The Role of Sphingosine-1-Phosphate in Adipogenesis of Graves’ Orbitopathy

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Submitted: August 3, 2015
Accepted: December 21, 2015
DOI:10.1167/iovs.15-17863

PURPOSE. To investigate the action of sphingosine-1-phosphate (S1P) in adipocyte differentiation of orbital fibroblasts and determine its putative role in the pathogenesis of Graves’ orbitopathy (GO).

METHODS. Primary preadipocyte orbital fibroblast cultures were stimulated for adipogenesis. Real-time PCR was performed to evaluate the expression of S1P receptor mRNA. To evaluate the effect of S1P and S1P receptor blockers (W146 and FTY720) on adipocyte differentiation, cultures were exposed to each receptor blocker for the first 4 days of the differentiation period. Differentiated cells were stained with Oil Red O, and the production of peroxisome proliferator activator gamma (PPARγ) and CCAAT-enhancer-binding proteins (C/EBP) α and β were determined by Western blot analysis.

RESULTS. Sphingosine-1-phosphate receptor 1, 2, and 3 mRNA expression levels were significantly higher in GO tissue samples than non-GO. Sphingosine-1-phosphate receptor 1 through 5 mRNA expression was significantly increased during the 10 days of adipogenesis. Sphingosine-1-phosphate treatment increased the size and number of adipocytes, and increased the expression of adipogenic transcriptional regulators. Treatment with S1P1 receptor inhibitor (W146) for 4 days after induction of adipogenesis attenuated adipocyte differentiation. 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 μM H2O2 or 2% cigarette smoke extract (CSE) treatment.

CONCLUSIONS. Sphingosine-1-phosphate could play a role in orbital adipocyte differentiation of GO. Modulation of S1P actions may provide a therapeutic target for the treatment of GO.

Keywords: sphingosine-1-phosphate, Graves’ orbitopathy, adipogenesis

Graves’ orbitopathy (GO) is an autoimmune disease that occurs in up to 50% of patients with Graves’ disease.1 Severe and potentially blinding orbital inflammation develops in approximately 5% to 6% of these patients.2 The inflammation causes muscle and soft tissue swelling, which, when progressive, can result in clinical features of proptosis, exposure keratopathy, and compressive optic neuropathy.

Current evidence points to orbital fibroblasts as the target cells in GO and suggests that their normal functions are dysregulated through autoimmune mechanisms.3 These orbital fibroblasts secrete large quantities of hyaluronan in response to various cytokines, and a subgroup of the orbital fibroblasts then differentiate into mature adipocytes that have increased thyrotropin receptor production.4,5 These cellular changes lead to enlargement of extraocular muscles and expansion of orbital fat in GO patients.

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.6,7 Sphingosine-1-phosphate is formed by the action of sphingosine kinase (SphK), which converts sphingosine and ATP to S1P. Most of its best-characterized actions are mediated through a family of five G protein-coupled receptors, the S1P1 through 5 receptors, which couple to a variety of G proteins allowing them to mediate many different biologic responses.5,8

Among the several interesting actions of S1P, a great deal of attention has been focused on its effects on the process of differentiation. The S1P/S1P1 receptor system plays a crucial role in promoting angiogenesis by regulating differentiation of vascular endothelial cells.9,10 Sphingosine-1-phosphate also promotes myogenic differentiation of the mouse C2C12 skeletal myocyte cell line,11 and modulates mouse osteoclast differentiation.12 Recently, Hashimoto et al.13 reported that sphingosine kinase and S1P are upregulated during adipogenesis and play a significant role in adipocyte differentiation in mouse 3T3-L1 cells. 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, is robustly induced when cells are treated with...
which acts as a functional antagonist of S1P, downregulates disease or GO and with no clinical evidence of GO (n = 5; AGC GGA CCA CGC AGT A-3).

To date, no studies have evaluated the expression of S1P receptors and the action of S1P in orbital fibroblasts and its putative role in adipocyte differentiation, one of the most important features of pathogenesis of GO. Here, we hypothesized that S1P could play a role in orbital adipocyte differentiation, and modulation of this bioactive mediator may provide a therapeutic target for the treatment of GO.

