Cigarette-Smoke-Extract-Induced Adipogenesis in Graves’ Orbital Fibroblasts Is Inhibited by Quercetin via Reduction in Oxidative Stress

Jin Sook Yoon, Hyun Jung Lee, Min Kyung Chae, Sang Yeul Lee, Eun Jig Lee

Institute of Vision Research, Departments of Ophthalmology, Yonsei University College of Medicine, Seoul, Korea (J.S.Y., M.K.C, S.Y.L.), Endocrinology, Brain Korea 21 Project for Medical Science, Institute of Endocrine Research, and Severance Integrative Research Institute for Cerebral & Cardiovascular Disease, Seoul, Korea (H.J.L., E.J.L.), Biochemistry and Molecular Biology, Yonsei University College of Medicine (E.J.L.)

Jin Sook Yoon and Hyun Jung Lee contributed equally to this work as first authors.

Address correspondence and reprint requests to Eun Jig Lee, MD, PhD
Department of Endocrinology, Yonsei University College of Medicine, 50 Yonsei-Ro, Seodaemun-Gu, Seoul 120-752, Korea
TEL: 82-2-2228-1983, FAX: 82-2-393-6884, E-mail: EJLEE423@yuhs.ac

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Abstract

Cigarette smoking is known to aggravate Graves’ orbitopathy (GO) severity by enhancing adipogenesis. We investigated the effect of quercetin, an antioxidant, on adipocyte differentiation induced by cigarette smoke extract (CSE) in primary cultured orbital fibroblasts (OFs) from GO patients. Freshly prepared CSE was added to the cells and H\textsubscript{2}O\textsubscript{2} was used as a positive control. Intracellular reactive oxygen species (ROS) generation and adipogenesis were measured. The expressions of proteins peroxisome proliferator-activated receptor (PPAR) \( \gamma \), CCAAT-enhancer-binding proteins (C/EBP) \( \alpha \) and \( \beta \), and heme oxygenase–1 (HO–1), an antioxidant enzyme, were examined during adipogenic differentiation. In result, CSE and H\textsubscript{2}O\textsubscript{2} dose-dependently stimulated intracellular ROS production in normal and Graves’ OFs. The effect of 2% CSE was similar to that of 10 \( \mu \)M H\textsubscript{2}O\textsubscript{2}; both concentrations were noncytotoxic and were used throughout the experiment. Quercetin pretreatment reduced the ROS generation stimulated by either CSE or H\textsubscript{2}O\textsubscript{2} in preadipocyte OFs. CSE and H\textsubscript{2}O\textsubscript{2} stimulated adipocyte differentiation in cultured OFs. The addition of quercetin (50 or 100 \( \mu \)M) suppressed adipogenesis. Quercetin also suppressed ROS generation in differentiating OFs during adipogenesis stimulated by CSE and H\textsubscript{2}O\textsubscript{2}. Additionally, the expressions of PPAR\( \gamma \), C/EBP\( \alpha \), and C/EBP\( \beta \) proteins were reduced in the quercetin-treated OFs. Quercetin also reduced the CSE- and H\textsubscript{2}O\textsubscript{2}-induced upregulation of ROS and HO–1 protein in differentiated OFs and preadipocyte OFs. As shown in this study, quercetin inhibited adipogenesis by reducing ROS \textit{in vitro}, supporting the use of quercetin in the treatment of GO.
Introduction

Oxidative stress is implicated in the pathogenesis of Graves’ orbitopathy (GO), and cigarette smoking is known to be a major environmental factor that affects GO. Cigarette smoking has been shown to influence the incidence, severity, and responses to treatment of GO, and appears to do so in a dose-dependent and temporal manner. Reportedly, smokers with Graves’ disease are approximately five times more likely to develop GO than nonsmokers with Graves’ disease (Bartalena, et al. 1989; Prummel and Wiersinga 1993; Winsa, et al. 1993). Smoking has also been shown to dose-dependently influence the course of GO during treatment, and responses to treatment have been shown to be delayed and considerably poorer in smokers (Eckstein, et al. 2003). Cigarette smoke is considered to act, in part, by enhancing the generation of reactive oxygen species (ROS) and increasing oxidative stress in the closed bony orbital environment, either through direct contact with the sinus and medial wall, or indirectly through the bloodstream. Several studies have shown evidence that ROS are present in the retro-orbital fibroblasts and plasma of GO patients (Lu, et al. 1999). Tsai et al. found that oxidative DNA damage, lipid peroxidation, and ROS production were increased in cultured GO fibroblasts relative to their levels in normal orbital fibroblasts (OFs) (Tsai, et al. 2010). Low doses of hydrogen peroxide (H$_2$O$_2$) have been shown to stimulate the proliferation of fibroblasts and to enhance heat shock protein 72 in GO fibroblasts (Heufelder, et al. 1992). However, the contribution of ROS to the pathogenesis of GO is unclear.

Oxidant stress in adipose tissue is emerging as an important mediator of adipocyte dysfunction in obesity (Espiritu and Mazzone 2008). Lee et al. recently reported that ROS facilitates adipocyte differentiation by accelerating the mitotic clonal expansion of 3T3-L1 preadipocytes (Lee, et al. 2009). Cell-cycle progression (from S to G$_2$/M phase) was markedly enhanced by H$_2$O$_2$, whereas an antioxidant caused S-phase arrest during mitotic clonal expansion. Similarly in GO, oxidative stress induced by cigarette smoking might mediate adipogenesis, as shown in an in vitro study by Cawood et al. (Cawood, et al. 2007). There are a number of in vitro methods for which to study the effects of...
cigarette smoke, using either a single compound, such as nicotine, or cigarette smoke extract (CSE) (Carnevali, et al. 2003; Kode, et al. 2008; Mortaz, et al. 2009). Although the use of CSE is limited by the quality of the extract, which may not reflect the full array of compounds in cigarette smoke, CSE has been shown to significantly enhance adipocyte differentiation and hyaluronan production in primary cultured OFs (Cawood et al. 2007). This may explain why smokers are more likely to develop more severe proptosis and myopathy in GO than nonsmokers.

