen species (ROS) production in orbital fibroblasts from Graves' ophthalmopathy. Sphingosine-1-phosphate receptor blocker also decreased reactive oxygen species (ROS) production in GO orbital fibroblasts and H2O2-stimulated HO-1 production in GO orbital fibroblasts. S1P1 receptor inhibitor reduced the number of adipocytes and suppressed the accumulation of lipid droplets induced by 10 lM H2O2 or 2% cigarette smoke extract (CSE) treatment.

S1P could play a role in orbital adipocyte differentiation and pathogenesis of Graves' ophthalmopathy and modulation of this bioactive mediator may provide a therapeutic target for the treatment of Graves' ophthalmopathy.

Key words : Sphingosine-1-phosphate, Graves' ophthalmopathy,
adipogenesis, S1P receptor antagonist

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I. INTRODUCTION

Graves' ophthalmopathy (GO) is an autoimmune disease that occurs in up to 50% of patients with Graves'disease. Severe, potentially blinding orbital inflammation develops in about 5–6% of these patients.[1] The inflammation causes muscle and soft tissue swelling, which when progressive, results in clinical features of proptosis, exposure keratopathy, and compressive optic neuropathy.

Current evidences point to orbital fibroblasts as the target cells in Graves' ophthalmopathy and suggest that their normal functions are dysregulated through autoimmune mechanisms.[2] These orbital fibroblasts secrete large quantities of hyaluronan in response to various cytokines, and a subgroup of orbital fibroblasts can differentiate into mature adipocytes that have increased expression of thyrotropin receptor.[3,4] These cellular changes lead to the characteristically enlarged eye muscles and expansion of orbital fat of patients with Graves' ophthalmopathy.

Until now, no reliable, specific, and safe medical therapeutic agent has yet been developed for Graves' ophthalmopathy. Glucocorticoids have been used for decades and are still indicated as the first-line treatment because of

their anti-inflammatory and immunosuppressive actions, either alone or in combination with orbital radiotherapy.[5,6] Glucocorticoids are mostly effective in patients with severe and active eye disease. However, proptosis and longstanding extraocular muscle involvement associated with fibrotic changes are poorly responsive.[5] A major drawback of systemic glucocorticoid therapy represented by its possible side effects and complications, including cushingoid features, diabetes, hypertension, infection, increased body weight and osteoporosis.

The challenges of developing specific therapies targeting pathways of inflammation, adipose tissue expansion, aberrant accumulation of extracellular matrix macromolecules, and fibrosis are mandatory.

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid that mediates diverse cellular responses such as proliferation, cytoskeletal organization and migration, immune cell trafficking, adherence and tight junction assembly, and differentiation.[7,8]. S1P is formed by an action of enzyme termed sphingosine kinase (SphK) that converts sphingosine and ATP to S1P. Most of its best-characterized actions are mediated through a family of five G protein-coupled receptors, $S1P_{1-5}$ receptors. The S1P receptors couple to a variety of G proteins, allowing them to mediate many different biologic responses. [7,9]

The cell-type-specific expression of combinations of S1P receptors, together with a differential coupling to heterotrimeric G-proteins, and down-stream signaling pathways dramatically enhance the repertoire of any S1P stimulation. The $S1P_1$ receptor is predominantly expressed by immune cells, neural cells, endothelial cells and smooth muscle cells. Genetic deletion of the $S1P_1$ receptor in mice suggests that it has a key role in angiogenesis and neurogenesis, as well as in the regulation of immune cell trafficking, endothelial barrier function. The $S1P_2$ receptor also shows widespread expression, and is essential for the proper functioning of the auditory and vestibular systems as

well as the development and/or mediation of neuronal excitability. $S1P_3$ receptor is expressed in the heart, lung, spleen, kidney and diaphragm. It may fine-tune some cardiovascular functions, including the regulation of heart rate. The $S1P_4$ receptor has a more restricted expression pattern and is detectable predominantly within immune compartments and on leukocytes, also on human airway smooth muscle cells. $S1P_4$ signaling is reported to be involved in the regulation of dendritic cell function and T_H17 T-cell differentiation. The $S1P_5$ receptor is predominantly expressed in oligodendrocytes in the white matter tracts of the CNS. However, its precise role in oligodendrocyte function remains to be determined.[8,10,11]

Among the several interesting actions of S1P in the context of cellular physiology, much attention has been focused on its effect on the process of differentiation. S1P/S1P1 receptor system plays a crucial role in promoting angiogenesis by regulating differentiation of vascular endothelial cells[12,13]. S1P also promotes myogenic differentiation of mouse C2C12 skeletal myocyte cell line[14] and modulates mouse osteoclast differentiation[15]. Lately, Hashimoto et al. reported that Sphingosine kinase and S1P were up-regulated during adipogenesis and played a significant role in adipocyte differentiation in mouse 3T3-L1 cells.[16] Also in 3T3-L1 preadipocytes, the expression of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which is known to contribute to adipose tissue differentiation and dysfunction, was robustly induced when cells were treated with S1P [17,18]. Conversely, FTY720, a synthetic analogue of S1P which acts as a functional antagonist of S1P, down-regulated adipogenic differentiation.[19]

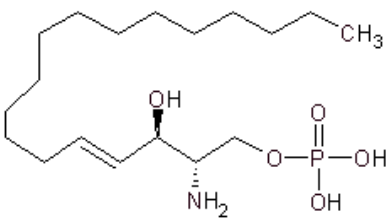
To date, no studies have evaluated the expression and action of S1P in orbital fibroblasts and adipocyte differentiation and its putative role in the pathogenesis of Graves' ophthalmopathy. Here we hypothesized that S1P could play a role in orbital adipocyte differentiation and pathogenesis of Graves' ophthalmopathy and modulation of this bioactive mediator may provide a

therapeutic target for the treatment of Graves' ophthalmopathy.

II. MATERIALS AND METHODS

1. Reagents and chemicals

Sphingosine-1-phosphate (S1P) and FTY720 were purchased from Sigma Aldrich, Inc (St. Louis, MO). Structure of S1P was shown in Figure 1. S1P receptor 1 antagonist, W146 (trifluoroacetate salt) and S1P receptor 2 antagonist, JTE013 were purchased from Cayman company (Ann Arbor, MI). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and gentamycin were purchased from Hyclone Laboratories, Inc. (Logan, UT, USA). The 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and Oil Red O were purchased from Sigma–Aldrich, Inc. Anti-peroxisome proliferator activator gamma (PPARγ) antibody, anti-CCAAT-enhancer-binding protein (C/EBP) α antibody, anti-C/EBP β antibody, anti-Heme oxygenase (HO)-1 antibody, anti-extracellular-regulated kinase (ERK) 1/2, anti-phospho ERK 1/2, anti-phospho-Akt, and anti-β-actin antibody were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA).



Sphingosine-1-phosphate

Figure 1. Chemical structure of S1P

2. Cell culture and differentiation protocol

Orbital adipose/connective tissue specimens were obtained during the course of orbital decompression surgery for severe GO (n=5; three women and two men, aged 29–59 years). The GO patients had not received steroid medication for at least 3 months before surgery, and were euthyroid at the time of surgery. The clinical activity scores (CAS) at the time of tissue harvest were less than four for all patients, indicating inactive inflammatory status, which was based on an original 10-point CAS developed by Mourits et al. (1997)[20]. None of the patients had been treated previously with orbital radiotherapy. Normal orbital adipose/connective tissue specimens were collected during the course of orbital surgery for other non-inflammatory problems from patients with no prior history of thyroid disease or GO and with no clinical evidence of GO (n=4; four women, aged 36–64 years). The study was approved by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine, and written informed consent was obtained from all participants.

Tissue explants were minced and placed directly in plastic culture dishes in DMEM:F12 (1:1) containing 20% FBS, penicillin (100 U/mL), and gentamycin (20 μg/mL), allowing preadipocyte fibroblasts to proliferate. After fibroblasts had grown out from the explants, monolayers were passaged serially by gently treating with trypsin/EDTA, and cultures were maintained in 80-mm flasks containing DMEM with 10% FBS and antibiotics. Cell cultures were grown in a humidified 5% CO_2 incubator at 37℃. The strains were stored in liquid N_2 until needed, and they were used between the second and fifth passage.