**MATERIALS AND METHODS**

**Reagents and Chemicals**

Sphingosine-1-phosphate (S1P) and FTY720 were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Sphingosine-1-phosphate receptor 1 antagonist, W146 ( trifluoracetate salt) was purchased from Cayman Chemical (Ann Arbor, MI, USA). 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-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and Oil Red O were purchased from Sigma-Aldrich, Inc. Anti-peroxisome proliferator activator gamma (PPARγ), anti-CCAAT-enhancer-binding protein (C/EBP) α, anti-C/EBP β, anti-heme oxygenase (HO)-1, anti-extra-cellular-regulated kinase (ERK) 1/2, anti-phospho ERK 1/2, anti-phospho-Akt, and anti-β-actin antibodies were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Orbital adipose/connective tissue specimens were obtained during the orbital decompression surgery for severe GO (n = 5; three women and two men, aged 29–59 years) (Table). 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 scale developed by Mourits et al.17 No patients had been harvested during the orbital decompression surgery for severe GO (n = 5; AGC GGA CCA CGC AGT A-3).

**Cell Culture and Differentiation Protocol**

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% CO2 incubator at 37°C. The strains were stored in liquid N2 until needed, and 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 was changed to serum-free DMEM supplemented with 33 μM biotin, 17 μM pantethinic 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 to 3 days. A PPARγ agonist, rosiglitazone (10 μM, Cayman, Ann Arbor, MI, USA), was added from day 1 for further stimulation of adipogenesis.18 To evaluate the effect of S1P and S1P receptor blockers (W146 and FTY720) on adipocyte differentiation, we exposed cultures to S1P and S1P receptor blockers for the first 4 days of the differentiation period.

**Real-Time PCR**

Total RNA (1 μg) was isolated and reverse transcribed into complementary DNA according to the manufacturer’s instructions (q74104; Qiagen, Valencia, CA, USA). The resulting cDNA was amplified using an ABI StepOnePlus real-time PCR thermocycler (Applied Biosystems, Carlsbad, CA, USA) using TaqMan universal PCR master mix and recommended PCR conditions to assess gene transcript levels quantitatively in the cell samples. All PCR reactions were performed in triplicate. Primer sequences are as follows: human S1P receptor 1, 5'-TGG GGG AAG GGA GTA TGT TT-3' (forward) and 5'-CCA TCC CCA CAC TCA AC-3' (reverse); 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); human S1P receptor 3, 5'-TGA TTG TGG TGA GCG TCT TCA-3' (forward) and 5'-GGC CAT AAT GAG GAA GAG-3' (reverse); human S1P receptor 4, 5'-CGG CAC AGC CTC CTC ATT GTC-3' (forward) and 5'-ACA GAC GGA TG CAG CAT ACA CAC-3' (reverse); for human S1P receptor 5, 5'-CGT CCT GCA TTA CTA CAC C-3' (forward) and 5'-GGC CAT AAT GAG GAA GAG-3' (reverse); and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) 5'-GCC AAG GTC ATC CAT GAC AAC-3' (forward) and 5'-AGA GCC CAG GTG TAC AGG AAG-3' (reverse).
were detected with horseradish peroxidase-conjugated secondary antibody in Tris Buffer Saline Tween 20, and washed three times with PBS. The cells were then trypsinized, washed, and resuspended in PBS. Thereafter, fluorescence intensity was measured using a flow cytometer (ELITE flow cytometer; Coulter Cytometry, Inc., Hialeah, FL, USA).

Preparation of CSE

Cigarette smoke extract was prepared by bubbling smoke from two commercially available filtered cigarettes (Marlboro 20 class A cigarettes, containing 8.0 mg of tar and 0.7 mg of nicotine; Philip Morris Korea, Inc., Seoul, Korea) through 20 mL prewarmed serum-free DMEM/F12 (1:1) at a rate of one cigarette per 2 minute, as described previously. The pH of the CSE was adjusted to 7.4 and the CSE was filter sterilized through a 0.2-μm filter (Sartorius Stedim Biotech, Gottingen, Germany), which was then standardized by measuring its absorbance (optical density = 0.65 ± 0.05 at 320 nm). The spectrophotographic pattern of absorbance at 320 nm showed very little variation between different CSE preparations. The CSE was freshly prepared within 1 hour of each experiment and diluted with culture medium, adjusted to a pH of 7.4, and filter sterilized as described for 10% CSE.