Until now, no reliable, specific, and safe medical therapeutic agent has been developed to treat GO. Glucocorticoids have been used for decades and are still indicated as the first-line treatment, either alone or in combination with orbital radiotherapy, because of their anti-inflammatory and immunosuppressive actions (Bartalena, et al. 2000; Kuriyan, et al. 2008). They are most effective in patients with severe and active eye disease (Bartalena et al. 2000). Soft-tissue inflammatory changes, recent-onset extraocular muscle involvement, and optic neuropathy are most responsive to glucocorticoids, whereas proptosis and long-standing extraocular muscle involvement associated with fibrotic changes are poorly responsive. A major drawback to systemic glucocorticoid therapy is its possible adverse effects and complications.

Previously, we reported that quercetin (3,3,4,5,7-pentahydroxy flavonone), a flavonoid phytoestrogen found abundantly in soybeans, vegetables, and fruits, significantly reduced the inflammation pathway, hyaluronan production, and adipogenesis in primary cultured OFs (Yoon, et al. 2011). Quercetin also inhibited fibrotic markers and affected matrix metalloproteinase-2 and -9 activities in both primary and orbital fat tissue cultures from GO patients at nontoxic concentrations (Yoon, et al. 2012a). In another recent report by Lisi et al, quercetin was shown to reduce cell proliferation and hyaluronan production (Lisi, et al. 2011). Quercetin has been shown to exhibit antioxidant, anti-inflammatory, and anti-adipogenic properties in other cell systems and animal models (Comalada, et al. 2005; Rogerio, et al. 2010; Rotelli, et al. 2009; Vasquez-Garzon, et al. 2009; Ying, et al. 2009). Here, we investigated the anti-adipogenic role of quercetin in OFs stimulated by
oxidants, such as CSE and H$_2$O$_2$, as well as associations between the anti-adipogenic and antioxidant
effects of quercetin, including its reduction of ROS production. Briefly, we found that quercetin
significantly suppressed adipogenesis induced by treatment with either CSE or H$_2$O$_2$ in OFs from
patients with GO, and inhibited the generation of ROS during adipogenesis. Consequently, not only
was the expression of adipogenic transcriptional regulator proteins significantly suppressed by
treatment with quercetin during adipogenesis, but also that of the antioxidant heme oxygenase−1
(HO−1) was as well, in both OFs and preadipocyte OFs.

Materials and Methods

Reagents

Quercetin (Q0125) and Oil Red O were purchased from Sigma-Aldrich, Inc. (St Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin, and gentamycin
were purchased from Hyclone Laboratories, Inc. (Logan, UT). The 3-(4,5-dimethylthiazol-2-yl)-2,5-
diphenyltetrazolium bromide (MTT) assay was purchased from Sigma-Aldrich. Anti-peroxisome
proliferator activator gamma (PPARγ) antibody, anti-CCAAT-enhancer-binding protein (C/EBP) α
antibody, anti-C/EBP β antibody, anti-HO−1 antibody, and anti-β-actin antibody were all obtained
from Santa Cruz Biotechnology (Santa Cruz, CA).

Subjects

Orbital adipose/connective tissue specimens were obtained during the course of orbital
decompression surgery for severe GO (n = 6; four women and two men, aged 34–55 years). The GO
patients had not received steroid medication for at least three 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 original 10-
point CAS by Mourits et al (Mourits, et al. 1997). 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 noninflammatory 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, in Seoul, Korea, and all study participants provided written informed consent.

**OF cultures**

OF cultures were established in accordance with published methods (Yoon et al. 2011). Orbital fat biopsies taken at surgery were minced and placed directly in plastic culture dishes, allowing preadipocyte fibroblasts to proliferate. The cells were incubated in DMEM containing 10% FBS, penicillin (100 U/mL), and gentamycin (20 µg/mL) in a humidified 5% CO₂ incubator at 37 °C. Then, monolayers were serially passaged by gently treating them with trypsin/EDTA. The strains were stored in liquid N₂ until needed and only strains between the third and seventh passages were used. We tried to use same passage of cell cultures for a same experiment to reduce bias caused by primary cultures from human samples.

Because treatment with ≤ 100 µM quercetin for 24 h did not reduce cell viability in Graves’ OFs to below 95% in an MTT analysis and did not induce significant apoptosis when assessed with an annexin V–fluorescein isothiocyanate (FITC) assay in our previous study (Yoon et al. 2011), 100 µM quercetin was used as the maximal nontoxic dose in the experiments of the present study.

**Preparation of CSE**

CSE was prepared by bubbling smoke from two commercially available, filtered cigarettes (Marlboro 20 class A cigarettes, made in Korea by Philip Morris Korea Inc., 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 (Kode et al. 2008). 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.

**Determination of noncytotoxic doses of CSE and H\textsubscript{2}O\textsubscript{2}**

Effects of cigarette smoke extract (CSE) and H\textsubscript{2}O\textsubscript{2} on cell viability in preadipocyte orbital fibroblasts were assessed. OFs (1 × 10\textsuperscript{5}) from normal subjects and GO patients were seeded into 24-well culture plates and treated with different concentrations of CSE (1%–5%) or H\textsubscript{2}O\textsubscript{2} (10–500 µM) for 24 h. After treatment, the cells were assayed with MTT to test their viability. The assays were performed at least three times in triplicate, expressed as the differences between the treated and untreated cells in the normal and GO OF samples.