After cells reached confluence in 6-well plates, differentiation of adipocytes was initiated by the following protocol. The culture medium were changed to serum-free DMEM supplemented with 33 μM biotin, 17 μM pantothenic acid, 10 μg/ml transferrin, 0.2 nM T3, 1 μM insulin

(Boehringer-Mannheim, Mannheim, Germany), and 0.2 μM carbaprostaglandin (cPGI2; Calbiochem, La Jolla, CA, USA). For the first 4 days, 1 μM insulin, 1 μM dexamethasone, and 0.1 mM isobutylmethylxanthine were included in the media. The differentiation was continued for 10 days, during which the media was replaced every 2-3 days. A PPARγ agonist, rosiglitazone (10 μM, Cayman, Ann Arbor, MI, USA), was added from day 1 for further stimulation of adipogenesis. To evaluate the effect of S1P and S1P receptor blockers (W146, JTE013, and FTY720) on adipocyte differentiation, we exposed cultures to S1P for the first 4 days of differentiation period.

3. Cell viability assay

Cell viability was assessed by using the MTT assay according to the manufacturer's (Sigma-Aldrich) protocol. To evaluate the effect of S1P on preadipocyte orbital fibroblast viability, orbital fibroblasts of GO patients were seeded into 24-well culture plates and treated with different concentrations of S1P (100, 250, 500, or 1000 ng/ml) for 24 h. After treatment, cells were washed, incubated with 5 mg/ml MTT solution for 4 h at 37℃, then solubilized in ice-cold isopropanol and analyzed spectrophotometrically. Absorbance of the dye was measured at 560 nm, with background subtraction at 630 nm, with a microplate reader (EL 340 Biokinetics Reader; Bio-Tek Instruments, Winooski, VT, USA). The optical density of formazan formed in control (medium alone) cells was taken as 100% of viability.

4. Semiquantitative RT-PCR

Total RNA (1 μg) was isolated and reverse transcribed into complementary DNA according to the manufacturer's instructions. The resulting cDNA was amplified on the ABI StepOnePlus™ real-time PCR themocycler

(Applied Biosystems, Carlsbad, California, USA) using TaqMan universal PCR master mix and recommended PCR conditions to assess gene transcript levels quantitatively in the cell samples. All PCR were performed in triplicate. Primers for human S1P receptor 1 were 5'-TGC GGG AAG GGA GTA TGT TT-3' (forward) and 5'-CCA TCC CCA CCA CAC TCA AC-3' (reverse); for human S1P receptor 2, 5'-GCC TCT CTA CGC CAA GCA TTA-3' (forward) and 5'-TTG AGC GGA CCA CGC AGT A-3' (reverse); for human S1P receptor 3, 5'-TGA TTG TGG TGA GCG TCT TCA-3' (forward) and 5'-GGC CAC ATC AAT GAG GAA GAG-3' (reverse); for human S1P receptor 4, 5′-CGG CAC AGC CTC CTC ATT GTC-3′ (forward) and 5′-ACA GAC CGA TGC AGC CAT ACA CAC-3′ (reverse); for human S1P receptor 5, 5'-CGT CCT GCA TTA CAA CTA CAC C-3' (forward) and 5'-GGC CAC ATC AAT GAG GAA GAG-3' (reverse). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primers were 5'-GCC AAG GTC ATC CAT GAC AAC-3' (forward) and 5'-GTC CAC CAC CCT GTT GCT GTA-3' (reverse). All samples were normalized to the values of GAPDH and the results were expressed as relative fold changes of thereshold cycle (Ct) value relative to the control group using the $2^{-\Delta\Delta Ct}$ method.[21] Amplification bands were quantified by densitometry and normalized against corresponding GAPDH bands to control for PCR variability.

5. Oil Red O stain

Cells were stained with Oil Red O as described by Green and Kehinde [22]. A 0.5% (w/v) stock solution of Oil Red O in isopropanol was prepared. For the working solution, 6 ml of the stock solution was mixed with 4 ml distilled water, left for 1 h at room temperature, and then filtered through a 0.2 μm filter. Cells were washed twice with 1X PBS, fixed with 3.7% (w/v) formalin in PBS for 1 h at 4℃ and stained with 300 μl of Oil Red O working solution for 1 h at room temperature. The dishes were washed with distilled water before being

visualized with an Axiovert (Carl Zeiss, Germany) light microscope and photographed at 40x, 100x and 400x magnification with an Olympus BX60 light microscope (Olympus; Melville, NY, USA). To measure lipid accumulation, cell-bound Oil Red O was solubilized with 100% isopropanol, and the optical density of the solution was measured with a spectrophotometer at 490 nm. Experiments for the quantitative assessment of adipogenic differentiation were performed in triplicate in cells from different donors, and results were normalized to the absorbance of untreated control differentiated cells.

6. Western blot assay

Differentiated cells were washed with ice-cold PBS and lysed on ice for 30 min in cell lysis buffer consisting of 20 mM HEPES (pH 7.2), 10% (/) glycerol, 10 mM Na_3VO_4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1% (/) Triton X-100. Reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). Lysates were centrifuged for 15 min at 14,000 rpm, and cell homogenate fractions were stored at -70℃ before use. Protein concentrations in supernatant fractions were determined by the Bradford assay (BioRad; Hercules, CA, USA). Equal amounts of protein (50 μg) were boiled in sample buffer and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis in 10% (w/) gels. The separated proteins were transferred to polyvinylidene fluoride membranes (Immobilon; Millipore, Billerica, MA, USA), probed overnight with primary antibodies in Tris Buffer Saline Tween 20, and washed three times with Tris Buffer Saline Tween 20. Immunoreactive bands were detected with horseradish peroxidase- conjugated secondary antibody, and the bound peroxidase was visualized using an enhanced chemiluminescence kit and exposure to X-ray film (GE healthcare; Piscataway, NJ, USA). The relative amount of each

immunoreactive band was quantified by densitometry and normalized to the β-actin level in the same sample.

7. Reactive Oxygen species (ROS) measurement

ROS release was determined with 5-(and 6)-carboxy-2',7'-dichloro-dihydrofluorescein diacetate (H_2DCFDA; Invitrogen, Eugene, OR, USA), an oxidant-sensitive fluorescent probe, as previously described [23]; H_2DCFDA is deacetylated intracellularly by esterase, forming H_2DCF, which is oxidized by ROS to 2',7'-dichlorofluorescein (DCF), a highly fluorescent compound. The cells were seeded at a density of $5x10^5$ cells per well in six-well plates to a total final volume of 2ml and pretreated with 10 μM W146, 10 μM JTE013, 1 μM FTY720 for 1h in the presence or absence of 2% cigarette smoke extract (CSE) or 200 μM H_2O_2 for 1 h. The culture medium was then removed, and the cells were washed with PBS, incubated with 10 μM H_2DCFDA at 37℃. The cells were then trypsinized, washed, and resuspended in PBS. Thereafter, fluorescence intensity was measured with a flow cytometer (ELITE flow cytometer; Coulter Cytometry, Inc., Hialeah, FL, USA).

Preparation of CSE

CSE was prepared by bubbling smoke from two commercially available, filtered cigarettes (Marlboro 20 class A cigarettes, made by Philip Morris Korea, Inc., Seoul, Korea, containing 8.0 mg of tar and 0.7 mg of nicotine) through 20 ml of prewarmed serum-free DMEM/F12 (1:1) at a rate of one cigarette per 2 min, as described previously [23]. The pH of the CSE was adjusted to 7.4 and the CSE was sterile filtered through a 0.2 μM filter (Sartorius Stedim Biotech, Goettingen, Germany). The CSE preparation was standardized by measuring its absorbance (optical density = 0.65 ± 0.05 at 320

nm). The spectrographic pattern of absorbance at 320 nm showed very little variation between different preparations of CSE. The CSE was freshly prepared within 1 h of each experiment and diluted with culture medium, adjusted to a pH of 7.4, and sterile filtered as described for 10% CSE.

8. Statistical analysis

All experiments were performed at least three times, and samples were assayed in duplicate each time. For statistical analysis of semiquantitative PCR assays and western blots, the mean value and standard deviation (SD) was calculated for normalized measurements of each mRNA or protein from multiple (≥3) samples harvested from different individuals. The one-way analysis of variance test (ANOVA) and Bonferroni method as a post-hoc test were performed to find differences between control and drug-treated samples. Data between or within cell groups at different drug concentrations and incubation times were analyzed by the t-test or analysis of variance using the SPSS program for Windows, version 16 (SPSS, Chicago, IL, USA). P-values less than 0.05 were considered to be significant.