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 SD was calculated for normalized measurements of each mRNA or protein from multiple (≥3) samples harvested from different individuals. Data between or within cell groups at different drug concentrations and incubation times were analyzed by the t-test or ANOVA test and Bonferroni method as a post hoc test using the SPSS program for Windows, version 16 (SPSS, Chicago, IL, USA). A P value less than 0.05 was considered significant.

RESULTS

S1P Receptor 1 Through 5 mRNA Expression in Orbital Fibroblasts

To compare the expression of S1P receptor mRNAs in GO and non-GO orbital tissues, we performed relative quantification of mRNA for each receptor by RT-PCR using whole orbital tissue explants obtained from five GO and five non-GO control patients. Sphingosine-1-phosphate 1, S1P2, and S1P3 mRNAs were expressed at relatively high levels in GO orbital tissues relative to non-GO tissues (P < 0.05); however, S1P receptor 4 and 5 gene levels were lower in GO tissues (Fig. 1A). We also measured S1P receptor 1 through 5 mRNA expression on days 0, 4, 7, and 10 of adipocyte differentiation of GO fibroblasts (n = 3) and non-GO fibroblast (n = 3) cultures. All S1P receptor types were significantly increased during the adipogenesis of GO fibroblasts. Receptor gene expression seemed to increase over time and was usually highest on day 7 (Fig. 1B). The receptor gene expression pattern during adipogenesis of non-GO fibroblast was similar to that of GO, yet the expression levels were comparatively low in non-GO cultures.

Effect of S1P on GO Orbital Fibroblast Adipogenesis

Confluent orbital fibroblasts from GO patients (n = 3) 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, the orbital fibroblasts lost their stellate fibroblastic appearance and converted to a spherical adipocytic shape under the control adipogenic conditions, and a fraction of these cells accumulated small lipid droplets. To examine whether treating with S1P had any effect on adipogenesis, different S1P concentrations (0–1000 ng/mL) were added to the adipogenic medium for 4 days during the differentiation period, being replaced whenever the medium was replaced. High-power (>200) microscopic examination of Oil Red O staining showed that S1P treatment increased the size and number of adipocytes and increased the accumulation of lipid droplets with the 250 and 500 ng/mL S1P treatments showing a prominent increase (Fig. 2A). Western blot analysis was performed to investigate whether S1P affects the production of adipogenic transcription factors during adipogenesis. Each experiment was performed in three GO cells from different patient samples and was assayed in duplicate.

**Figure 1.** Expression of S1P receptor mRNAs in GO and non-GO orbital tissues, and differentiating Graves' orbital fibroblast cultures. (A) Comparison of S1P receptor 1 through 5 mRNA expression using real-time PCR between non-GO controls (n = 5) and GO (n = 5) tissue samples. Sphingosine-1-phosphate receptor 1, 2, and 3 mRNA expression levels were all significantly higher in GO tissues samples relative to non-GO tissues (*P < 0.05). (B) Sphingosine-1-phosphate receptor 1 through 5 mRNA expression was significantly increased during the 10 days of adipogenesis in orbital fibroblast cultures from different non-GO (n = 3, white columns) and GO orbital tissues (n = 3, black columns). The data in the columns are the mean relative fold of mRNA ± SD of three experiments (*P < 0.05 versus control undifferentiated cells).
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Oxidative Stress Conditions

Expression of S1P Receptors 1 Through 5 Under Oxidative Stress Conditions

Effect of S1P on ERK1/2 Activation

Signaling Pathway of S1P Action Under H2O2-Induced Oxidative Stress

S1P receptor blocker treatment decreased ROS production in orbital fibroblasts from GO. When pretreated with or without 200 μM H2O2 for 1 hour, treatment of orbital fibroblasts with 10 μM W146 or 1 μM FTY720 for 1 hour significantly suppressed HO-1 production in cells stimulated with 200 μM H2O2 (Fig. 4C). In addition, we examined whether S1P receptor blockers decrease ROS production in orbital fibroblasts from GO. When pretreated with or without 200 μM H2O2 for 1 hour, treatment of orbital fibroblasts with 10 μM W146 or 1 μM FTY720 significantly decreased ROS levels detected by the ROS-sensitive fluorescent probe, DCFDA (Fig. 4D). Each experiment was performed in three GO cells from different patient samples and was assayed in duplicate per each cell sample.

