Therapeutic Effect of Resveratrol on Oxidative Stress in Graves’ Orbitopathy Orbital Fibroblasts

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PURPOSE. Multiple causative factors complicate the pathogenesis in Graves’ orbitopathy (GO). It has been suggested that oxidative stress contributes to the development and progression of GO. Therefore, we investigated the therapeutic effect of resveratrol, a potent antioxidant, upon oxidative stress levels in GO orbital fibroblasts in vitro.

METHODS. Orbital fibroblasts were cultured from orbital connective tissues obtained from GO patients. Intracellular reactive oxygen species (ROS) levels and the expression of heme oxygenase-1 (HO-1), superoxide dismutase (SOD), catalase, and thioredoxin (Trx), were measured after resveratrol treatment. Adipogenesis was induced, and ROS levels were examined during adipogenic differentiation. Western blot assay was performed to evaluate the effects of resveratrol on the expression of antioxidants levels and transcriptional regulators.

RESULTS. Treatment with 30 or 50 μM resveratrol reduced ROS production and HO-1 level induced by oxidative stress. Levels of Cu/Zn-SOD, catalase, and Trx were also reduced, while Mn-SOD increased with 50 μM resveratrol treatment. Resveratrol suppressed adipogenesis, reducing the number of adipocytes and suppressing the accumulation of lipid droplets. Treatment with 50 μM resveratrol also decreased ROS levels during adipogenesis. Expression of the transcriptional regulators phosphor–extracellular signal-regulated kinase and phospho–Jun NH(2)-terminal kinase significantly increased after treatment with 50 μM resveratrol, and decreased in response to inhibitors of each protein. Phosphonuclear factor kappa-light-chain-enhancer of activated B cells p65 levels also increased after treatment with 50 μM resveratrol.

CONCLUSION. Resveratrol reduced ROS levels and inhibited adipogenesis in GO orbital fibroblasts in vitro. This study supports the potential use of resveratrol in GO treatment.

Keywords: adipogenesis, Graves’ orbitopathy, orbital fibroblast, oxidative stress, reactive oxygen species, resveratrol

Graves’ disease is a representative autoimmune disease affecting the thyroid gland. It is the most common cause of hyperthyroidism and in 25% to 50% of cases, the eye is involved as well.1 Graves’ orbitopathy (GO) manifests in various clinical signs and symptoms, ranging from mild ocular discomfort to severe pain and diplopia, eyelid retraction, chemosis, edema and erythema of periorbital tissues, proptosis, and even compressive optic neuropathy. The primary causes of GO pathogenesis are abnormal excessive hyaluronic acid accumulation and orbital adipogenesis resulting from autoimmune processes through thrytropin receptors (TSHRs) and insulin-like growth factor 1 receptors.1 Enlargement of the extraocular muscles and expansion of the orbital fatty connective tissues follow; orbital fibroblasts are known to be the primary target cells of the disease process.1-3

Graves’ orbitopathy causes patients physical pain and considerable distress. It is difficult to manage patients with GO because multiple causative factors complicate its pathogenesis. Glucocorticoids, the most common treatment for GO, are not very effective for the treatment of proptosis and the longstanding extraocular muscle involvement associated with fibrotic changes; they are also associated with many side effects.4 Well-known and occasionally serious complications include cushingoid features, diabetes, hypertension, and osteoporosis. Thus, more effective, safer long-term therapeutic agents for GO are required.

A large number of studies suggest that oxidative stress is associated with GO pathogenesis. The hypothesis that the formation of oxygen radicals is involved in the initiation of the immune response in Graves disease was introduced in the 1980s.5 Subsequently, a study in an experimental animal model showed that thyroid hormone enhances reactive oxygen species (ROS) generation and produces changes in tissue antioxidant systems.6 Burch et al.7 have suggested that oxygen free radicals contribute to retroocular fibroblast proliferation in GO. Bednarek et al.8 have also proposed that thyroid hormones accelerate basal metabolic rate and oxidative metabolism via the induction of mitochondrial enzymes, and thyroid metabolic status influences changes in blood extracellular indices of ROS generation and free radical scavenging in Graves disease. Indeed, a number of recent studies have demonstrated that oxidative stress may play a role in the pathogenesis of GO.9-11
Moreover, the hypothesis that GO orbital fibroblasts are hypersensitive to oxidative stress has been proposed, and several studies have demonstrated ROS in the retroocular fibroblasts and plasma of patients with GO. Finally, Tsai et al. have detected oxidative DNA damage in the urine of GO patients and demonstrated a positive correlation between this damage and clinical GO activity. Resveratrol is a well-known polyphenolic flavonoid with potent antioxidant activity. It is derived from red grapes, berries, knotweed, peanuts, and other plants. Resveratrol has a number of desirable biological properties; it is anticarcinogenic, anti-inflammatory, free-radical scavenging, inhibits/induces apoptosis, and inhibits platelet aggregation. Resveratrol has been shown to mediate death in a wide variety of cells and to have health benefits, especially in common age-related diseases such as cancer, type 2 diabetes, arthritis, and cardiovascular and neurologic diseases.