Ⅲ. RESULTS

1. The effect of S1P on the viability of GO orbital fibroblasts

The cytotoxicity of S1P to GO orbital fibroblasts was examined through the loss of cell viability using MTT assay. As shown in Figure 2, more than 90% of cells were viable after challenging with 100 - 1000 ng/ml S1P for both 6 and 24 h in orbital fibroblasts of Graves' ophthalmopathy patients (Figure 2).

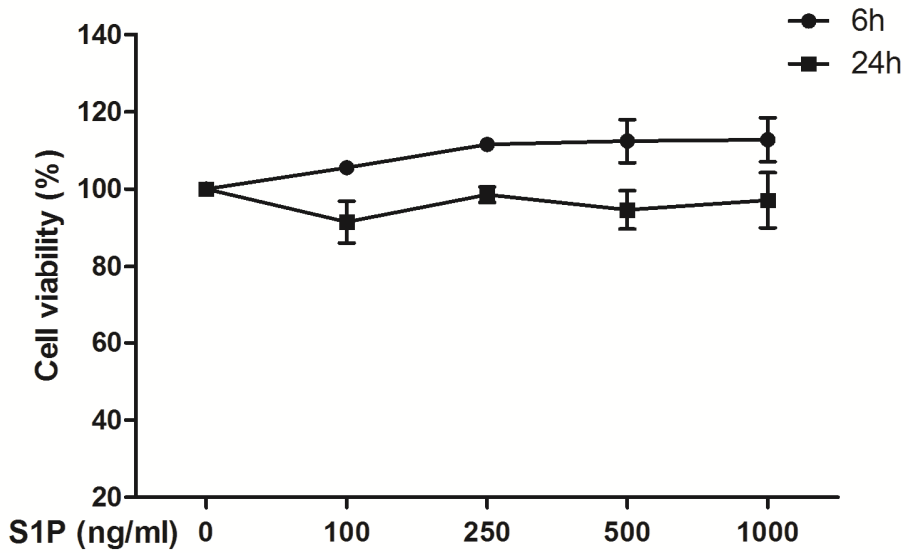


Figure 2. Effect of S1P on cell viability in Graves' ophthalmopathy orbital fibroblasts

Orbital fibroblasts (1 x 10^5) of Graves' ophthalmopathy patients were seeded into 24-well culture plate and treated with different concentrations of S1P (100 - 1000 ng/ml) for 6 and 24 h. After treatment, assays with 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-
tetrazolium bromide (MTT) were performed to test for viability. Assays were performed at least three times in triplicate. Results are expressed as percentage of untreated control values presented as mean ± standard deviation (SD).

2. S1P receptor expression in orbital fibroblasts of Graves' ophthalmopathy (GO) and non-GO controls.

To compare which type of S1P receptors are expressed in orbital fibroblasts of Graves' ophthalmopathy (GO) and non-GO controls, we performed relative quantification of mRNA for each receptor by RT-PCR. S1P1, S1P2 and S1P3 mRNA were expressed in relatively high level in GO orbital fibroblasts than in non-GO tissues.

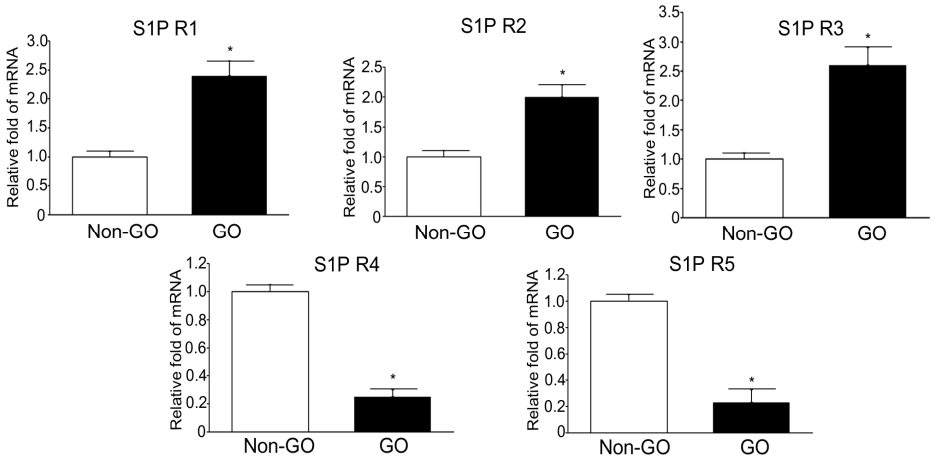


Figure 3. Comparison of tissue expression of S1P receptor 1-5 mRNA using real time RT-PCR between Non-GO (n=3) and GO (n=5) samples.

S1P receptor 1, 2 and 3 mRNA expression levels were all significantly higher in GO tissues samples (* $p<0.05$) than non-GO tissues. However S1P receptor 4 and 5 gene levels were lower in GO tissues conversely.

3. Expression of S1P receptor 1-5 during adipogenesis

We measured S1P receptor 1-5 mRNA expression on days 0, 4, 7, and 10 of adipocyte differentiation in GO fibroblasts. All types of S1P receptors were significantly increased during the adipogenesis.

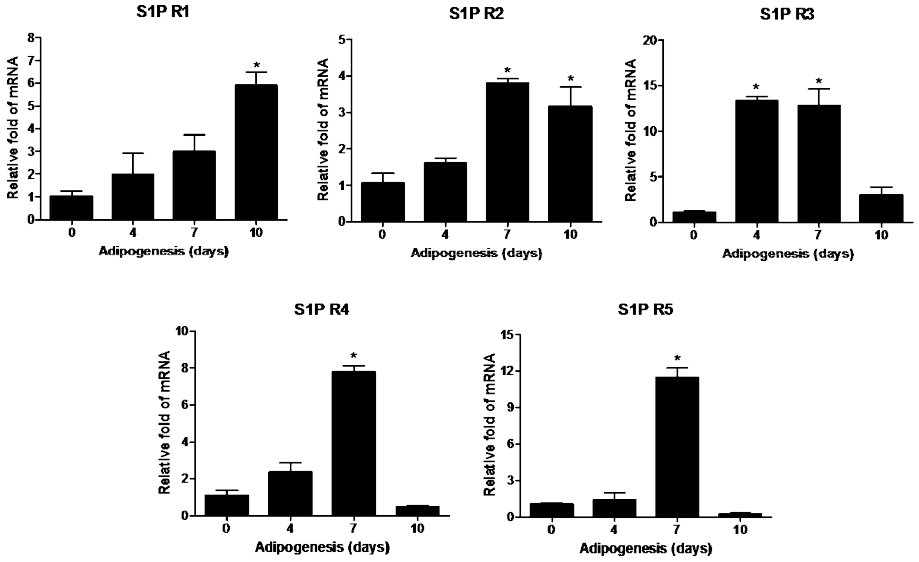


Figure 4. Expression of S1P receptor 1-5 during adipogenesis.

S1P receptor 1-5 mRNA expression was significantly increased during 10 days of adipogenesis. The receptor gene expression seemed to increase over time during adipogenesis and were usually highest on day 7 (*$p<0.05$ vs. day 0)

4. Effect of S1P on adipogenesis of GO orbital fibroblasts

Confluent orbital fibroblasts from GO patients were subjected to an adipocyte differentiation protocol for 10 days. The cells were examined under light microscopy and then stained with Oil Red O. As previously reported, under the control adipogenic conditions, the orbital fibroblasts lost their stellate fibroblastic appearance and converted to a spherical adipocytic shape, and a fraction of these cells accumulated small lipid droplets [24].

To examine whether treating with S1P had any effects on adipogenesis, different concentration of S1P (0 ~ 1000 ng/ml) were added to the adipogenic medium for 4 days during the differentiation period, being replaced whenever media was replaced. High power (x200) microscopic examination of Oil red O

staing showed that S1P treatment increased the size and number of adipocytes and increased accumulation of lipid droplets. Especially in 250 and 500 ng/ml S1P, the increase was prominent. The optical density of stained cell lysates was measured to evaluate adipocyte differentiation quantitatively (Figure 5). 250 and 500 ng/ml S1P treated cells showed significantly increased absorbance at 490 nm (* $p<0.05$).