**Adipogenesis**

OFs were exposed to a differentiation protocol according to our previous report (Yoon et al. 2011) to enhance adipogenesis. The cells were grown to confluence in six-well plates, and then exposed to differentiation medium for 10 days. The culture medium was then 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, Manheim, Germany), and 0.2 µM carbaprostaglandin (Calbiochem, La Jolla, CA). For the first four days, 1 µM insulin, 1 µM dexamethasone, and 0.1 mM isobutylmethylxanthine were included in the medium. Differentiation was allowed to continue for 10 days, during which period the medium was replaced every 3–4 days. A PPAR\textgreekgamma agonist, rosiglitazone (10 µM; Cayman, Ann Arbor, MI), was added on day 1 to further stimulate adipogenesis. To evaluate
the effects of CSE and H$_2$O$_2$ on adipogenesis, low sublethal concentrations of those compounds, were added to the cultures for the first three days of adipogenesis only. Treatment with 2% CSE or 10 µM H$_2$O$_2$ for the first three days during adipogenesis did not decrease cell viability according to an MTT analysis on day 3 of adipogenesis (data not shown). Also, cell viability was not affected by quercetin treatment for three days during adipogenesis, according to an MTT analysis performed in our previous study (Yoon et al. 2011). Therefore, we exposed the cultures to quercetin for the first three days of the differentiation period to determine the suppressive effects of quercetin on adipocyte differentiation and ROS production during adipogenesis.

**Intracellular ROS measurement**

ROS release was determined with 5-(and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA; Molecular Probes, Inc., OR), an oxidant-sensitive fluorescent probe, as previously described (Kode et al. 2008); H$_2$DCFDA is deacetylated intracellularly by esterase, forming H$_2$DCF, which is oxidized by ROS to 2',7'-dichlorofluorescein (DCF), a highly fluorescent compound. The cells were seeded at a density of 5 × 10$^5$ cells per well in six-well plates to a total final volume of 2 mL and treated with various concentrations of CSE (1%–5%) or H$_2$O$_2$ (10–500 µM) for 30 min to evaluate the effects of these compounds on ROS production in normal and Graves’ OFs. To determine the effect of quercetin on ROS production stimulated by CSE (2%) or H$_2$O$_2$ (10 µM) for 30 min, the cells were pretreated with 100 µM quercetin for 24 h. The culture medium was then removed, and the cells were washed with phosphate-buffered saline (PBS), incubated with 10 µM H$_2$DCFDA at 37 °C for 30 min, and then stimulated with CSE or H$_2$O$_2$ for 30 min. The cells were then trypsinized, washed, and resuspended in PBS. Thereafter, fluorescence intensity was measured with an IX71-F22PH inverted fluorescence microscope (Olympus, Japan) and a flow-cytometric analysis was performed (ELITE flow cytometer, Coulter Cytometry, Inc., Hialeah, FL). For each sample, ≥ 10,000 events were acquired. Cells were gated out and the analysis was performed using only live
populations. The fluorescently stained cells were also examined microscopically (×100 magnification).

Oil Red O staining

The cells were stained with Oil Red O, as described by Green and Kehinde, on day 10 of differentiation (Green and Kehinde 1975). A stock solution of Oil Red O (0.5% Oil Red O in isopropanol) was prepared. To prepare the working solution, 6 mL of the stock solution was mixed with 4 mL of distilled water, left for 1 h at room temperature, and filtered through a 0.2 µm filter. The cells were washed twice with PBS, fixed with 3.7% formalin in PBS for 1 h at 4 °C, and stained with 300 µL of the Oil Red O working solution for 1 h at room temperature. The dishes were washed with distilled water before they were inspected under an Axiovert light microscope (Carl Zeiss) and photographed at ×40 and ×400 magnification with an Olympus BX60 light microscope (Olympus, Melville, NY).

Western blot assay

The differentiated cells were washed with ice-cold PBS and lysed with cell lysis buffer (20 mM HEPES [pH 7.2], 10% [v/v] glycerol, 10 mM Na3VO4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 1 µg/mL leupeptin, 1 µg/mL pepstatin, and 1% [v/v] Triton X-100; Sigma-Aldrich) on ice for 30 min. The lysates were then centrifuged for 10 min at 12,000 × g, and the cell homogenate fractions were stored at −70 °C until ready for use. The protein concentrations in the supernatant fractions were determined using the Bradford assay. Equal amounts of protein (50 µg) were boiled in sample buffer and resolved by 10% (w/v) SDS–PAGE. The proteins were then transferred to polyvinylidene difluoride membranes (Immobilon, Millipore, Billerica, MA), probed overnight with primary antibodies in TBST, and washed three times with TBST. The immunoreactive bands were detected with horseradish-peroxidase-conjugated secondary antibody, developed with an
enhanced chemiluminescence kit (Amersham Pharmacia Biotech), and exposed to X-ray film (Amersham Pharmacia Biotech).

Statistical analysis

All experiments were performed at least three times using different strains, and the samples were always assayed in duplicate. To statistically analyze ROS generation and the results of the protein analysis by western blotting, the means and standard deviations (SD) were calculated from the normalized values for each ROS and protein measured in at least three samples harvested from different individuals. Groups containing multiple comparisons were analyzed by analysis of variance (ANOVA) with Tukey’s multiple-comparison test, as a post hoc test. Comparisons of data between cell groups or within cell groups at different concentrations of compound or at different times were analyzed with a \( t \) test or ANOVA using SPSS software package for Windows, version 12.0.1 (SPSS, Chicago, IL). A \( P \) value of < 0.05 was considered significant.

