

Cigarette smoke extract-induced adipogenesis in Graves' orbital fibroblasts is inhibited by quercetin via reduction in oxidative stress

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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 H₂O₂ 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) γ , CCAAT-enhancer-binding proteins (C/EBP) α and β , and heme oxygenase-1 (HO-1), an antioxidant enzyme, were examined during adipogenic differentiation. In result, CSE and H₂O₂ dose-dependently stimulated intracellular ROS production in normal and Graves' OFs. The effect of 2% CSE was similar to that of 10 μ M H₂O₂; both concentrations were noncytotoxic and were used throughout the experiment. Quercetin pretreatment reduced the ROS generation stimulated by either CSE or H₂O₂ in preadipocyte OFs. CSE and H₂O₂ stimulated adipocyte differentiation in cultured OFs. The addition of quercetin (50 or 100 μ M) suppressed adipogenesis. Quercetin also suppressed ROS generation in differentiating OFs during adipogenesis stimulated by CSE and H₂O₂. Additionally, the expressions of PPAR γ , C/EBP α , and C/EBP β proteins were reduced in the quercetin-treated OFs. Quercetin also reduced the CSE- and H₂O₂-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 *in vitro*, supporting the use of quercetin in the treatment of GO.

Key Words

- ▶ quercetin
- ▶ Graves' orbitopathy
- ▶ heme oxygenase-1
- ▶ cigarette smoke extract
- ▶ orbital fibroblasts
- ▶ adipogenesis
- ▶ reactive oxygen species

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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 & 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 (OFs) and plasma of GO patients (Lu *et al.* 1999). Tsai *et al.* (2010) found that oxidative DNA damage, lipid peroxidation, and ROS production were increased in cultured GO fibroblasts relative to their levels in normal OFs. Low doses of hydrogen peroxide (H_2O_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 & Mazzone 2008). Lee *et al.* (2009) recently reported that ROS facilitates adipocyte differentiation by accelerating the mitotic clonal expansion of 3T3-L1 preadipocytes. Cell cycle progression (from S to G₂/M phase) was markedly enhanced by H_2O_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.* (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 of 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 at nontoxic concentrations also inhibited fibrotic markers and affected matrix metalloproteinase-2 and -9 activities in both primary and orbital fat tissue cultures from GO patients (Yoon *et al.* 2012a). In another recent report by Lisi *et al.* (2011) quercetin was shown to reduce cell proliferation and hyaluronan production. Quercetin has been shown to exhibit antioxidant, anti-inflammatory, and anti-adipogenic properties in other cell systems and animal models (Comalada *et al.* 2005, Rotelli *et al.* 2009, Vasquez-Garzon *et al.* 2009, Ying *et al.* 2009, Rogerio *et al.* 2010). Here, we investigated the anti-adipogenic role of quercetin in OFs stimulated by oxidants, such as CSE and H_2O_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_2O_2 in OFs from patients with GO, and inhibited the generation of ROS during adipogenesis. Consequently, treatment with quercetin during adipogenesis not only significantly suppressed the expression of adipogenic transcriptional regulator proteins but also that of the antioxidant heme oxygenase-1 (HO-1) as well, in both OFs and preadipocyte OFs.

Materials and methods

Reagents

Quercetin (Q0125) and Oil Red O were purchased from Sigma-Aldrich, Inc. 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

was purchased from Sigma–Aldrich. Anti-peroxisome proliferator activator gamma (PPAR γ) 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.

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 3 months before surgery, and were euthyroid at the time of surgery. The clinical activity scores (CASs) at the time of tissue harvest were less than four for all patients, indicating inactive inflammatory status, which was based on an original 10-point CAS developed by Mourits *et al.* (1997). None of the patients had been treated previously with orbital radiotherapy. Normal orbital adipose/connective tissue specimens were collected during the course of orbital surgery for other non-inflammatory problems from patients with no prior history of thyroid disease or GO and with no clinical evidence of GO ($n=4$; four women, aged 36–64 years). The study was approved by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine, 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 μ g/ml) in a humidified 5% CO $_2$ incubator at 37 $^{\circ}$ C. Then, monolayers were serially passaged by gently treating them with trypsin/EDTA. The strains were stored in liquid N $_2$ until needed and only strains between the third and seventh passages were used. We tried to use the same passage of cell cultures for a same experiment to reduce bias caused by primary cultures from human samples.