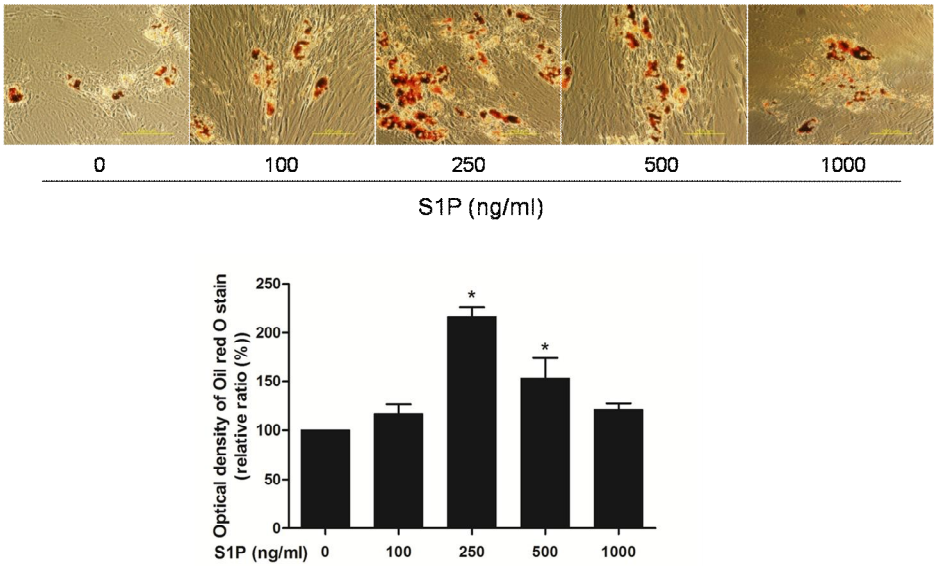


Figure 5. Effect of S1P on adipogenesis of GO orbital fibroblasts

Different concentration of S1P was treated during the first 4 days of adipogenic differentiation. Cells were stained with Oil red O and examined microscopically (x200). Adipogenesis was increased by S1P treatment compared to control. The increased adipocyte differentiation was prominant on oil red O stain when 250 and 500 ng/ml S1P was treated. The optical density of stained cell lysates was measured to evaluate adipocyte differentiation quantitatively. 250 and 500 ng/ml S1P treated cells showed significantly increased absorbance at 490 nm (* $p<0.05$ vs untreated control).

5. Effect of S1P on the expression of transcriptional regulators of adipogenesis

Same as the previous experiment, different concentration of S1P (0 ~ 1000 ng/ml) were added to the adipogenic medium for 4 days during the differentiation period, being replaced whenever media was replaced. Western blot analysis was performed to investigate whether S1P affects the expression of adipogenic transcription factors during adipogenesis. As shown in Figure 6, PPARγ and C/EBPα were enhanced in cells treated with S1P. However, C/EBPβ did not increased significantly.

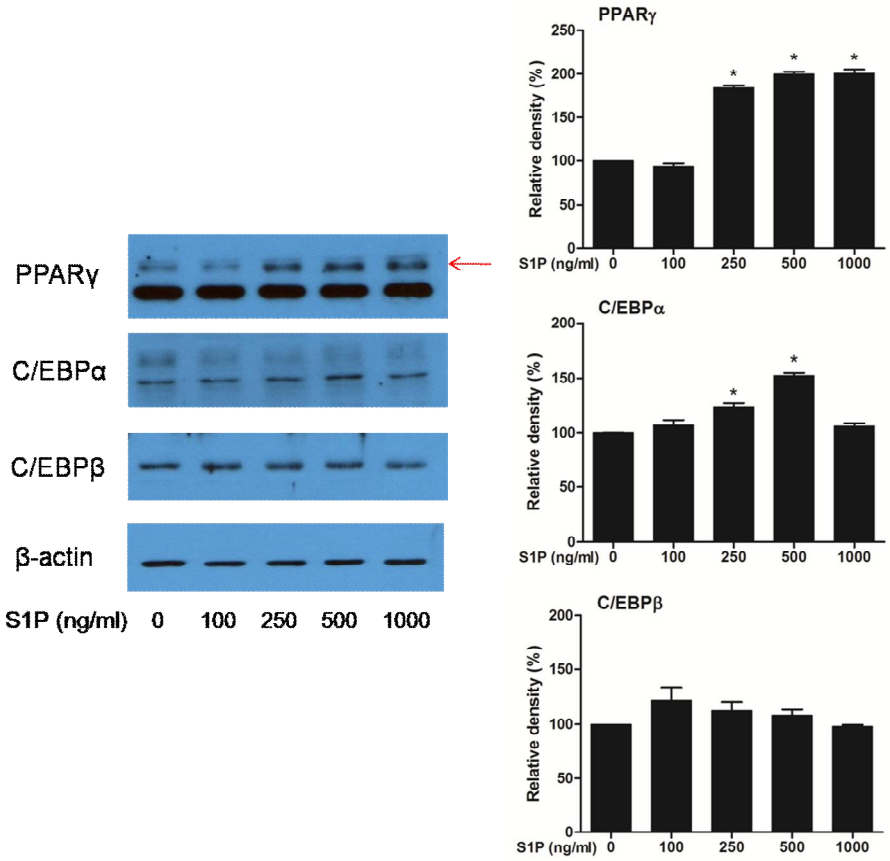


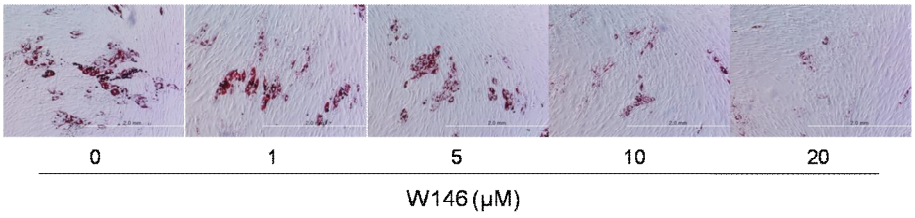
Figure 6. Effect of S1P on the expression of adipogenic transcriptional regulators in differentiated orbital fibroblasts from GO patients
Different concentration of S1P (100, 250, 500, or 1000 ng/ml) were treated to each cell culture for the first 4 days after initiation of 10-day adipogenesis. After 10 days, cell lysates were subjected to western blot analysis of PPARγ, C/EBPα and C/EBPβ protein expression. The experiments were performed in triplicate with cells from three different donors. Quantification of the PPARγ, C/EBPα and C/EBPβ by densitometry, normalized to the level of β-actin in the same sample, is shown. The data in the columns are the mean relative density ratios (%) ± SD of three experiments. (* $P<0.05$ vs. untreated control differentiated cells.)

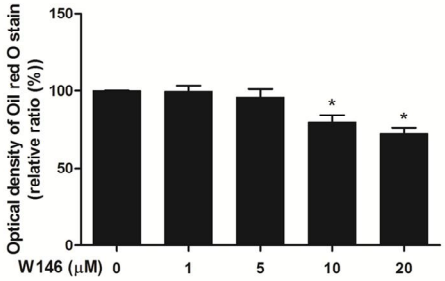
6. Effect of S1P receptor 1 blocker (W146) on Adipogenesis in GO OFs

To examine whether W146, S1P receptor 1 blocker, has any suppressive effect on adipogenesis, different concentrations of W146 were added to the adipogenic medium for the first 4 days during the differentiation period. W146 dose-dependently reduced the number of adipocytes and suppressed the accumulation of lipid droplets, as visualized by microscopic examination after Oil Red O staining (Figure 7A). The optical density of stained cell lysates was also measured to evaluate adipocyte differentiation quantitatively. The 10, and 20 μM W146 treated cells showed significantly decreased absorbance at 490nm ($p<0.05$).

Western blot analysis was then performed to investigate whether W146 affects the expression of adipogenic transcription factors, PPARγ, C/EBPα and C/EBPβ, during adipogenesis. PPARγ and C/EBPα proteins were significantly attenuated by 20 μM W146 treatment as shown in Figure 7B.

A.