S1P Receptor Blockers Inhibit HO-1 Production and ROS Generation in GO Orbital Fibroblasts

Production of the antioxidant enzyme hemeoxygenase-1 (HO-1) in GO fibroblasts was significantly upregulated by treatment with 200 μM H2O2 for 4 hours. Pretreatment with S1P receptor antagonists 10 μM W146 or 1 μM FTY720 for 1 hour significantly suppressed HO-1 production in cells stimulated with 200 μM H2O2 (Fig. 4A). In addition, we examined whether S1P receptor blockers decrease ROS production in orbital fibroblasts from GO. When pretreated with or without 200 μM H2O2 for 1 hour, treatment of orbital fibroblasts with 10 μM W146 or 1 μM FTY720 significantly decreased ROS levels detected by the ROS-sensitive fluorescent probe, DCFDA (Fig. 4D). Each experiment was performed in three GO cells from different patient samples and was assayed in duplicate per each cell sample.

Effect of S1P on ERK1/2 Activation

Sphingosine-1-phosphate at 100 ng/mL and above provoked a rapid and relevant activation of ERK 1/2 in GO cells, whose phosphorylation was maximal at 30 minutes and declined to basal levels at 180 minutes (Fig. 5A).

**FIGURE 2.** Effect of S1P on adipogenesis of Graves’ orbital fibroblasts. (A) Different S1P concentrations (100, 250, 500, or 1000 ng/mL) were used to treat orbital fibroblasts during the first 4 days of adipogenic differentiation. Cells were stained with Oil Red O and examined microscopically (×200). Adipogenesis was increased by S1P treatment compared with the control. The increased adipocyte differentiation was prominent on Oil Red O staining when exposed to 250 and 500 ng/mL S1P treatments. The optical density of stained cell lysates also showed significantly increased absorbance at 490 nm with 250 and 500 ng/mL S1P treatment (*P < 0.05 versus untreated control). (B) Western blot analysis for PPARγ, C/EBPα, and C/EBPβ protein production after 10 days of adipogenic differentiation. The experiments were performed in triplicate with cells from three different donors. Quantification of the PPARγ, C/EBPα, and C/EBPβ levels by densitometry were normalized to the level of β-actin in the same sample. The data in the columns are the mean relative density ratios (%) ± SD of three experiments (*P < 0.05 versus untreated differentiated control cells).

**Effect of the W146 S1P Receptor 1 Blocker on Adipogenesis in GO Orbital Fibroblasts**

S1P1 receptor inhibitor, a S1P receptor 1 blocker, was added at different concentrations to the adipogenic medium for the first 4 days during the differentiation period to examine whether W146 has a suppressive effect on adipogenesis. S1P1 receptor inhibitor reduced the number of adipocytes and suppressed the accumulation of lipid droplets, as visualized by microscopic examination after Oil Red O staining (Fig. 3A). The optical density of stained cell lysates was also measured to evaluate adipocyte differentiation quantitatively. At 10 and 20 μM concentrations, W146 treated cells showed significantly decreased absorbance at 490 nm (P < 0.05). Western blot analyses were then performed to investigate whether W146 affects the production of the adipogenic transcription factors PPARγ, C/EBPα, and C/EBPβ during adipogenesis. PPARγ and C/EBPβ proteins were significantly attenuated by 20 μM W146 treatment (Fig. 3B). Each experiment was performed in three GO cells from different patient samples and was assayed in duplicate per each cell sample.

**Expression of S1P Receptors 1 Through 5 Under Oxidative Stress Conditions**

We measured S1P receptor 1 through 5 mRNA expression under oxidative stress conditions. Orbital fibroblasts of GO (n = 3) and non-GO (n = 5) patients were exposed to 200 μM H2O2 or 2% CSE for 0, 1, 3, and 6 hours. All S1P receptor types were significantly increased under the oxidative stress conditions induced with H2O2 by 6 hours (Fig. 4A). However, the expression levels were comparatively low in non-GO cultures. Two percent of CSE also induced expression of S1P receptor 1, 2, 3, and 5 mRNA in GO fibroblast cultures by 6 hours, yet the elevation levels in non-GO cultures were not statistically significant (Fig. 4B).