Because treatment with ≤ 100 μ M quercetin for 24 h did not reduce cell viability in Graves' OFs to below 95% as shown in an MTT analysis and did not induce significant apoptosis when assessed with an annexin V-FITC assay in our previous study (Yoon *et al.* 2011), 100 μ M quercetin were used as the maximal nontoxic dose in the experiments of this study.

Preparation of CSE

CSE was prepared by bubbling smoke from two commercially available, filtered cigarettes (Marlboro 20 class A cigarettes, made by Philip Morris Korea, Inc., Seoul, Korea, containing 8.0 mg of tar and 0.7 mg of nicotine) through 20 ml of prewarmed serum-free DMEM/F12 (1:1) at a rate of one cigarette per 2 min, as described previously (Kode *et al.* 2008). The pH of the CSE was adjusted to 7.4 and the CSE was sterile filtered through a 0.2 μ M 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 $_2$ O $_2$

Effects of CSE and H $_2$ O $_2$ on cell viability in preadipocyte OFs were assessed. OFs (1×10^5) from normal subjects and GO patients were seeded into 24-well culture plates and treated with different concentrations of CSE (1–5%) or H $_2$ O $_2$ (10–500 μ M) 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 μ M biotin, 17 μ M pantothenic acid, 10 μ g/ml transferrin, 0.2 nM T $_3$, 1 μ M insulin (Boehringer-Mannheim), and 0.2 μ M carbaprostaglandin (Calbiochem, La Jolla, CA, USA). For the first 4 days, 1 μ M insulin, 1 μ M 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 to 4 days. A PPAR γ agonist, rosiglitazone (10 μ M; Cayman, Ann Arbor, MI, USA), 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 3 days of adipogenesis only. Treatment with 2% CSE or 10 μM H_2O_2 for the first 3 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 3 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 3 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_2DCFDA ; Invitrogen, Eugene, OR, USA), an oxidant-sensitive fluorescent probe, as previously described (Kode *et al.* 2008); H_2DCFDA is deacetylated intracellularly by esterase, forming H_2DCF , which is oxidized by ROS to 2',7'-dichlorofluorescein (DCF), a highly fluorescent compound. The cells were seeded at a density of 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_2O_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_2O_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 PBS, incubated with 10 μM H_2DCFDA at 37 °C for 30 min, and then stimulated with CSE or H_2O_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, USA). For each sample, $\geq 10\,000$ events were acquired. Cells were gated out and the analysis was performed using only live populations. The fluorescently stained cells were also examined microscopically ($\times 100$ magnification).

Oil Red O staining

The cells were stained with Oil Red O, as described by Green & Kehinde (1975), on day 10 of differentiation. A stock solution of Oil Red O (0.5% Oil Red O in isopropanol) was prepared. To prepare the working solution, 6 ml stock solution was mixed with 4 ml 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 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 $\times 40$ and $\times 400$ magnification with an Olympus BX60 light microscope (Olympus, Melville, NY, USA).

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 Na_3VO_4 , 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ 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, USA), probed overnight with primary antibodies in TBST, and washed three times with TBST. The immunoreactive bands were detected with HRP-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 s.d.s 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 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). A *P* value of <0.05 was considered significant.

Results

Effects of CSE and H₂O₂ 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-CSE 2%, $P=0.021$; control-CSE 2.5%, $P=0.015$; control-CSE 5%, $P=0.024$; **Supplementary Figure 1A**, see section on supplementary data given at the end of this article). Similarly, treatment with another oxidant, H₂O₂, at 50 and 100 μM for 24 h increased cell proliferation in GO cells (control-H₂O₂ 50, $P=0.022$; control-H₂O₂ 100, $P=0.01$), and treatment with 500 μM H₂O₂ significantly reduced cell viability ($P<0.01$; **Supplementary Figure 1B**); treatment with 10 μM H₂O₂ did not affect cell viability on MTT analysis (GO control-H₂O₂ 10, $P=0.129$; normal control-H₂O₂ 10, $P=0.415$). In normal cells, treatment with 100 μM H₂O₂ for 24 h increased cell proliferation ($P=0.031$) and treatment with 500 μM H₂O₂ significantly reduced cell viability ($P<0.01$).