Results

Effects of CSE and \( \text{H}_2\text{O}_2 \) on cell proliferation

On MTT analysis treating normal cells with various concentrations of CSE (1%–5%) for 24 h did not alter the viability, whereas 2%–5% CSE induced proliferation (about 125%) in GO cells (control-CSE2%; \( p=0.021 \), control-CSE2.5%; \( p=0.015 \), control-CSE5%; \( p=0.024 \)) (Supplementary Fig. 1A). Similarly, treatment with another oxidant, \( \text{H}_2\text{O}_2 \), at 50 and 100 µM for 24 h increased cell proliferation in GO cells (control- \( \text{H}_2\text{O}_2 \) 50; \( p=0.022 \), control- \( \text{H}_2\text{O}_2 \) 100; \( p=0.01 \)), and treatment with 500 µM \( \text{H}_2\text{O}_2 \) significantly reduced cell viability (\( p<0.01 \)) (Supplementary Fig. 1B); treatment with 10 µM \( \text{H}_2\text{O}_2 \) did not affect cell viability on MTT analysis (GO control- \( \text{H}_2\text{O}_2 \) 10; \( p=0.129 \), normal control- \( \text{H}_2\text{O}_2 \) 10; \( p=0.415 \)). In normal cells, treatment with 100 µM \( \text{H}_2\text{O}_2 \) for 24 h increased cell proliferation (\( p=0.031 \)), and treatment with 500 µM \( \text{H}_2\text{O}_2 \) significantly reduced cell viability (\( p<0.01 \)).
Measurement of intracellular ROS in normal and GO cells stimulated with CSE or H$_2$O$_2$

Basal levels of intracellular ROS were significantly higher in GO cells (n=3) (149.4±6.2%) than in normal cells (n=3) (p=0.005). Intracellular ROS in both normal and GO preadipocyte OFs were significantly increased in a dose-dependent manner by stimulation with CSE or H$_2$O$_2$ for 30 min, and this increase was far greater in the GO cells (GO control-CSE 2%; p=0.014, GO control-CSE 2.5%; p<0.01, GO control-CSE 5%; p<0.01, Normal control-CSE 5; p=0.025) (GO control-H$_2$O$_2$ 10, 50, 10 and 500; all p<0.01, Normal control-H$_2$O$_2$ 100; p=0.026, Normal control-H$_2$O$_2$ 500; p=0.011) (Fig. 1A and B). Only high concentrations of CSE (5%) or H$_2$O$_2$ (100 and 500 µM) increased ROS production in the normal cells relative to the control levels.

Quercetin treatment reduced CSE- and H$_2$O$_2$-stimulated ROS production in preadipocyte fibroblasts

As previously reported, the treatment of preadipocyte OFs with 10–100 µM quercetin for 24 h did not affect cell viability according to previous MTT assay and annexin V-fluorescence isothiocyanate apoptosis assay (Yoon et al. 2011). Therefore, a maximal concentration of 100 µM quercetin was used in the experiments of the present study. ROS production in cells stimulated with either 2% CSE or 10 µM H$_2$O$_2$ for 30 min and in the control cells was significantly and dose-dependently suppressed by quercetin pretreatment (all p<0.05) (Fig. 2).

Effect of quercetin on adipogenesis in GO OFs

Confluent OFs from GO were subjected to the adipocyte differentiation protocol for 10 days. The cells were first examined under light microscopy and then stained with Oil Red O. As we previously reported, under control adipogenic conditions, the OFs lost their stellate fibroblastic appearance and adopted a spherical adipocytic shape, and a fraction of these cells accumulated small lipid droplets (Yoon et al. 2011). The lipid droplets were visible from day 3, and increased in number
and size over the 10 days of differentiation. The addition of 2% CSE or 10 µM H₂O₂ for the first three days of differentiation significantly increased adipogenesis, assessed with light microscopy, compared with that observed under control conditions (Fig. 3). Combined treatment with CSE and rosiglitazone or H₂O₂ and rosiglitazone significantly upregulated cellular differentiation compared with the differentiation stimulated by CSE or H₂O₂ alone. When quercetin was added to the adipogenic medium for three days during the differentiation period, as visualized by Oil Red O staining, quercetin dose-dependently reduced the number of adipocytes and suppressed the accumulation of lipid droplets induced by 2% CSE or 10 µM H₂O₂ treatment. The experiments were performed in triplicate with cells from three different donors, and all the findings were similar. Figure 3 shows representative data of GO OFs.

**Effects of quercetin on the generation of ROS during adipocyte differentiation**

When intracellular ROS was measured on days 0, 1, 4, 7, and 10 of adipocyte differentiation in GO cells, the increase in ROS was greatest on day 1, and was maintained continuously at about 200% of the control level throughout the differentiation period (Fig. 4A). Treatment with 2% CSE or 10 µM H₂O₂ in the first three days of differentiation further increased ROS production relative to that in the control cultures, especially on day 10 (control-CSE 2%; p<0.01, control-H₂O₂ 10; p<0.01) (Fig. 4B and C). The addition of 100 µM quercetin to the adipogenic medium suppressed intracellular ROS production, predominantly on days 7 and 10 of differentiation, in both the unstimulated cultures and those stimulated with CSE or H₂O₂ (all p<0.01 on day 7 and 10) (Fig. 4A–C). Similarly, when the ROS in the differentiated cells were fluorescently stained on day 10 of adipogenesis, a significant reduction in fluorescently stained cells was observed after treatment with 100 µM quercetin (×100; Fig. 4A–C). In normal OFs (n=3), treatment with 100 µM quercetin also suppressed intracellular ROS production stimulated by either 2% CSE or 10 µM H₂O₂ on day 10 of adipogenesis (all p<0.01 (Supplementary Fig. 2).
Effects of quercetin on the expression of the transcriptional regulators of adipogenesis and HO−1 during adipocyte differentiation

In preadipocyte fibroblasts, the expression of HO−1 protein was significantly upregulated by treatment with 2% CSE for 8–48 h, or with 1%–5% CSE for 24 h, and by treatment with 10 µM H2O2 for 8–48 h or 50 µM or 100 µM H2O2 for 24 h (p<0.05 vs. CSE or H2O2-untreated cells) (Fig. 5A–D, F, G). Pretreatment with quercetin for 24 h significantly and dose-dependently suppressed HO−1 expression in cells stimulated with 2% CSE or 10 µM H2O2 for 24 h (p<0.05 vs. quercetin-untreated cells), but it did not affect the expression of HO−1 in the unstimulated control cells from patients with GO (Fig. 5E and H).