Given the focus on oxidative stress in GO pathogenesis, in this study we investigated the therapeutic effect of resveratrol (3,5,4′-trihydroxystilbene, Fig. 1), a potent antioxidant, in an in vitro model of GO.

Materials and Methods

Reagents

Resveratrol, the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, the fluorescent probe propidium iodide (PI), and oil red O were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin, and gentamycin were purchased from Hyclone Laboratories, Inc. (Logan, UT, USA). Fluorescent probes 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA), an oxidant-sensitive fluorescent probe, and dihydroethidine (DHE) were purchased from Molecular Probes, Invitrogen (Eugene, OR, USA). Insulin, carbprostaglandin, and rosiglitazone were purchased from Boehringer-Mannheim (Manheim, Germany); Calbiochem (La Jolla, CA, USA); and Cayman (Ann Arbor, MI, USA), respectively. Anti–heme oxygenase (HO)-1, antimanganese (Mn)-superoxide dismutase (SOD), anticopper and zinc (Cu/Zn)-SOD, anticalcatase, antithioredoxin (Trx), anti-extracellular signal-regulated kinase (ERK), anti-phospho-ERK, anti-c-Jun NH2-terminal kinase (JNK), anti–phospho-JNK, antinuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) p65, anti–phospho-NF-kB p65, and anti–β-actin antibodies were all obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Inhibitors of ERK (PD98059 and U0126), JNK (SP600125), protein kinase B (Akt/PKB; LY294002), and p38 (SB203580) were also purchased from Santa Cruz Biotechnology, Inc.

Patients

The research was approved by the Institutional Review Board (IRB)/Ethics Committee. This study followed the tenets of the Declaration of Helsinki, and written informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study.

Orbital connective tissue was obtained from six GO patients who underwent orbital decompression surgery for severe proptosis. The patients did not have a history of radiotherapy and did not take steroid medication for at least 3 months prior to the surgery. They had euthyroid status and a clinical activity score <4 at the time of surgery. Four control tissue samples were obtained from patients who underwent orbital wall reconstruction surgery for orbital wall fracture. Controls did not have a history of GO or hyperthyroidism.

Orbital Fibroblast Culture

Orbital fibroblasts were cultured using published methods. Tissue explants were minced and placed directly in culture dishes with DMEM containing 20% FBS, penicillin (100 U/mL), and gentamycin (20 μg/mL). Cells were incubated in a humidified 5% CO2 incubator at 37°C and maintained in two 80-mm flasks with DMEM containing 10% FBS and antibiotics. Monolayers of proliferated orbital fibroblasts were serially passaged by gently treating with trypsin/EDTA. Strains were stored in liquid N2 and only used between the 3rd and 10th passages.

Cell Viability (MTT) Assay

Cell viability was assessed using the MTT assay, according to the manufacturer’s (Sigma-Aldrich Corp.) protocol. Orbital fibroblasts from control and GO patients were seeded into 24-well culture plates (1 × 105 cells/well). Then, different concentrations of resveratrol (10, 30, 50, or 100 μM) were added for 24 hours. After treatment, cells were washed and treated with MTT (5 mg/mL) solution for 4 hours at 37°C. Then, the medium was removed and the converted dye was solubilized in ice-cold isopropanol. Absorbency was measured at 560 nm with background subtraction at 650 nm using a microplate reader (EL 340 Bio Kinetics Reader; Bio-Tek Instruments, Winooski, VT, USA).

Cigarette Smoke Extract (CSE) Preparation and Dose Determination for CSE and H2O2

We prepared CSE from two commercially available filtered cigarettes containing 8.0 mg tar and 0.7 mg nicotine (Marlboro 20 class A; Philip Morris Korea, Inc., Seoul, Korea) using published methods. In a previous study, we found that treatment with 1% to 5% CSE did not alter the viability of normal cells, while treatment with 2% to 5% CSE induced proliferation in GO cells. In addition, 10 μM H2O2 did not affect the viability of GO cells. Therefore, 2% CSE and 10 μM H2O2 were used in our experiments.