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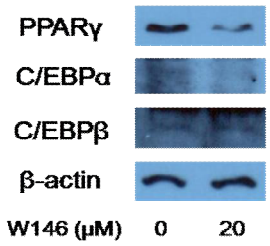
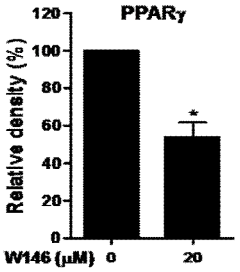
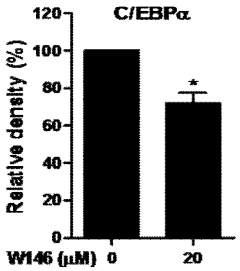






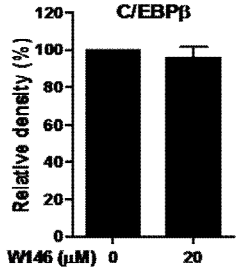


Figure 7. Adipogenesis after S1P receptor 1 blocker (W146) treatment.

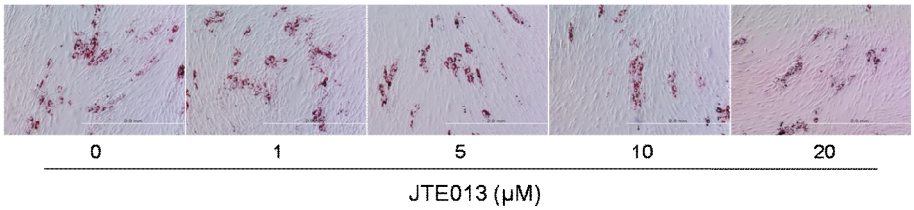
Treatment of S1P receptor 1 inhibitor (W146) for 4 days after induction of adipogenesis attenuated adipocyte differentiation by dose dependent manner as shown with microscopic examination (x100) of Oil red O staining. The optical density of stained cell lysates was also measured to evaluate adipocyte differentiation quantitatively. The 10, and 20 μM W146 treated cells showed significantly decreased absorbance at 490 nm (* $p<0.05$ vs untreated control) (A). Representative figure of western blot analysis of PPARγ, C/EBPα, C/EBPβ protein expressions after 20 μM W146 treatment is shown in (B). PPARγ and C/EBPα proteins were significantly attenuated by 20 μM W146 treatment, However, C/EBPβ did not significantly affected. Quantification of the PPARγ, C/EBPα and C/EBPβ by densitometry, normalized to the level of β-actin in the same sample, is shown. The data in the columns are the mean relative density ratios (%) ± SD of three experiments. (* $P<0.05$ vs. untreated control differentiated cells.)

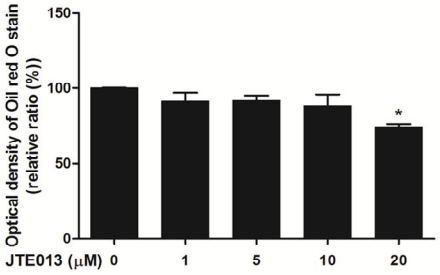
7. Effect of S1P receptor 2 blocker (JTE013) on Adipogenesis in GO OFs

In the same manner as previous experiment, different concentrations of JTE013, a S1P receptor 2 blocker, was added to the adipogenic medium for the first 4 days during the differentiation period. JTE013 also reduced the number of adipocytes and suppressed the accumulation of lipid droplets, as visualized by Oil Red O staining (Figure 8A). The optical density of stained cell lysates was also measured to evaluate adipocyte differentiation quantitatively. The 20 μM JTE013 treated cells showed significantly decreased absorbance at 490 nm ($p<0.05$, vs control).

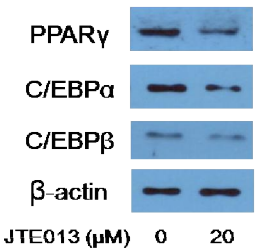
Western blot analysis was then performed to investigate whether JTE013 affects the expression of adipogenic transcription factors, PPARγ, C/EBPα and C/EBPβ, during adipogenesis. PPARγ, C/EBPα, and C/EBPβ proteins were significantly attenuated by 20 μM JTE013 treatment as shown in Figure 8B.

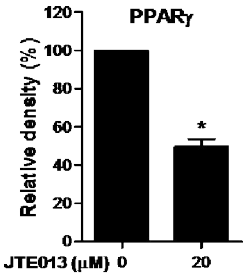
A.

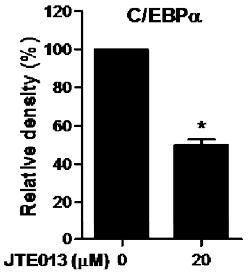




B.







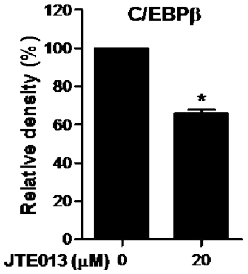


Figure 8. Adipogenesis after S1P receptor 2 blocker (JTE013) treatment.

Treatment of S1P receptor 2 inhibitor (JTE013) for 4 days after induction of adipogenesis attenuated adipocyte differentiation as shown with microscopic examination (x100) of Oil red O staining. The optical density of stained cell lysates was also measured to evaluate adipocyte differentiation quantitatively. The 20 μM JTE013 treated cells showed significantly decreased absorbance at 490 nm (* $p<0.05$ vs control). (A) Western blot analysis for PPARγ, C/EBPα and C/EBPβ protein expressions after JTE013 treatment showed that those proteins were significantly attenuated by 20 μM JTE013 treatment. Quantification of the PPARγ, C/EBPα and C/EBPβ by densitometry, normalized to the level of β-actin in the same sample, is shown. The data in the columns are the mean relative density ratios (%) ± SD of three experiments. (* $P<0.05$ vs. untreated control differentiated cells.)

8. Expression of S1P receptor 1-5 under oxidative stress condition

We measured S1P receptor 1-5 mRNA expression under oxidative stress condition. 200μM H_2O_2 or 2% CSE was exposed to orbital fibroblasts of Graves' ophthalmopathy for 0, 1, 3 and 6 hours. All types of S1P receptor were significantly increased in the oxidative stress condition induced with H_2O_2 (Figure 9(A)). S1P receptor 2, 3, and 5 were significantly increased in the oxidative stress condition induced with 2% CSE (Figure 9(B)).