As we previously reported, PPARγ, C/EBPα, and C/EBPβ are all strongly expressed in differentiated adipocytes (Fig. 6A–D). The addition of either 2% CSE or 10 µM H2O2 for the first three days of differentiation caused not only a further increase in adipogenesis, but also significantly increased PPARγ, C/EBPα, and C/EBPβ expression in GO OFs (all p<0.05 vs. unstimulated differentiated cells) (Fig. 6A–D). Treatment with quercetin for the first three days of differentiation significantly and dose-dependently attenuated the expression of PPARγ, C/EBPα, and C/EBPβ in differentiated fibroblasts after stimulation with either CSE or H2O2 (p<0.05; quercetin 100 µM- vs quercetin-untreated cells) (Fig. 6A–D). In differentiated normal OFs, quercetin 100 µM suppressed PPARγ and C/EBPα protein production (Supplementary Fig. 3). However, C/EBPβ protein was neither increased by stimulation with CSE or H2O2 nor suppressed by quercetin treatment in normal OFs. HO−1 expression was significantly upregulated in the differentiated fibroblasts. This expression was further increased by stimulation with 2% CSE or 10 µM H2O2 and suppressed dose-dependently by treatment with quercetin (p<0.05 vs. quercetin-untreated cells) (Fig. 6A and E), similar to the results for preadipocyte fibroblasts shown in Fig. 5.
Discussion

We previously reported that quercetin blocks three major pathogenic processes—inflammation, aberrant accumulation of extracellular matrix macromolecules, and adipose tissue expansion—stimulated by proinflammatory cytokines in Graves’ OFs. In this study, we found that adipogenesis in Graves’ OFs was upregulated by the oxidant H$_2$O$_2$, and CSE, and that this was suppressed in a dose-dependent manner by quercetin, in parallel with a reduction in ROS and the protective antioxidant enzyme HO–1 during adipogenesis. The suppression of adipogenesis by quercetin might be strongly associated with the antioxidant effects of quercetin.

There is growing evidence that oxidative stress plays an important role in the pathogenesis of GO (Bartalena, et al. 2003; Bednarek, et al. 2005; Tsai, et al. 2009). Superoxide radicals stimulate OFs to proliferate and to produce glycosaminoglycan (Burch, et al. 1997), and H$_2$O$_2$ is known to induce the expression of HLA-DR and heat shock protein 72 (Heufelder et al. 1992). Smoking is the strongest known environmental factor that stimulates the occurrence and aggravation of GO by enhancing the generation of ROS and reducing antioxidant production (Stan and Bahn 2010). In examining the mechanism that underlies the aggravation of GO due to smoking, Cawood et al. reported that CSE increases hyaluronan production and adipogenesis in OFs, and that the effects of interleukin 1 and CSE on adipogenesis are synergistic (Cawood et al. 2007). Because superoxide radicals and tissue hypoxia can induce the proliferation of OFs (Burch et al. 1997; Hsu, et al. 2009), smoke containing a variety of oxidants and free radicals should stimulate cell proliferation during adipogenesis. In this study, we used CSE to mimic smoking conditions in vitro, which strongly induced adipogenesis, as in a previous study by Cawood et al. (Cawood et al. 2007). We decided to use CSE rather than a single compound, such as nicotine, because cigarette smoke contains more than 4,800 compounds, and the active compound in GO has not yet been identified. In our study, CSE and H$_2$O$_2$ stimulated cell proliferation in a dose-dependent manner. Baseline ROS levels were also nearly
150% significantly higher in GO than in normal OFs. Even by stimulation with low noncytotoxic concentrations of CSE or H$_2$O$_2$, ROS were significantly increased in preadipocyte OFs, predominantly more in GO than in normal OFs. Also, in differentiating OFs, ROS significantly increased in the late phase of adipogenesis, compared to non-stressed control conditions, which were correlated with an increase in adipogenesis. This *in vitro* finding was consistent with the clinical data presented in a journal review that provided strong evidence for a causal association between smoking and the development of GO, with risk ratios/odds ratios of > 2 in most studies (Thornton, et al. 2007). We recently reported that smoking status is a predictive risk factor of GO severity and the development of optic neuropathy in Korean populations, with odds ratios of 6.6 and 10.0, respectively (Lee, et al. 2010a).