Measurement of intracellular ROS in normal and GO cells stimulated with CSE or H₂O₂

Basal levels of intracellular ROS were significantly higher in GO cells ($n=3$) ($149.4\pm 6.2\%$) 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 H₂O₂ 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%, $P=0.025$; Fig. 1A) (GO control-H₂O₂ 10, 50, 100, and 500, all $P<0.01$; normal control-H₂O₂ 100, $P=0.026$; normal control-H₂O₂ 500, $P=0.011$; Fig. 1B). Only high concentrations of CSE (5%) or H₂O₂ (100 and 500 μM) increased ROS production in the normal cells relative to the control levels.

Quercetin treatment reduced CSE- and H₂O₂-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 a previous MTT assay and annexin V-FITC apoptosis assay (Yoon *et al.* 2011). Therefore, a maximal concentration of 100 μM quercetin was used in the experiments of this study. ROS production in cells

stimulated with either 2% CSE or 10 μM H₂O₂ 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

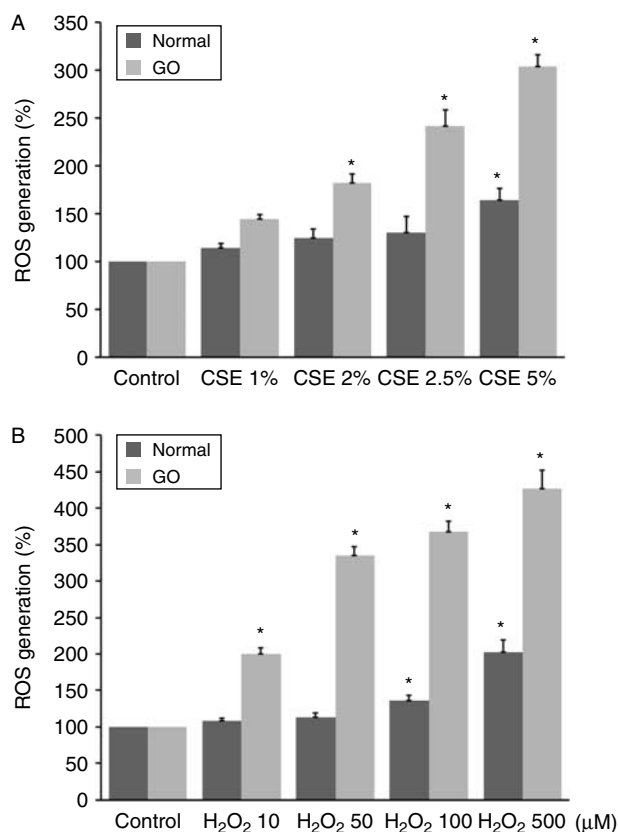
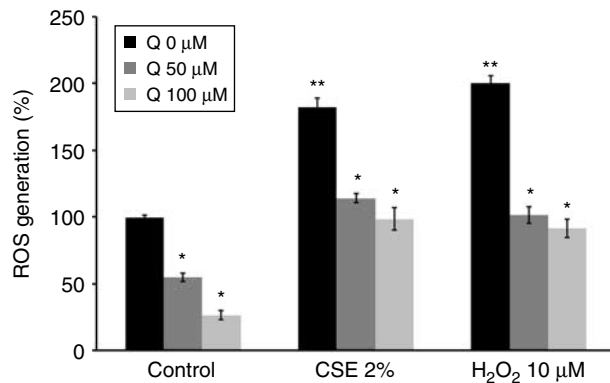