Measurement of Intracellular ROS in GO Cells Stimulated With CSE or H2O2

Levels of ROS were measured using 5-(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA), an oxidant-sensitive fluorescent probe. Cells were seeded at a density of 5 × 105 cells per well in six-well plates to a total final volume of 2 mL and then treated with 2% CSE or 10 μM H2O2 for 30 minutes. To evaluate the effect of resveratrol on ROS levels, cells were pretreated with 30 μM or 50 μM resveratrol for 24 hours. After the medium was removed, cells were washed with PBS and incubated with 10 μM H2DCFDA at 37°C for 30 minutes, and then 2% CSE or 10 μM H2O2 was added for 30
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minutes. Subsequently, cells were trypsinized, washed, and resuspended in PBS. Thereafter, fluorescence intensity was measured with an IX71-F22PH inverted fluorescence microscope (Olympus Corp., Tokyo, Japan) and flow-cytometric analysis was performed (ELITE flow cytometer; Coulter Cytometry, Inc., Hialeah, FL, USA). For each sample, more than 10,000 events were acquired. Cells were gated and the analysis was performed using only live populations. Fluorescent cells were also examined microscopically (>100 magnification).

Western Blot Assay

Cells were washed with ice-cold PBS and lysed on ice for 30 minutes in cell lysis buffer consisting of 20 mM HEPES (pH 7.2), 10% glycerol (vol/vol), 10 mM Na3VO4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1% (vol/vol) Triton X-100. Lysates were centrifuged at 12,000 × g for 10 minutes, and cell homogenate fractions were stored at −70°C before use. The protein concentration of supernatant fractions was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Then, samples containing 50 μg protein were boiled in buffer and resolved by SDS-PAGE, 10% (wt/vol). Proteins were transferred to polyvinylidene fluoride membranes (Immobilon; Millipore Corp., Billerica, MA, USA), probed overnight with primary antibodies in Tris-Buffered Saline and Tween 20 (TBST), and washed three times with TBST. Immunoreactive bands were detected with horseradish peroxidase-conjugated secondary antibody, developed with the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Inc.; Hercules, CA, USA), and exposed using x-ray film (Amersham Pharmacia Biotech, Inc.). The relative amount of each immunoreactive band was quantified via densitometry and normalized to the level of β-actin in the sample.

Adipogenesis

Undifferentiated cells were forced to differentiate using a previously-published protocol.4 Cells were grown to confluence in six-well plates. Then, culture medium was changed to serum-free DMEM supplemented with 33 μM biontin, 17 μM pantothenic acid, 10 μg/mL transferrin, 0.2 mM T3, 1 μM insulin, and 0.2 μM carbaprostaglandin. For the first 4 days, cells were incubated in 1 mM insulin, 1 μM dexamethasone and 0.1 mM isoamylxanthine. Differentiation was continued for 10 days, and media was replaced every 3 days. To further stimulate adipogenesis, 10 μM resiglitazone, a peroxisome proliferator-activated receptor-gamma agonist, was added on day 1. To evaluate the effect of resveratrol on adipocyte differentiation, cells were treated with resveratrol (10, 50, or 100 μM) for the entire 10-day differentiation period.

Oil Red O Staining

Cells were stained with oil red O as described by Green and Kehinde.19 A stock solution of oil red O (0.5% of oil red O in isopropanol) was prepared. To make the working solution, 6 mL stock solution was mixed with 4 mL distilled water, left for 1 hour at room temperature, and filtered through a 0.2-μm filter. Cells were washed twice with PBS, fixed with 5.7% formalin in PBS for 1 hour at 4°C, and stained with 500 μL oil red O working solution for 1 hour at room temperature. After dishes were washed with distilled water, they were inspected using a light microscope (Axiovert; Carl Zeiss AG, Oberkochen, Germany) and photographed at ×40 and ×100 magnification using a light microscope (Olympus BX60; Olympus Corp., Melville, NY, USA).

Statistical Analysis

All experiments were performed using at least three cells strains from different individuals, and samples were assayed in duplicate. For statistical analysis of ROS generation and Western blots, means and standard deviations were calculated from normalized measurements taken from at least three samples harvested from different individuals. Multiple comparisons were performed using ANOVA with Tukey’s multiple-comparison test, as a post hoc test. The Wilcoxon signed-rank test was used to perform comparisons between cell groups or within cell groups treated with different concentrations of a compound or at different times. The test was two-sided with α = 0.05 and was performed using statistical software (IBM SPSS Statistics version 20; IBM, Corp., Armonk, NY, USA).