HO is a rate-limiting enzyme in the oxidative degradation of heme into biliverdin, carbon monoxide, and ferrous iron. Among the isoforms of HO, only HO−1 is induced by xenobiotics and by a variety of agents that cause oxidative stress, such as CSE, hypoxia, and cytokines (Baglole, et al. 2008; Lee, et al. 2010b; Shih, et al. 2011). The induction of HO−1 is considered to be part of the generalized protective response to oxidative stress, as an active defense mechanism. To the best of our knowledge, this is the first study to show that an antioxidant enzyme, HO−1, is upregulated in OFs by oxidative stress and adipogenic stimuli. Treatment with CSE or H$_2$O$_2$ induced HO−1 protein in preadipocyte OFs in a dose- and time-dependent manner. It is clear that antioxidant enzymes are upregulated by oxidative stress. Hondur et al. reported that the activities of antioxidant enzymes, such as superoxide dismutase, glutathione reductase, and glutathione peroxidase, as well as lipid peroxidase levels, were all elevated in fibroadipose tissue from GO patients relative to their levels in tissues from normal subjects (Hondur, et al. 2008). HO−1 was also induced in cells cultured in adipogenic medium, which was subsequently reduced, in parallel with the suppression of adipogenesis, by treatment with quercetin during adipogenesis. It has been reported that quercetin induces HO−1 in a variety of cell lines (Chow, et al. 2005; Kim, et al. 2010; Yao, et al. 2007).
However, we found that HO−1 expression was not upregulated in OFs by quercetin treatment, but was reduced by quercetin treatment in both oxidant-stimulated preadipocyte OFs and fully differentiated OFs. We believe that this reduction in HO−1 is a consequence of the reduction in intracellular ROS caused by quercetin in the final stage of adipogenesis. The mechanisms underlying the antioxidant and antiadipogenic effects of quercetin might be associated via different pathways from HO−1.

In the present study, a significant increase in ROS by oxidative stress and reduction in ROS produced by quercetin was observed in differentiating fibroblasts, predominantly in the late stage of adipogenesis, which seemed to correlate with a reduction in intracytoplasmic oil droplets. We believe that the antioxidant effect of quercetin is probably associated with its antiadipogenic effect. Because oxidative stress, including cigarette smoke, is known to be a strong risk factor for GO, antioxidant treatment has been tested as a supportive treatment with glucocorticoid therapy or in the treatment of mild GO, for which steroid therapy is ineffective. Several nonrandomized and/or uncontrolled studies of antioxidants, such as nicotinamide, allopurinol, and pentoxifylline, have demonstrated favorable results in GO patients, although the therapeutic effects were not fully convincing because the study designs were inadequate (Balazs, et al. 1997; Bouzas, et al. 2000; Hiromatsu, et al. 1998). Recently, a randomized, double-blind, placebo-controlled trial was undertaken to determine the effects of selenium or pentoxifylline on patients with mild GO (Marcocci, et al. 2011). At the six-month evaluation, treatment with selenium, but not with pentoxifylline, was associated with an improved quality of life, less eye involvement, and a slower progression of GO compared with those of patients treated with a placebo. As far as we know, the present study is the first in vitro study of the antioxidant effects of quercetin on Graves’ OFs. It may be premature to suggest that this agent can be used as an alternative to corticosteroids as a therapeutic drug for GO, because these are only in vitro results; however, together with our previous favorable results regarding quercetin on GO, these new data lend rationale for future randomized clinical studies to determine whether this compound might prove useful in GO treatment, as an anti-inflammatory, antiadipogenic, and antioxidant agent.
Although baseline and stimulated ROS levels by CSE and H\textsubscript{2}O\textsubscript{2} were significantly higher in GO than in normal OFs, the inhibitory effect of quercetin on ROS production and adipogenesis was similar in both GO and normal OFs. Similar to data in GO cells, intracellular ROS as well as PPAR\textgreek{\textgamma} and C/EBP\textalpha production were increased by addition of 2% CSE or 10 \textmu M H\textsubscript{2}O\textsubscript{2}, and were suppressed by treatment with 100 \textmu M quercetin in normal cells. In our previous report, we experienced that primary cultured Graves’ and normal orbital fibroblasts responded similarly to quercetin treatment.(Yoon et al. 2012a; Yoon et al. 2011) Adipogenesis induced by adipogenic stimuli was also similar between Graves’ and normal orbital fibroblasts (Yoon et al. 2011). The action of quercetin in an \textit{in vitro} condition does not seem to be specific to only GO cells. Recently, van Steensel et al. introduced a whole tissue orbital tissue culture system for GO research which may better mimic the clinical environment thereof than single cell culture (van Steensel, et al. 2011). Using this tissue culture model, we previously showed that cytokines, such as IL-1\beta, IL-6 and TNF\alpha, protein levels were significantly higher in GO tissue cultures than in normal tissue, and that the production of proinflammatory cytokines was significantly inhibited by quercetin (Yoon, et al. 2012b).

Many questions regarding flavonoids remain to be investigated. It is not yet known whether flavonoids contribute to the clinical benefits seen in epidemiological studies. Further research and more clinical studies are required to ensure the safety of quercetin and to ascertain the optimum doses for the prevention and treatment of GO.

\section*{Declaration of interest}

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure legend

Figure 1 Intracellular reactive oxygen species (ROS) measured in normal and GO cells stimulated with CSE or H$_2$O$_2$. Orbital fibroblasts (OFs) from normal (n=3) and GO subjects (n=3) were treated with 1%-5% CSE (A) or 10-500 µM H$_2$O$_2$ (B) for 30 min. ROS were measured by flow cytometry using 5-(and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA). The results are expressed as percentages of the untreated control values in each Graves’ and normal cells, presented as means ± SD. Assays were performed at least three times in triplicate with cells from different donors; data from a representative experiment are shown, and are expressed as the differences between treated and untreated cells in normal and GO OF samples (*P < 0.05).

Figure 2 Effects of quercetin on cigarette smoke extract (CSE)- and H$_2$O$_2$-induced generation of intracellular reactive oxygen species (ROS) in preadipocyte orbital fibroblasts. Orbital fibroblasts (OFs) from GO patients (n=3) were treated with 2% CSE or 10 µM H$_2$O$_2$ for 30 min with or without pretreatment with quercetin (50 or 100 µM) for 24 h. ROS were measured with flow cytometry using 5-(and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA). The results are expressed as percentages of the untreated control values, presented as means ± SD. Assays were performed at least three times in triplicate with cells from three different GO samples; data from a representative experiment are shown, and expressed as the differences between the quercetin-treated and -untreated cells (*P < 0.05) and between the CSE- or H$_2$O$_2$-stimulated and -unstimulated control cells (**P < 0.05).