Figure 1

Intracellular ROS measured in normal and GO cells stimulated with CSE or H₂O₂. 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 H₂DCFDA. The results are expressed as percentages of the untreated control values in each Graves' and normal cells, presented as means \pm s.d. 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**

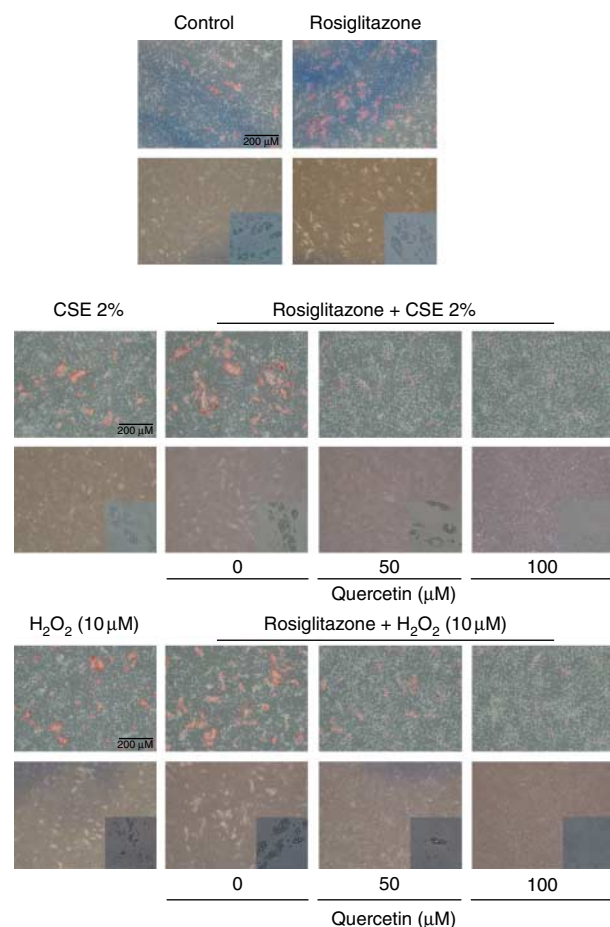
Effects of quercetin (Q) on CSE- and H₂O₂-induced generation of intracellular ROS in preadipocyte OFs. OFs from GO patients ($n=3$) were treated with 2% CSE or 10 μM H₂O₂ for 30 min with or without pretreatment with quercetin (50 or 100 μM) for 24 h. ROS were measured with flow cytometry using H₂DCFDA. The results are expressed as percentages of the untreated control values, presented as means \pm s.d. 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 H₂O₂-stimulated and -unstimulated control cells (** $P<0.05$).

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 3 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 3 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.