RESULTS

Effect of Resveratrol on Cell Viability

We used the MTT assay to detect resveratrol cytotoxicity in GO and non-GO orbital fibroblasts. Cell viability did not fall below 95% in GO and non-GO orbital fibroblasts treated with ≤50 μM resveratrol for 6 hours. However, a significant decrease in viability was observed after treatment with 100 μM resveratrol for 6 hours (P < 0.05). For 24 hours, more than 85% of GO cells were viable without a significant decrease in viability compared with control after treatment with ≤50 μM resveratrol, but a significant decrease in viability was observed after treatment with 100 μM resveratrol (P < 0.05). In non-GO cells, more than 90% of cells were viable when treated with ≤30 μM resveratrol for 24 hours, but there was a significant decrease in viability when non-GO cells were treated with ≥50 μM resveratrol for 24 hours (P < 0.05, Fig. 2).

Resveratrol Decreased ROS Levels Induced by 2% CSE or 10 μM H2O2

Intracellular ROS levels induced by 2% CSE or 10 μM H2O2 were reduced by pretreatment of 30 or 50 μM resveratrol for 24 hours. Treatment with 50 μM resveratrol significantly decreased ROS levels (P < 0.05, Fig. 3).

HO-1 Levels Induced by 2% CSE or 10 μM H2O2 Lowered After Resveratrol Treatment

Intracellular levels of HO-1 were higher in GO preadipocyte orbital fibroblasts treated with 2% CSE or 10 μM H2O2 than in GO cells that were not treated. In cells pretreated with 30 or 50 μM resveratrol for 24 hours, there was a significant reduction in HO-1 protein levels (P < 0.05, Fig. 4).

Effects of Resveratrol on SOD, Catalase, and Trx

The relative level of Mn-SOD was significantly induced after treatment with 50 μM resveratrol in GO preadipocyte orbital fibroblasts when treated with 2% CSE (P < 0.05). Treatment with 50 μM resveratrol also induced the level of Mn-SOD in cells treated with 10 μM H2O2, but there was no statistical significance. We found Cu/Zn-SOD, catalase, and Trx were significantly lowered in cells treated with 2% CSE after treatment with 50 μM resveratrol (P < 0.05). In cells treated with 10 μM H2O2, 50 μM resveratrol reduced the levels of catalase (P < 0.05, Fig. 5).
Effects of Resveratrol on Adipogenesis of GO Orbital Fibroblasts

Undifferentiated orbital fibroblasts from GO patients were differentiated to adipocytes, stained with oil red O, and examined with light microscopy. The number and size of differentiated cells increased over 10 days of differentiation. Treatment with 10 μM rosiglitazone significantly upregulated cellular differentiation. When resveratrol was added to the adipogenic medium for 3 days during the differentiation period, the number of adipocytes and the accumulation of lipid droplets induced by 10 μM rosiglitazone decreased in a dose-dependent fashion (Fig. 6A).

Intracellular ROS generation was measured on days 0, 1, 4, 7, and 10 of adipocyte differentiation in GO cells. The increase in ROS levels was greatest on day 1, and subsequently ROS levels remained at roughly 200% of the level on day 0. Treatment with 50 μM resveratrol reduced ROS levels at days 7 and 10 compared with untreated and 50 μM resveratrol-treated cells, respectively (P < 0.05). GO, Graves’ ophthalmopathy; MTT, 3-(4,5-dimethyl-2-tetrazolium)-2,5-diphenyl-tetrazolium bromide; h, hour(s).

Effects of Resveratrol on Expression of HO-1 and Transcriptional Regulators

Treatment with 50 μM resveratrol for 24 hours significantly attenuated expression of HO-1 in GO preadipocyte orbital fibroblasts (P < 0.05). When cells were pretreated with PD (PD98059, inhibitor of ERK) and SP (SP600125, inhibitor of JNK) for 1 hour, reduced expression of HO-1 was recovered (P < 0.05, Fig. 7A). Expression of the transcriptional regulators phospho-ERK and phospho-JNK significantly increased after treatment with 50 μM resveratrol, and decreased in response to inhibitors of each protein (P < 0.05, PD and SP respectively, Fig. 7B).

Phospho-NF-κB p65 levels increased after treatment with 50 μM resveratrol in GO cells, which was also observed in GO cells treated with 2% CSE (P < 0.05, Supplementary Fig. S1).
DISCUSSION

We showed that resveratrol treatment decreased ROS levels in an in vitro model of GO. ROS, induced by CSE or H₂O₂, decreased when primary cultured orbital fibroblasts from GO patients were treated with resveratrol. Cigarette smoke extract and H₂O₂ are potent oxidants, and cigarette smoking is a well-known risk factor affecting GO incidence, severity, and response to treatment. We also found resveratrol treatment significantly reduced ROS generation during adipogenesis.