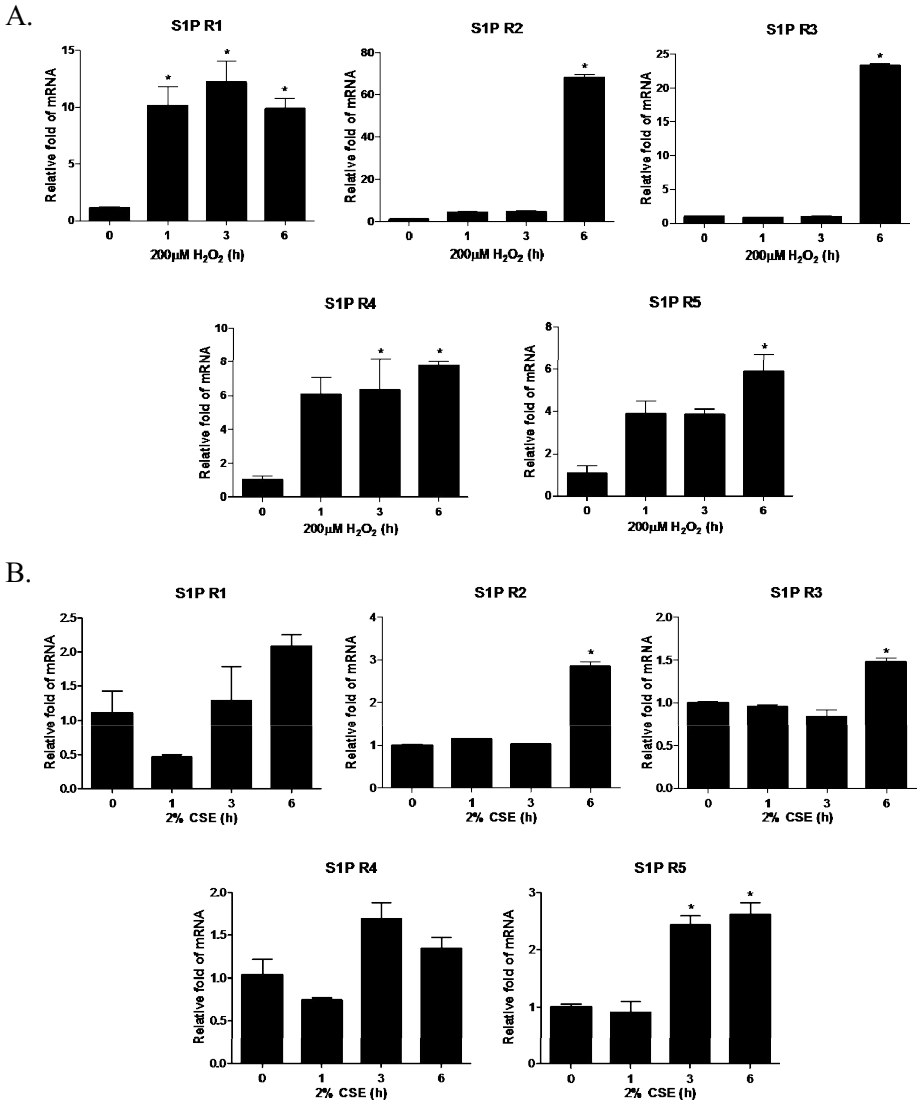


Figure 9. S1P receptor 1-5 gene expression under oxidative stress condition.
After treatment of 200 μM H_2O_2 for 0, 1, 3 and 6 hours, S1P receptor 1-5 gene expression was increased compared to control (*$p<0.05$ vs. control). Significant increase was detected after 6 h treatment of H_2O_2. (A)
After treatment of 2% CSE for 0, 1, 3 and 6 hours, S1P receptor 1-5 gene expression showed increasing tendency compared to control. The increase of S1P receptor 2, 3, and 5 were statistically significant (B).

9. S1P receptor blockers inhibit HO-1 expression and ROS generation in orbital fibroblasts.

The expression of antioxidant enzyme Hemeoxygenase-1 (HO-1) protein was significantly upregulated by treatment with 200 μM H_2O_2 for 4 h. Pretreatment with S1P receptor antagonists, W146 and FTY720 for 1 h significantly suppressed HO-1 expression in cells stimulated with 200 μM H_2O_2 (Figure 10A).

Additionally, we examined whether S1P receptor blockers may decrease reactive oxygen species (ROS) production in orbital fibroblasts from Graves' ophthalmopathy. When pretreated with or without 200 μM H_2O_2 for 1 h, treatment of orbital fibroblasts with S1P receptor 1 blocker (W146) and S1P receptor functional antagonist (FTY720) significantly decreased ROS levels detected by a ROS-sensitive fluorescent probe, DCFDA. (Figure 10B).

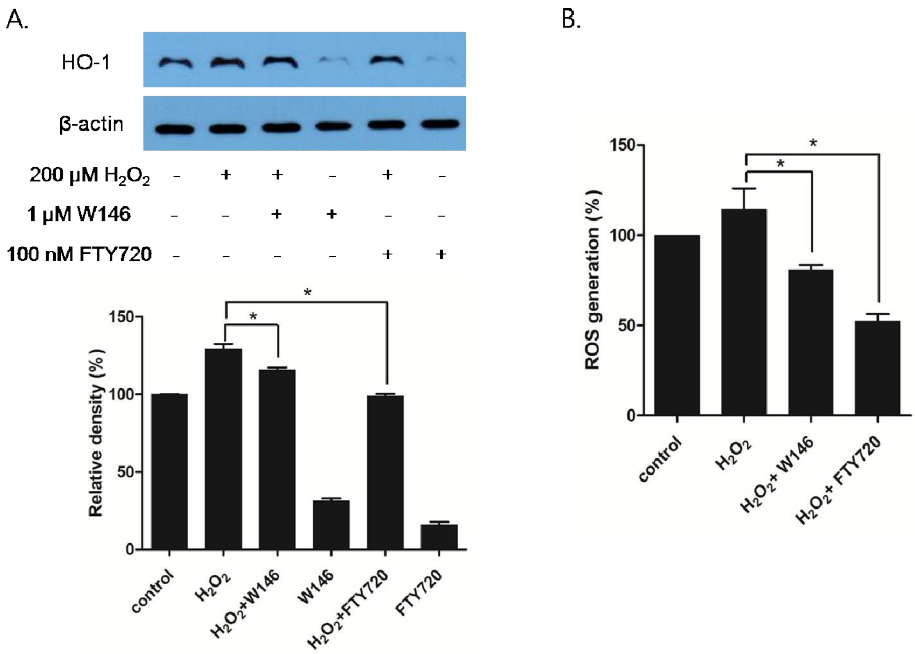


Figure 10. Effects of S1P receptor blockers on H_2O_2-stimulated HO-1 expression and ROS generation in GO orbital fibroblasts.

(A) Effects of S1P receptor blockers, W146 and FTY720 on H_2O_2-stimulated HO-1 expression in preadipocyte orbital fibroblasts, analyzed with western blotting.

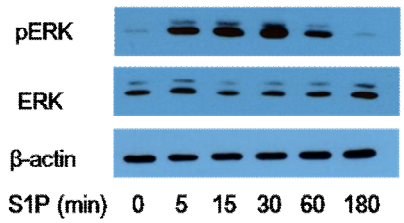
OFs ($5x10^5$) from GO patients (n=3) were pretreated with 1 μM W146 and 100 nM FTY720 for 1 h, and then stimulated with 200 μM H_2O_2 for 4 h to determine the effect of the S1P receptor antagonists on H_2O_2-stimulated HO-1 protein expression. Quantification of HO-1 by densitometry, normalized to the level of β-actin in the same sample, is also shown below. The data in the columns are the mean relative density ratios ± SD of three experiments. (*P<0.05 vs untreated control cells.)

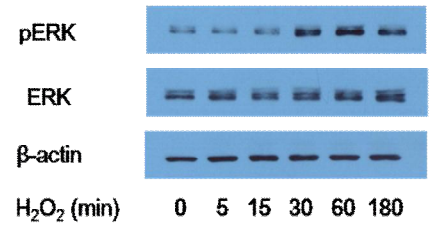
(B) S1P receptor antagonists (W146 and FTY720) decreased ROS levels in orbital fibroblasts from Graves' ophthalmopathy.

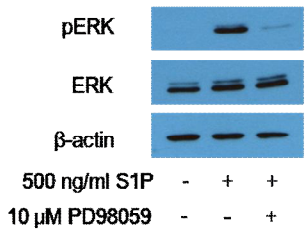
Orbital fibroblasts were incubated with H_2DCFDA 10μM for 24 h with 10 μM W146, and 1 μM FTY720 in the presence or absence of 200 μM H_2O_2 for 1 h and the fluorescence intensities were analyzed using FACS. Results are expressed as percentage of untreated control values presented as mean ± standard deviation (SD) of three separate experiments (* p<0.05 vs. cells treated with 200 μM H_2O_2 only).

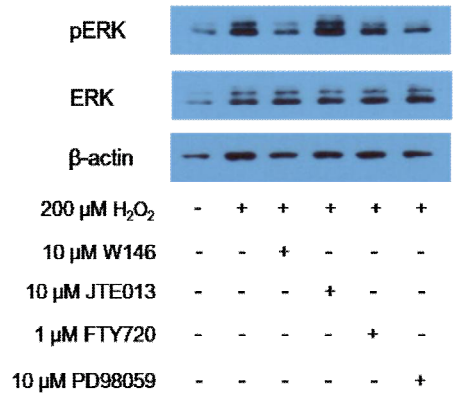
10. Signaling pathway of S1P action under H_2O_2 induced oxidative stress

To investigate the molecular mechanism of S1P in orbital fibroblasts under oxidative stress condition, we observed the effect of S1P on ERK1/2 activation. 500ng/ml S1P provoked a rapid and relevant activation of ERK1/2, whose phosphorylation was maximal at 30 minutes in GO cells, and declined to basal levels at 180 minutes. The phosphorylation of ERK1/2 was also strongly enhanced at 30- to 60-minute challenge with H_2O_2. The PD98059 significantly blocked phosphorylation of ERK 1/2 in GO orbital fibroblasts pretreated with 200 μM H_2O_2 for 6 hours. When cells were pretreated with W146 and FTY720, phospho-ERK was also significantly inhibited, suggesting that activation of the ERK signaling pathway is required for the oxidative stress induced proliferation of orbital fibroblasts.