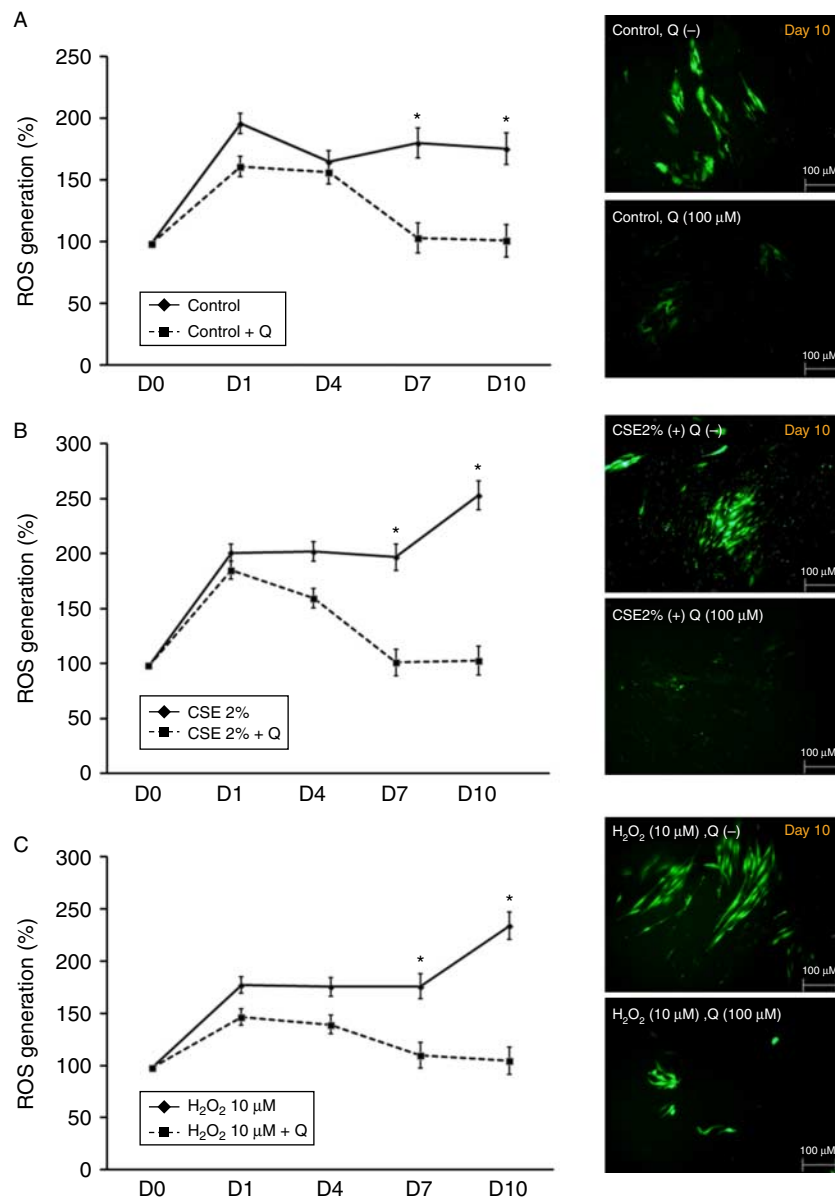
Effects of quercetin on the generation of ROS during adipocyte differentiation

When intracellular ROS were 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 3 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, B and C). Similarly, when the ROS in the differentiated cells were fluorescently stained on day 10 of

**Figure 3**

Effects of quercetin on CSE- or H₂O₂-stimulated adipogenesis. Cells from GO patients were treated with 2% CSE or 10 μM H₂O₂ for the first 3 days of the 10-day period of adipogenesis in adipogenic medium containing 10 μM rosiglitazone. To determine the suppressive effect of quercetin on adipogenesis, quercetin (50 or 100 μM) was also added for the first 3 days of differentiation. The cells were stained with Oil Red O and examined microscopically ($\times 40$; inset $\times 400$). The experiments were performed in triplicate with cells from different GO donors, and the representative figures are shown.