Figure 3 Effects of quercetin on cigarette smoke extract (CSE)- or H$_2$O$_2$-stimulated adipogenesis. Cells from GO patient were treated with 2% CSE or 10 µM H$_2$O$_2$ for the first three days of the 10-day period of adipogenesis in adipogenic medium containing 10 µM rosiglitazone. To determine the suppressive effect of quercetin on adipogenesis, quercetin (50 or 100 µM) was also added for the first
three days of differentiation. The cells were stained with Oil Red O and examined microscopically (×40; inset ×400). The experiments were performed in triplicate with cells from different GO donors, and the representative figures are shown.

**Figure 4** Effect of quercetin on the cigarette smoke extract (CSE)- or H$_2$O$_2$-stimulated generation of intracellular reactive oxygen species (ROS) in differentiating orbital fibroblasts during adipogenesis. Confluent fibroblasts from GO patients were subjected to a differentiation protocol that included adipogenic supplements for 10 days (A), and then further stimulated with 2% CSE (B) or 10 µM H$_2$O$_2$ (C) for the first three days of adipogenesis. To determine the suppressive effect of quercetin on adipogenesis, quercetin (100 µM) was also added during the first three days of differentiation. ROS were measured by flow cytometry using 5-(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) on days 0, 1, 4, 7, and 10 of adipogenesis. The results are expressed as percentages of the untreated control values, and presented as means ± SD. The assays were performed at least three times in triplicate with cells from three different GO samples; data from a representative experiment are shown, and expressed as the differences between the quercetin-treated and -untreated cells (*$P < 0.01$). On day 10 of adipogenesis, the cells were stained with H$_2$DCFDA and examined microscopically (×100).

**Figure 5** Effects of quercetin on cigarette smoke extract (CSE)- or H$_2$O$_2$-stimulated heme oxygenase−1 (HO−1) expression in preadipocyte orbital fibroblasts, analyzed with western blotting. Orbital fibroblasts (5 × 10$^5$) from GO patients (n=3) were treated with 2% CSE for 0–48 h (A), 0–5% CSE for 24 h (B), 10 µM H$_2$O$_2$ for 0–48 h (C), or 0–500 µM H$_2$O$_2$ for 24 h (D) and their HO−1 protein expression was determined by western blotting. The cells were pretreated with 50 or 100 µM quercetin for 24 h, and then stimulated with 2% CSE or 10 µM H$_2$O$_2$ for 24 h (E) to determine the effect of quercetin on CSE- or H$_2$O$_2$-stimulated HO−1 protein expression. Quantification of HO−1 by
densitometry, normalized to the level of β-actin in the same sample, is shown (F–H). The data in the columns are the mean relative density ratios ± SD of three experiments. *P < 0.05 vs. untreated control cells.

**Figure 6** Effects of quercetin on adipogenesis-related transcription factors and heme oxygenase–1 (HO–1) expression in differentiated adipocytes stimulated with either cigarette smoke extract (CSE) or H$_2$O$_2$. Cells from GO patients were treated with quercetin (50 or 100 µM) for the first three days of a 10-day period of adipogenesis in adipogenic medium containing 10 µM rosiglitazone alone or combined with 2% CSE or 10 µM H$_2$O$_2$ (A). After 10 days, the cell lysates were subjected to western blotting analysis for PPARγ, C/EBPα, C/EBPβ, and HO–1 protein expression. The experiments were performed in triplicate with cells from three different GO donors. Quantification of PPARγ (B), C/EBPα (C), C/EBPβ (D), and HO–1 (E) by densitometry, normalized to the β-actin levels in the same samples, is shown. The data in the columns are the mean relative density ratios ± SD of three experiments. *P < 0.05 vs. untreated differentiated control cells.

**Supplementary Fig. 1.** Effects of cigarette smoke extract (CSE) and H$_2$O$_2$ on cell viability in preadipocyte orbital fibroblasts (OFs). OFs (1 × 10$^5$) from normal subjects (n=3) and Graves’ orbitopathy (GO) patients (n=3) were seeded into 24-well culture plates and treated with different concentrations of CSE (1%–5%) (A) or H$_2$O$_2$ (10–500 µM) (B) for 24 h. After treatment, the cells were assayed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to test their viability. The results are expressed as percentages of the untreated control values, presented as means ± SD. The assays were performed at least three times in triplicate with cells from different samples;
data from a representative experiment are shown, and expressed as the differences between the treated and untreated cells in the normal and GO OF samples (*P < 0.05).

**Supplementary Fig. 2.** Effect of quercetin on cigarette smoke extract (CSE)- or H$_2$O$_2$-stimulated generation of intracellular reactive oxygen species (ROS) in differentiating orbital fibroblasts from normal tissue samples during adipogenesis. Confluent fibroblasts from normal patients were subjected to a differentiation protocol that included adipogenic supplements for 10 days, and then further stimulated with 2% CSE or 10 µM H$_2$O$_2$ for the first three days of adipogenesis. To determine the suppressive effect of quercetin on adipogenesis, quercetin (100 µM) was also added during the first three days of differentiation. ROS were measured by flow cytometry using 5-(and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) on day 10 of adipogenesis. The results are expressed as percentages of the untreated control values, and presented as means ± SD. The assays were performed at least three times in triplicate with cells from three different normal samples, and expressed as the differences between the quercetin-treated and -untreated cells (*P < 0.01).