S1P Receptor Blockers Inhibit HO-1 Production and ROS Generation in GO Orbital Fibroblasts

Production of the antioxidant enzyme hemeoxygenase-1 (HO-1) in GO fibroblasts was significantly upregulated by treatment with 200 μM H2O2 for 4 hours. Pretreatment with S1P receptor antagonists 10 μM W146 or 1 μM FTY720 for 1 hour significantly suppressed HO-1 production in cells stimulated with 200 μM H2O2 (Fig. 4A). In addition, we examined whether S1P receptor blockers decrease ROS production in orbital fibroblasts from GO. When pretreated with or without 200 μM H2O2 for 1 hour, treatment of orbital fibroblasts with the 10 μM W146 or 1 μM FTY720 significantly decreased ROS levels detected by the ROS-sensitive fluorescent probe, DCFDA (Fig. 4D). Each experiment was performed in three GO cells from different patient samples and was assayed in duplicate per each cell sample.

**Signaling Pathway of S1P Action Under H2O2-Induced Oxidative Stress**

To investigate the molecular mechanism of S1P in GO orbital fibroblasts under oxidative stress conditions, we observed the effect of S1P on ERK1/2 activation. Sphingosine-1-phosphate at 500 ng/mL provoked a rapid and relevant activation of ERK 1/2 in GO cells, whose phosphorylation was maximal at 30 minutes and declined to basal levels at 180 minutes (Fig. 5A).
FIGURE 3. Adipogenesis after S1P receptor blocker treatment. (A) Treatment with the S1P receptor 1 inhibitor W146 for 4 days after induction of adipogenesis was shown to attenuate adipocyte differentiation as shown with microscopic examination (×100) of Oil Red O staining. The optical density of stained cell lysates was also measured to evaluate adipocyte differentiation quantitatively. The W146-treated cells at 10 and 20 μM concentrations showed significantly decreased absorbance at 490 nm (*P < 0.05 versus untreated control). (B) A representative Western blot of PPARγ, C/EBPα, and C/EBPβ protein production after 20 μM W146 treatment. PPARγ and C/EBPα proteins were significantly attenuated by 20 μM W146 treatment; however, C/EBPβ was not significantly affected. Quantification of PPARγ, C/EBPα, and C/EBPβ protein levels 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 versus untreated differentiated control cells).

Treatment with PD98059 significantly blocked the S1P-provoked phosphorylation of ERK 1/2 (Fig. 5B). The phosphorylation of ERK 1/2 was also strongly enhanced at 30- to 60-minutes when challenged with 200 μM H2O2 (Fig. 5C). When cells were pretreated with W146 and FTY720, H2O2-induced phospho-ERK was significantly inhibited (Fig. 5D). Each experiment was performed in three GO cells from different patient samples and was assayed in duplicate per each cell sample.

Effects of W146 on H2O2- or CSE-Stimulated Adipogenesis of GO Orbital Fibroblasts
To determine the additive effect of W146 on adipogenesis and against oxidative stress, we stimulated adipogenesis by addition of 10 μM H2O2 or 2% CSE along with W146 for the first 3 days of differentiation. As previously reported by Yoon et al.,25 the addition of 10 μM H2O2 or 2% CSE significantly increased adipogenesis compared with that observed under control conditions, which was assessed with light microscopy (×40) after Oil Red O staining (Fig. 6). When W146 (1–20 μM) was added to the adipogenic medium for the first 4 days during the differentiation period, W146 reduced the number of adipocytes and suppressed the accumulation of lipid droplets induced by 10 μM H2O2 or 2% CSE treatment (Fig. 6). The optical density of stained cell lysates was also measured to evaluate adipocyte differentiation quantitatively. The W146-treated cells showed a decreased absorbance at 490 nm (*P < 0.05, in contrast to cells treated with 10 μM H2O2 or 2% CSE only).