The autoimmune process induces an inflammatory reaction that produces ROS. In turn, ROS result in oxidative damage to DNA in various autoimmune diseases. To date, there have been many reports of the role of oxidative stress in the pathogenesis of Graves disease. Generation of ROS and
increased oxidative stress has also been suggested as a mechanism for the development and progression of GO.\textsuperscript{21} Since ROS may contribute to the pathogenesis of GO, the possibility of treating GO with an antioxidant is attractive. Quercetin (3,3,4,5,7-penta-hydroxy flavonone), a flavonoid phytoestrogen with potent antioxidant activity, has been reported to inhibit inflammation, hyaluronan production, and adipogenesis in GO in vitro.\textsuperscript{4,18} Another study has demonstrated the protective effects of N-acetylcysteine and vitamin C in early stages of GO. Pretreatment with these antioxidants inhibits the stimulation of GO orbital fibroblast proliferation and the production of proinflammatory cytokines in vitro.\textsuperscript{26} A recent randomized, double-blind, placebo-controlled clinical trial in 159 patients with mild GO demonstrated that selenium supplementation was associated with improved quality of life, less eye involvement, and slowed progression of GO,\textsuperscript{27} although there is still controversy about the efficacy and safety of selenium supplementation.\textsuperscript{28}

We also found that resveratrol suppressed the adipogenesis of GO orbital fibroblasts. Our findings that resveratrol reduced the number of adipocytes and the accumulation of lipid droplets was similar to previously reported findings on the effect of quercetin in GO orbital fibroblasts.\textsuperscript{18} Moreover, there is another report that resveratrol inhibits adipogenesis and induces apoptosis in 3T3-L1 mouse embryo fibroblasts.\textsuperscript{29}

\textbf{FIGURE 5.} Effects of resveratrol on SOD, catalase, and Trx. (A) Results of western blot assays and (B) their quantification. Experiments were performed three times using different strains, and samples were assayed in duplicate. Results are expressed as the relative density of each protein and presented as means \pm SDs. GO cells neither treated with 2\% CSE nor 10 \mu M H\textsubscript{2}O\textsubscript{2} were used as control. *\textit{P} < 0.05 between control and 2\% CSE-treated cells. **\textit{P} < 0.05 between RV-treated and untreated cells.
Heme oxygenase is a rate-limiting enzyme in the pathway by which heme is degraded into biliverdin/bilirubin. There are two isoforms of HO, HO-1, and HO-2. Heme oxygenase 1 is induced by a variety of agents that cause oxidative stress. Heme oxygenase 1 inhibits inducible nitric oxide synthase activity and protects against stress conditions. It is upregulated in orbital fibroblasts by oxidative stress and adipogenic stimuli. In our experiments, the levels of HO-1 induced by CSE or H₂O₂ in GO orbital fibroblasts were significantly lowered by treatment with resveratrol. However, the attenuation of HO-1 expression by resveratrol might not be due to the inhibition of CSE or H₂O₂ stimulation but from another mechanism because resveratrol reduced HO-1 expression in the absence of CSE or H₂O₂. Presumably, resveratrol reduces the need for HO-1 by decreasing ROS levels.

Superoxide dismutase, cytosolic dimeric Cu/Zn-SOD (SOD-1), and mitochondrial tetrameric Mn-SOD (SOD-2) contribute to the first line of antioxidant defenses by catalyzing the conversion of O₂⁻ into H₂O₂. Catalase belongs to the secondary line of antioxidant defenses by catalyzing the conversion of H₂O₂ into H₂O. Thioredoxin also acts as an antioxidant by facilitating the reduction of other proteins via cysteine thiol-disulfide exchange. There were various reports demonstrate regulatory effects of resveratrol on antioxidant enzymes in various cells. In a study using Rotifer model, resveratrol increased expression of Mn-SOD, but inhibit expression of Cu/Zn-SOD and catalase. In GO cell, antiox-
FIGURE 7. Effects of resveratrol on the expression of (A) HO-1 and (B) transcriptional regulators in GO preadipocyte orbital fibroblasts. Experiments were performed three times using different strains, and samples were assayed in duplicate. Results are expressed as percentages of the control and presented as means ± SDs. *P < 0.05 between RV-treated and RV-untreated cells. **P < 0.05 between RV-treated and RV with inhibitor treated cells. LY (LY294002), inhibitor of protein kinase B; PD (PD98059), inhibitor of ERK; SB (SB203580), inhibitor of p38; SP (SP600125), inhibitor of JNK; U (U0126), inhibitor of ERK.
resveratrol alone are responsible for the content and writing of the paper.

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