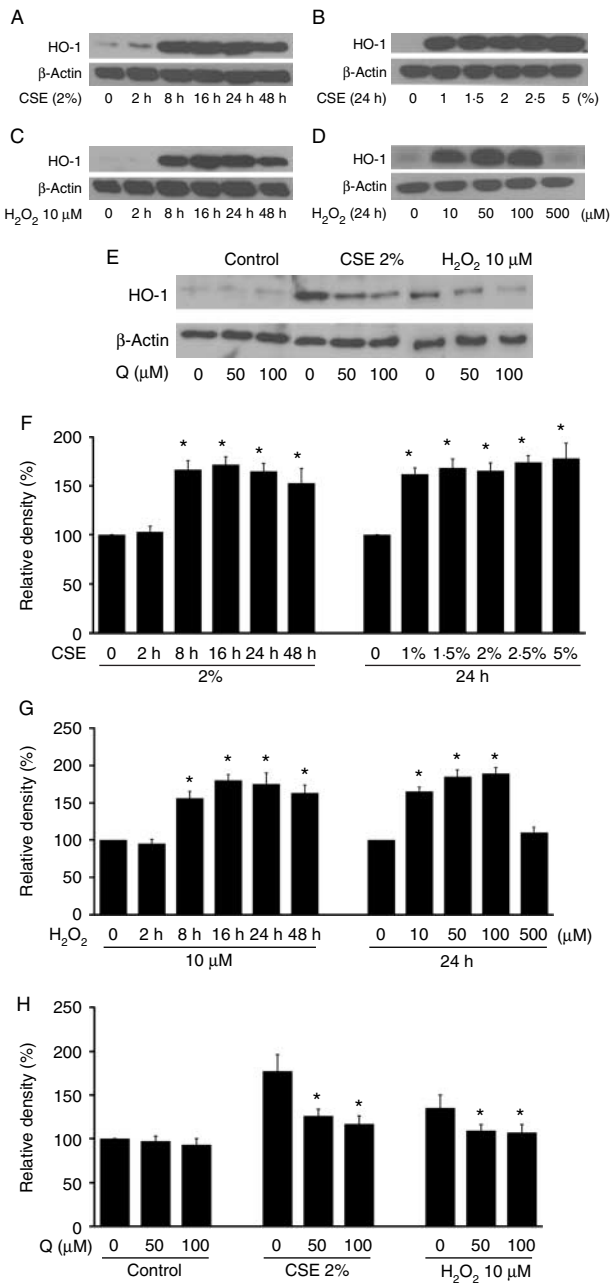
**Figure 4**

Effect of quercetin (Q) on the CSE- or H₂O₂-stimulated generation of intracellular ROS in differentiating OFs 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 H₂O₂ (C) for the first 3 days of adipogenesis. To determine the suppressive effect of quercetin on adipogenesis, quercetin (100 μM) was also added during the first 3 days of differentiation. ROS were measured by flow cytometry H₂DCFDA 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 ± s.d. 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 H₂DCFDA and examined microscopically (×100). Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-12-0257>.

adipogenesis, a significant reduction in fluorescently stained cells was observed after treatment with 100 μM quercetin (×100; Fig. 4A, B and 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 Figure 2**, see section on supplementary data given at the end of this article).

**Figure 5**

Effects of quercetin on CSE- or H₂O₂-stimulated HO-1 expression in preadipocyte OFs, analyzed with western blotting. OFs (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, and 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, G and H). The data in the columns are the mean relative density ratios \pm s.d. of three experiments. * $P < 0.05$ vs untreated control cells.

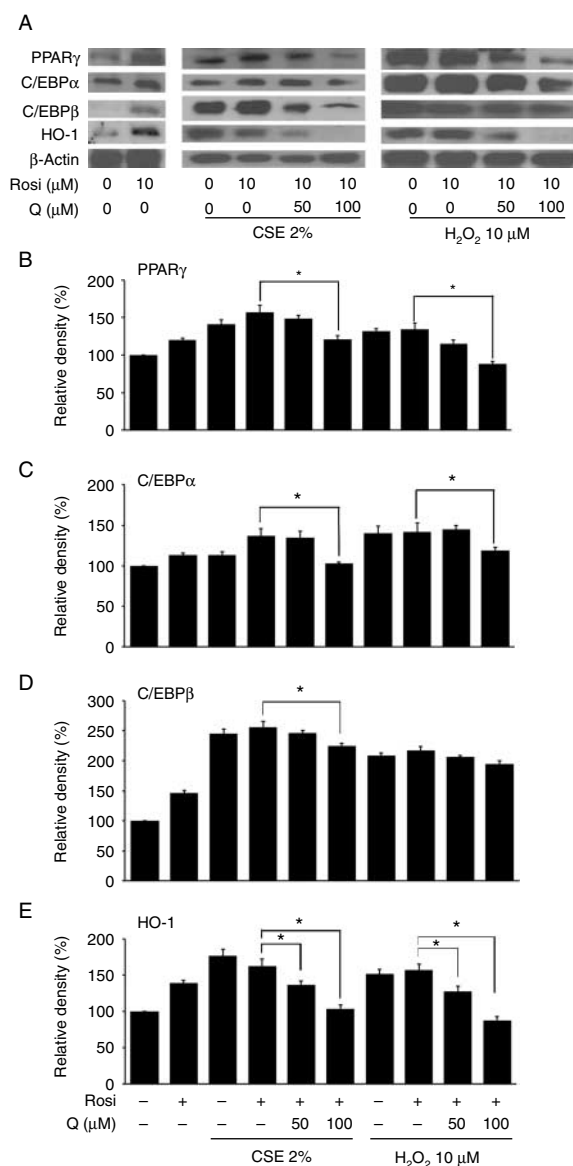
Effects of quercetin on the expression of the transcriptional regulators of adipogenesis and HO-1 during adipocyte differentiation