DISCUSSION
Sphingosine-1-phosphate is involved in many biological activities including cell proliferation, survival, migration, cytoskeletal organization, and morphogenesis.6,24 In this study, we focused on the role of S1P in GO adipogenesis. Previous research with 3T3-L1 cells showed that S1P plays a significant role during adipocyte differentiation.13 FTY720, a functional antagonist of S1P, was found to downregulate the adipogenesis of 3T3-L1 preadipocytes.16

In our study, we first identified the distribution of S1P receptor isoforms in GO tissues in comparison with non-GO orbital fat tissue, and which showed higher level of S1P receptor 1, 2, and 3 in GO than in non-GO. Sphingosine-1-phosphate receptor mRNA expression was significantly increased in GO cells overtime during adipogenic differentiation. The receptor gene expression patterns of non-GO orbital fibroblasts during adipogenesis and under oxidative conditions were similar to that of GO, yet the expression levels were comparatively low in non-GO cultures. Therefore, we could assume that the action of S1P and S1P receptor blocker in adipogenesis and oxidative stress condition would be specific in GO.

Interestingly, as shown in Figure 1B, all S1P receptor mRNA expression was increased during the adipogenic differentiation, however S1P receptor 2 to approximately 5 mRNA decreased from day 7 to day 10. We consider it to be due to the maturation of preadipocyte to adipocyte at the end of adipogenic differentiation. Sphingosine-1-phosphate may affect adipogenesis during the active differentiating phase of adipogenesis. There is a similar report that activity of sphingosine kinase, which are responsible for S1P biosynthesis from sphingosine, was increased in early adipogenic differentiation and decreased after day 6 of adipogenesis and S1P receptor 1 to approximately 5 mRNA levels were high in preadipocyte state and declined after full differentiation.25 Exogenous S1P treatment enhanced adipocyte differentiation as shown in Oil red O stain, and also increased the expression...
of adipogenetic transcriptional markers. Conversely, adipogenesis was significantly attenuated by blockade of S1P1 receptor.

In this study, we found that W146 suppressed adipogenesis in orbital fibroblasts and reduced the protein levels of adipogenesis-related transcriptional factors, PPARc and C/EBPa. PPARc and C/EBP transcription factors are produced at distinct phases during adipogenesis, which have been shown to play important roles. Sphingosine-1-phosphate 1 receptor antagonist, W146 that exhibits no agonist or antagonist activity at S1P2, S1P3, or S1P5 receptors is known to cause in vivo skin capillary leakage in murine lung and skin as well as inhibition of S1P1 agonist-induced lymphocyte sequestration. Our data suggest that W146 exert antiadipogenic effect by suppressing these adipogenic transcription factors.

Oxidative stress plays an important role in the pathogenesis of GO. Oxygen free radicals contribute to the proliferation of orbital fibroblasts and glycosaminoglycan production. Smoking is the strongest known environmental factor, which stimulates the development and deterioration of GO by enhancing ROS generation and adipogenesis. H2O2-induced elevation of superoxide anions can be abolished by antioxidant treatment, however. Recently, selenium (an antioxidant) was successfully applied in patients with mild GO in a large, multicenter, randomized, placebo-controlled trial in Europe. Antioxidants may exert their actions through antioxidative or anti-inflammatory effects.

There is a growing evidence that the sphingolipid rheostat also plays a pivotal role in cellular response to oxidative stress, determining whether cells survive or proceed to apoptosis. Moderate levels of oxidative stress activate Sphingosine kinase 1 (SphK1), an enzyme which phosphorylates sphingosine to S1P, making a shift in the ceramide-S1P balance toward S1P, and that suppress apoptosis and maintain cell survival and proliferation. However, excessive oxidative stress leads to SphK1 degradation, which decreases S1P, shifting the balance of the rheostat toward apoptosis. As shown in this study, S1P

![Figure 4](image-url)