11. Effects of W146 on H_2O_2- or CSE-stimulated adipogenesis

To determine the additive effect of W146 on adipogenesis and against oxidative stress, we stimulated adipogenesis by adding 10 μM H_2O_2 or 2% CSE for the first 3 days of differentiation and W146 was also treated to the adipogenic medium. As previously reported by Yoon et al[25], the addition of 10 μM H_2O_2 or 2% CSE significantly increased adipogenesis compared to that observed under control conditions, which was assessed with light microscopy (x100) after Oil red O staining (Figure 11 and Figure 12). When W146 (1~20 μM) was added to the adipogenic medium for the first 4 days during the differentiation period, W146 dose dependently reduced the number of adipocytes and suppressed the accumulation of lipid droplets induced by 10 μM H_2O_2 or 2% CSE treatment. The experiments were performed in triplicate with cells from three different donors, and all the findings were similar. Figure 11 and 12 shows representative data of GO OFs. The optical density of stained cell lysates was also measured to evaluate adipocyte differentiation quantitatively. The W146 treated cells showed dose-dependantly decreased absorbance at 490nm (* $p<0.05$, in contrast to cells treated with 10 μM H_2O_2 or 2% CSE only).

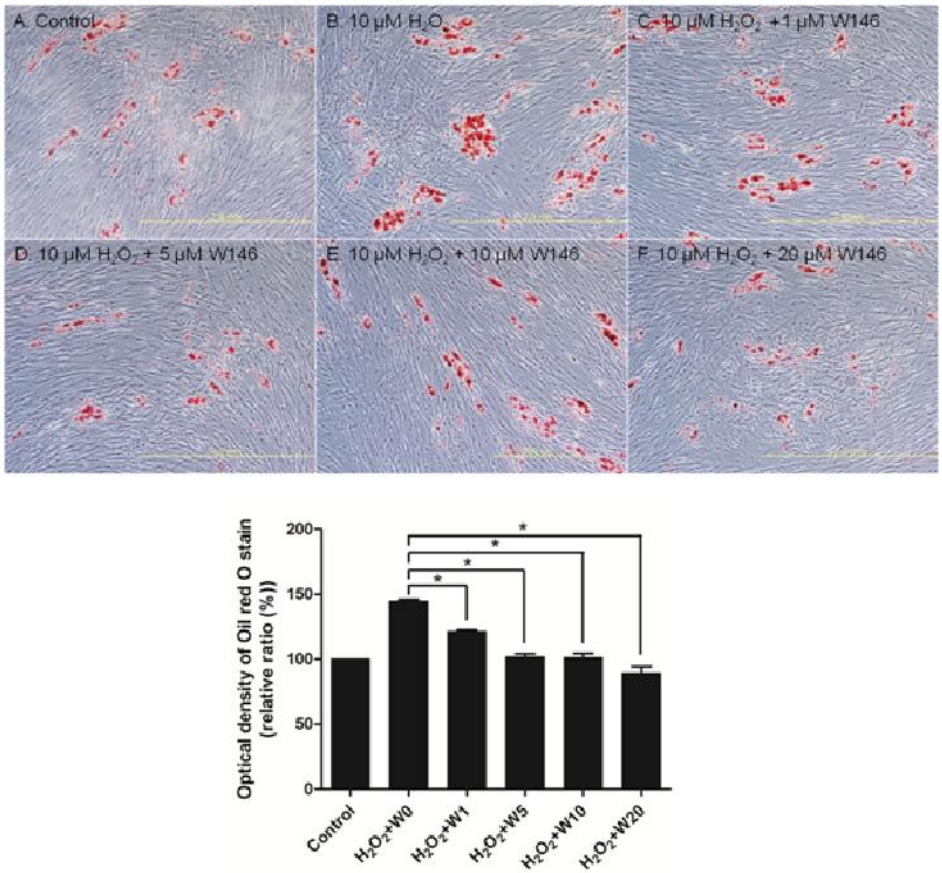


Figure 12. Effects of W146 on H_2O_2 stimulated adipogenesis.

Cells from GO patients were treated with 2% CSE or 10μM H_2O_2 for the first 3 days of the 10-day period of adipogenesis in adipogenic medium containing 10 μM rosiglitazone. To determine the suppressive effect of W146 on adipogenesis, W146 (1~20 μM) was also added for the first 4 days of differentiation. The cells were stained with Oil Red O and examined microscopically (x100). The experiments were performed in triplicate with cells from different GO donors, and the representative figures are shown. The optical density of stained cell lysates was also measured (* $p<0.05$, in contrast to cells treated with 10 μM H_2O_2 only).

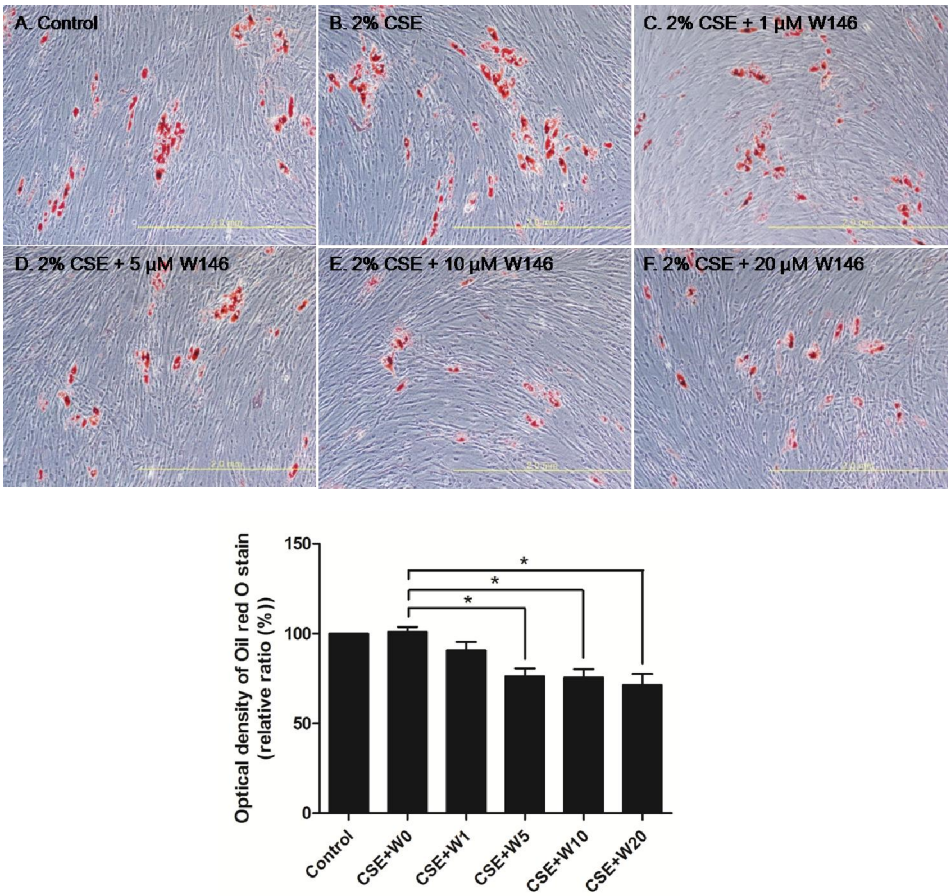


Figure 13. Effects of W146 on CSE-stimulated adipogenesis

Cells from GO patients were treated with 2% CSE for the first 3 days of the 10-day period of adipogenesis in adipogenic medium containing 10 μM rosiglitazone. To determine the suppressive effect of W146 on adipogenesis, W146 (1~20 μM) was also added for the first 4 days of differentiation. The cells were stained with Oil Red O and examined microscopically (x100). The experiments were performed in triplicate with cells from different GO donors, and the representative figures are shown. The optical density of stained cell lysates was also measured (* $p<0.05$, in contrast to cells treated with 2% CSE only).

Ⅳ. DISCUSSION

Adipogenesis of orbital fibroblast is one of the major pathogenic mechanisms in development of GO. The orbital volume is largely increased by adipogenesis, which is the differentiation of predominantly Thy-1 negative fibroblasts (preadipocytes) into adipocytes. Not only increasing the orbital volume, it also elevates auto-antigen abundance since TSHR expression is upregulated during adipogenesis.[26] Kumar et al. also reported that TSHR gene expression occurs together with expression of fat differentiation genes: leptin, adiponectin and PPARγ.[27]

Among the diverse spectrum of biological activities of S1P, including cell proliferation, survival, migration, cytoskeletal organization, and morphogenesis[7,28], we focused to its role in adipogenesis in GO. As for previous study with 3T3-L1 cell, S1P played a significant role during adipocyte differentiation in mouse 3T3-L1 cells.[16] Fingolimod, a functional antagonist of S1P, down-regulated the adipogenesis of 3T3-L1 preadipocytes vice versa.[19]

However there had been no report of S1P involving GO pathogenesis. Thus, we first compared the distribution of S1P receptor isoforms in GO and normal control orbital fat tissue, and which showed higher level of S1P receptor 1,2 and 3 in GO than in control. S1P receptor mRNA expression was significantly increased over time during adipogenic differentiation. Exogenous S1P treatment enhanced adipocyte differentiation as shown in Oil red O stain, and also increased the expression of adipogenetic transcriptional markers. Conversly, adipogenesis was attenuatated by blockade of S1P1 and S1P2 receptors.