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 or 100 μM H₂O₂ for 24 h ($P < 0.05$ vs CSE or H₂O₂-untreated cells) (Fig. 5A, B, C, D, F and 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, PPAR γ , C/EBP α , and C/EBP β are all strongly expressed in differentiated adipocytes (Fig. 6A, B, C and D). The addition of either 2% CSE or 10 μM H₂O₂ for the first 3 days of differentiation caused not only a further increase in adipogenesis but also significantly increased PPAR γ , C/EBP α , and C/EBP β expression in GO OFs (all $P < 0.05$ vs unstimulated differentiated cells) (Fig. 6A, B, C and D). Treatment with quercetin for the first 3 days of differentiation significantly and dose dependently attenuated the expression of PPAR γ , C/EBP α , and C/EBP β in differentiated fibroblasts after stimulation with either CSE or H₂O₂ ($P < 0.05$; quercetin 100 μM vs quercetin-untreated cells) (Fig. 6A, B, C and D). In differentiated normal OFs, quercetin 100 μM suppressed PPAR γ and C/EBP α protein production (Supplementary Figure 3, see section on supplementary data given at the end of this article). However, C/EBP β 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

**Figure 6**

Effects of quercetin (Q) on adipogenesis-related transcription factors and HO-1 expression in differentiated adipocytes stimulated with either CSE or H₂O₂. Cells from GO patients were treated with quercetin (50 or 100 μM) for the first 3 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 PPAR γ , C/EBP α , C/EBP β , and HO-1 protein expression. The experiments were performed in triplicate with cells from three different GO donors. Quantification of PPAR γ (B), C/EBP α (C), C/EBP β (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 \pm s.d. of three experiments. * $P < 0.05$ vs untreated differentiated control cells.

oxidant H₂O₂, 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 H₂O₂ 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 & Bahn 2010). In examining the mechanism that underlies the aggravation of GO due to smoking, Cawood *et al.* (2007) reported that CSE increases hyaluronan production and adipogenesis in OFs, and that the effects of interleukin 1 and CSE on adipogenesis are synergistic. 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.* (2007). We decided to use CSE rather than a single compound, such as nicotine, because cigarette smoke contains more than 4800 compounds, and the active compound in GO has not yet been identified. In our study, CSE and H₂O₂ 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 H₂O₂, 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 with 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.* 2010).

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 (Baglolle *et al.* 2008, Lee *et al.* 2010, 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 H₂O₂ 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.* (2008) 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. 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, Yao *et al.* 2007, Kim *et al.* 2010). 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 anti-adipogenic effects of quercetin might be associated via different pathways from HO-1.

In this 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, Hiromatsu *et al.* 1998, Bouzas *et al.* 2000). 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 6-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, this study is the first *in vitro* study on 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 H₂O₂ 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 γ and C/EBP α production was increased by addition of 2% CSE or 10 μ M H₂O₂, and was suppressed by treatment with 100 μ M quercetin in normal cells. In our previous report, we experienced that primary cultured Graves' and normal OFs responded similarly to quercetin treatment (Yoon *et al.* 2011, 2012a). Adipogenesis induced by adipogenic stimuli was also similar between Graves' and normal OFs (Yoon *et al.* 2011). The action of quercetin in an *in vitro* condition does not seem to be specific to only GO cells. Recently, van Steensel *et al.* (2011) introduced a whole orbital tissue culture system for GO research, which may better mimic the clinical environment than single cell culture. Using this tissue culture model, we previously showed that cytokines such as IL1 β , IL6, and TNF α , 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.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-12-0257>.

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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