**Figure 4.** Effect of oxidative stress on S1P receptors and the S1P receptor blockers on ROS production. (A) After treatment with 200 μM H2O2 for 0, 1, 3, or 6 hours, S1P receptor 1 through 5 gene expression was increased in both non-GO (n = 3, white column) and GO (n = 3, black column) orbital fibroblasts (*P < 0.05). A significant increase was detected after a 6-hour H2O2 treatment. (B) After treatment with 2% CSE for 0, 1, 3, and 6 hours, S1P receptor 1 through 5 gene expression showed a significant increase in GO orbital fibroblasts (black column; *P < 0.05 versus control). However, the increase was not statistically significant in non-GO cultures (white column). (C) Effects of the W146 and FTY720 S1P receptor blockers on H2O2-stimulated HO-1 production in preadipocyte orbital fibroblasts, analyzed with Western blotting. Orbital fibroblasts (5 × 105) from GO patients (n = 3) were pretreated with 10 μM W146 or 1 μM FTY720 for 1 hour, and then stimulated with 200 μM H2O2 for 4 hours to determine the effect of the S1P receptor antagonists on H2O2-stimulated HO-1 protein production. Quantification of HO-1 by densitometry, normalized to the level of β-actin in the same sample, is shown. (D) GO orbital fibroblasts were incubated with H2DCFDA 10 μM for 24 hours with 10 μM W146, and 1 μM FTY720 in the presence or absence of 200 μM H2O2 for 1 hour and the fluorescence intensities were analyzed using FACS W146 and FTY720 decreased ROS levels significantly in orbital fibroblasts from GO. Results are expressed as a percentage of untreated control values presented as the mean ± SD of three separate experiments (*P < 0.05 versus cells treated with 200 μM H2O2 only).
seems to play a role in the response to oxidative stress in GO orbital fibroblasts. In our study, W146 and FTY720 significantly inhibited H2O2-induced antioxidant enzyme HO-1 production and ROS generation in GO orbital fibroblasts (Fig. 4). The induction of HO-1 is considered a part of the protective response to oxidative stress, as an active defense mechanism.23

Antioxidant enzyme hemeoxygenase-1 was upregulated in orbital fibroblasts by oxidative stress and pretreatment with S1P receptor antagonists reduced the production of HO-1. This reduction in HO-1 seems to be a consequence of the reduction in intracellular ROS induced by S1P receptor antagonist activity.
Figure 6. Effects of W146 on H$_2$O$_2$-stimulated adipogenesis. Cells from GO patients ($n=3$) were treated with 10 µM H$_2$O$_2$ (A) or 2% CSE (B) for the first 3 days of the 10-day adipogenesis period 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 ($\times40$). The experiments were performed in triplicate with cells from three different GO donors, and representative images are shown. The optical density of stained cell lysates was also measured (*$P<0.05$, in contrast to cells treated with 10 µM H$_2$O$_2$ only).
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We also identified one signaling cascade that appears to control the action of S1P under oxidative stress. Our results suggest that H2O2-induced oxidative stress activates the ERK pathway (Fig. 5). Blockage of the S1P receptor by W146 or FTY720 strongly attenuated phospho-ERK expression, similar to blockage by PD98059, implying the involvement of S1P in the activation of ERK signal by oxidants such as H2O2. In this study, we used two housekeeping genes, GAPDH for RT-PCR and β-actin for Western blot assay. In our laboratory setting, both RNAs were usually constant during adipogenesis of GO orbital fibroblasts and in oxidative stress condition.

Oxidative stress in adipose tissue is emerging as an important mediator of adipocyte dysfunction in obesity, which also enhances adipogenesis in GO. As we previously reported, adipogenesis in GO cells was upregulated by the oxidants H2O2 and CSE, and that this was suppressed by the S1P1 receptor blocker W146. This suppression of adipogenesis is overtly due to the composite antiadipogenic and antioxidative effects of W146.

Our results imply that the S1P1 receptor might be a potentially valuable therapeutic target for designing new GO drugs, especially targeting adipogenesis and oxidative stress. Given that S1P receptor patterns may be complicated by the presence of multiple isoforms with opposing actions on the cell surface, receptor affinity specificity is a key element in successful S1P receptor-based therapeutic interventions. So far, S1P1 antagonists have not reached clinical development, yet they are shown to be effective in various disease models and we are willing to suggest GO as one of new target diseases.

CONCLUSIONS

Sphingosine-1-phosphate plays a significant role in orbital adipocyte differentiation in GO and modulation of this bioactive mediator and its receptor signaling may be a viable therapeutic target for the treatment of GO.

Acknowledgments

Supported by grants from the Basic Science Research Program through the National Research Foundation of Korea (NRF; Seoul, Korea) funded by the Ministry of Education, Science and Technology, grant number (NRF-2013R1A1A2007190; Seoul, Korea).

Disclosure: S.E. Kim, None; J.H. Lee, None; M.K. Chae, None; E.J. Lee, None; J.S. Yoon, None

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