**Supplementary Fig. 3.** Effects of quercetin on adipogenesis-related transcription factors in differentiated adipocytes from normal samples stimulated with either cigarette smoke extract (CSE) or H$_2$O$_2$. Cells from normal patients were treated with quercetin (100 µM) for the first three days of a 10-day period of adipogenesis in adipogenic medium containing 10 µM rosiglitazone alone or combined with 2% CSE or 10 µM H$_2$O$_2$. After 10 days, the cell lysates were subjected to western blotting analysis for PPARγ, C/EBPα and C/EBPβ protein expression (A). The experiments were performed in triplicate with cells from three different normal donors. Quantification of PPARγ, C/EBPα and C/EBPβ by densitometry, normalized to the β-actin levels in the same samples, is shown (B). The data in the columns are the mean relative density ratios ± SD of three experiments in cells from three different normal samples. The assays were performed at least three times in triplicate with cells from three different normal samples, and expressed as the differences between the quercetin-treated and -
untreated cells (*$P < 0.05$) in each control, CSE or H$_2$O$_2$ stimulated condition.
Figure 1. Intracellular reactive oxygen species (ROS) measured in normal and GO cells stimulated with CSE or H2O2. Orbital fibroblasts (OFs) from normal (n=3) and GO subjects (n=3) were treated with 1%–5% CSE (A) or 10–500 µM H2O2 (B) for 30 min. ROS were measured by flow cytometry using 5-(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA). The results are expressed as percentages of the untreated control values, presented as means ± SD. Assays were performed at least three times in triplicate with cells from different donors; data from a representative experiment are shown, expressed as the differences between treated and untreated cells in normal and GO OF samples (*P < 0.05).
Figure 2. Effects of quercetin on cigarette smoke extract (CSE)- and H2O2-induced generation of intracellular reactive oxygen species (ROS) in preadipocyte orbital fibroblasts. Orbital fibroblasts (OFs) from GO patients (n=3) were treated with 2% CSE or 10 µM H2O2 for 30 min with or without pretreatment with quercetin (50 or 100 µM) for 24 h. ROS were measured with flow cytometry using 5-(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA). The results are expressed as percentages of the untreated control values, presented as means ± SD. Assays were performed at least three times in triplicate with cells from three different GO samples; data from a representative experiment are shown, expressed as the differences between the quercetin-treated and -untreated cells (*P < 0.05) and between the CSE- or H2O2-stimulated and -unstimulated control cells (**P < 0.05).
Figure 3. Effects of quercetin on cigarette smoke extract (CSE)- or H2O2-stimulated adipogenesis. Cells were treated with 2% CSE or 10 µM H2O2 for the first three days of the 10-day period of adipogenesis in adipogenic medium containing 10 µM rosiglitazone. To determine the suppressive effect of quercetin on adipogenesis, quercetin (50 or 100 µM) was also added for the first three days of differentiation. The cells were stained or not stained with Oil Red O and examined microscopically (×40; inset ×400). The experiments were performed in triplicate with cells from different donors, and the representative figures are shown.

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Figure 4. Effect of quercetin on the cigarette smoke extract (CSE)- or H2O2-stimulated generation of intracellular reactive oxygen species (ROS) in differentiating orbital fibroblasts during adipogenesis. Confluent fibroblasts were subjected to a differentiation protocol that included adipogenic supplements for 10 days (A), and then further stimulated with 2% CSE (B) or 10 µM H2O2 (C) for the first three days of adipogenesis. To determine the suppressive effect of quercetin on adipogenesis, quercetin (100 µM) was also added during the first three days of differentiation. ROS were measured by flow cytometry using 5-(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) on days 0, 1, 4, 7, and 10 of adipogenesis. The results are expressed as percentages of the untreated control values, presented as means ± SD. The assays were performed at least three times in triplicate with cells from three different GO samples; data from a representative experiment are shown, expressed as the differences between the quercetin-treated and -untreated cells (*P < 0.05). On day 10 of adipogenesis, the cells were stained with H2DCFDA and examined microscopically (×100).
Figure 5. Effects of quercetin on cigarette smoke extract (CSE)- or H2O2-stimulated heme oxygenase−1 (HO−1) expression in preadipocyte orbital fibroblasts, analyzed with western blotting. Orbital fibroblasts (5×10^5) from GO patients (n=3) were treated with 2% CSE for 0–48 h (A), 0–5% CSE for 24 h (B), 10 µM H2O2 for 0–48 h (C), or 0–500 µM H2O2 for 24 h (D) and their HO−1 protein expression was determined by western blotting. The cells were pretreated with 50 or 100 µM quercetin for 24 h, then stimulated with 2% CSE or 10 µM H2O2 for 24 h (E) to determine the effect of quercetin on CSE- or H2O2-stimulated HO−1 protein expression. Quantification of HO−1 by densitometry, normalized to the level of β-actin in the same sample, is shown (F–H). The data in the columns are the mean relative density ratios ± SD of three experiments. *P < 0.05 vs untreated differentiated control cells.
Figure 6 Effects of quercetin on adipogenesis-related transcription factors and heme oxygenase–1 (HO–1) expression in differentiated adipocytes stimulated with either cigarette smoke extract (CSE) or H2O2. Cells were treated with quercetin (50 or 100 µM) for the first three days of a 10-day period of adipogenesis in adipogenic medium containing 10 µM rosiglitazone alone or combined with 2% CSE or 10 µM H2O2 (A). After 10 days, the cell lysates were subjected to western blotting analysis for PPARγ, C/EBPα, C/EBPβ, and HO–1 protein expression. The experiments were performed in triplicate with cells from three different GO donors. Quantification of PPARγ (B), C/EBPα (C), C/EBPβ (D), and HO–1 (E) by densitometry, normalized to the β-actin levels in the same samples, is shown. The data in the columns are the mean relative density ratios ± SD of three experiments. *P < 0.05 vs untreated differentiated control cells.