W146 is a $S1P_1$ receptor antagonist that exhibits no agonist or antagonist activity at $S1P_2$, $S1P_3$, or $S1P_5$ receptors. W146 is active *in vivo* causing skin capillary leakage in murine lung and skin as well as inhibition of $S1P_1$ agonist-induced lymphocyte sequestration. [29] JTE-013 is a potent,

selective $S1P_2$ receptor antagonist that reverses the inhibitory effects of S1P on cell migration of vascular endothelial cells and smooth muscle cells.[30] In this study, we found the anti-adipogenic effect of these S1P receptor antagonists which was more prominent with W146 then JTE013 as shown in Oil red O stain. (Figure 7, 8)

The microscopic results with Oil Red O staining show that W146 and JTE013 suppressed adipogenesis in orbital fibroblasts and reduced the protein levels of adipogenesis-related transcriptional factors, PPARγ and C/EBPα, and their upstream regulator, C/EBPβ. The inhibitory effect of S1P receptor antagonists on adipogenesis is not associated with nonspecific drug cytotoxicity, as shown by MTT analysis of cell viability. It is known that the PPARγ and C/EBP transcription factors are expressed at distinct phases during adipogenesis, and they have been shown to play important roles. Our data suggest that W146 and JTE013 exerted antiadipogenic effects by suppressing these adipogenic transcription factors.

Oxidative stress plays an important role in the pathogenesis of GO.[31,32] Oxygen free radicals contribute to the proliferation of orbital fibroblasts and glycosaminoglycan production.[33] Smoking is the strongest known environmental factor stimulating the development and deterioration of GO by enhancing the generation of ROS and enhance adipogenesis.[25,34] So as to the treatment of GO, H_2O_2-induced elevation of the superoxide anions was abolished by the antioxidant treatment.[35] Recently, selenium (an antioxidant) was successfully applied in patients with mild GO in a large, multicenter, randomized, placebo-controlled trial in Europe.[36] Antioxidants may exert their actions through anti-oxidative or anti-inflammatory effects.

There is growing evidence that the sphingolipid rheostat also plays a pivotal role in cellular reponse to oxidative stress, determining whether cells survive of proceed to apoptosis.[37,38] Moderate levels of oxidative stress activate SphK1, thus shifting the ceramide-S1P balance toward S1P, suppressing

apoptosis and maintaining cell survival and proliferation. However excessive oxidative stress leads to SphK1 degradation, which decreases S1P, tipping the balance of the rheostat toward apoptosis.

As shown in this study, S1P seems to play a role in the response to oxidative stress in GO orbital fibroblasts. In our study, W146 and FTY720 significantly inhibited H_2O_2 induced antioxidant HO-1 production and ROS generation in orbital fibroblasts (Figure 10).

The induction of antioxidant enzyme HO-1 is considered to be a part of protective response to oxidative stress, as an active defense mechanism.[25] HO-1 was upregulated in OFs by oxidative stress and pre-treatment with S1P receptor antagonists reduced the expression of HO-1. This reduction in HO-1 seems to be a consequence of the reduction in intracellular ROS induced by S1P receptor antagonists activity.

We also examined to identify which signal cascade controlled the action of S1P under oxidative stress. The results suggest that H_2O_2 induced oxidative stress activates ERK pathway rather than JNK 1/2, p38 MAPK, or PI3-K. The blockade of the S1P receptor by W146 or FTY720 strongly attenuated phospho-ERK expression same as blokade by PD98059, implying the involvement of the ERK signaling pathway in this antioxidant action of S1P receptor blockers.

Oxidative stress in adipose tissue is emerging as an important mediator of adipocyte dysfunction in obesity[39] and which also enhances adipogenesis in GO. In this study, we found that adipogenesis in Graves' orbital fibroblasts was upregulated by the oxidant H_2O_2, and CSE, and that this was suppressed in a dose-dependent manner by S1P1 receptor blocker W146. This suppression of adipogenesis is overtly due to the compositive effect of both anti-adipogenesis and anti-oxidative effect of W146.

Given that S1P receptor patterns may be complicated by the presence of multiple isoforms with opposing actions on cell surface, and thus specificity

of receptor affinity is a key element in successful S1P receptor-based therapeutic interventions[40], our results implicate the S1P1 receptor as a potentially valuable therapeutic target for designing new drugs for GO, especially targeting for adipogenesis.

Ⅴ. CONCLUSION

S1P plays a significant role in orbital adipocyte differentiation and pathogenesis of Graves' ophthalmopathy. Modulation of this bioactive mediator and its receptor signaling may provide a therapeutic target for the treatment of Graves' ophthalmopathy.

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ABSTRACT(IN KOREAN)

갑상선안병증의 지방분화에서 Spingosine-1-phosphate의 역할

<지도교수 윤 진 숙>

연세대학교 대학원 의학과

김 성 은

갑상선안병증은 자가면역 염증질환으로, 안와섬유모세포의 염증반응, Hyaluronan 생성 및 지방분화의 증가로 인한 안와 내 지방결체조직과 외안근의 팽창이 주요기전으로 이해되고 있다.
Spingosine-1-phosphate(S1P)는 증식, 면역 세포 유도, 분화 등의 다양한 세포 반응을 매개하는 생체 활성 Sphingolipid이다. 본 연구자는 갑상선안병증의 안와섬유모세포의 지방분화에 S1P가 어떠한 역할을 하는지 연구하고자 하였다.

갑상선안병증 환자와 정상인의 안와 지방결체조직으로부터 안와섬유모세포를 일차 배양하고, 지방분화 유도 배양액에서 지방분화를 촉진하였다. Semiquantitive RT-PC R을 이용하여 S1P 수용체의 mRNA 발현을 측정하였다. 지방분화에 대한 S1P 및 S1P 수용체 차단제 (W146, JTE013 및 FTY720)의 효과를 평가하기 위해, 지방분화기간의 첫 4 일 동안 이들을 처리하고, 지방분화가 끝난 후 Oil red O 염색 및 western blot assay를 이용하여 지방분화정도 및 PPARɣ, C/EBP 단백질 발현을 확인하였다.

S1P 수용체 1, 2, 3 mRNA 발현 수준은 모든 갑상선안병증 조직 샘플에서 정상조직에 비해 유의하게 높았다. S1P 수용체 1-5 mRNA 발현은 10일 간의 지방분화 과정동안 유의하게 증가하였다. S1P를 처리한 경우, 지방 세포의 크기 및 수가 증가하였으며, 지방 방울의 축적이 증가하였고, 지방 세포 전사 조절제의 발현도 증가하였다. S1P 수용체 1 억제제 (W146) 및 S1P 수용체 2 차단제 (JTE013)를 지방분화유도 후 4 일 동안 처리한 경우, 지방분화가 억제됨을 관찰하였다. 또한, S1P 수용체 차단제가 갑상선

안병증의 안와 섬유아세포에서 활성 산소종 (ROS)의 생산을 감소시킬 수 있는지 여부를 연구하였다. S1P 수용체 차단제는 갑상선안병증 안와 섬유아세포에서 과산화수소에 의해 자극된 HO-1의 발현 및 활성산소종의 생성을 감소시켰다. 또한 S1P1 수용체 차단제는 10 μM H2O2와 2 % 담배 연기 추출물 (CSE) 처리에 의해 자극된 지방세포 분화 및 지방질의 축적을 억제하였다.

본 연구자는 S1P가 갑상선안병증의 지방분화에 작용하여 갑상선안병증의 병리기전에 영향을 미칠 수 있음을 확인하였으며, 추후 S1P 수용체 차단제를 이용하여 갑상선안병증의 치료에 적용할 수 있을 것으로 기대한다.

핵심되는 말 : 스핑고신-1-인산염, 갑상선안병증, 지방분화,
S1P 수용체 차단제