Anti-oxidative and anti-adipogenic effects of caffeine in an in vitro model of Graves’ orbitopathy

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Abstract. Oxidative stress and adipogenesis play key roles in the pathogenesis of Graves’ orbitopathy (GO). In this study, the therapeutic effects of caffeine on the reduction of oxidative stress and adipogenesis were evaluated in primary cultured GO orbital fibroblasts in vitro. Orbital fibroblasts were cultured from orbital connective tissues obtained from individuals with GO. Intracellular reactive oxygen species (ROS) levels induced by hydrogen peroxide or cigarette smoke extract and the expression of anti-oxidative enzymes were measured after caffeine treatment. After adipogenic differentiation and caffeine treatment, cells were stained with Oil Red O and the levels of peroxisome proliferator activator γ (PPARγ), C/EBPa, and C/EBPβ were determined by western blot analysis. Hydrogen peroxide and cigarette smoke extract increased the levels of intracellular ROS and anti-oxidative enzymes, which decreased in a dose-dependent manner upon pretreatment with caffeine in GO orbital fibroblasts. Oil Red-O staining results revealed a decrease in lipid droplets; furthermore, PPARγ, C/EBPa, and C/EBPβ protein expression levels were inhibited upon treatment with caffeine during adipocyte differentiation. In conclusion, caffeine decreased oxidative stress and adipogenesis in GO orbital fibroblasts in vitro. These findings may contribute to the development of new types of caffeine-containing pharmacological agents for use in the management of GO.

Key words: Graves’ orbitopathy, Thyroid eye disease, Oxidative stress, Adipogenesis, Caffeine

GRAVES’ ORBITOPATHY (GO) is an autoimmune disorder of the orbit involving the infiltration of T cells, B cells, plasma cells, and macrophages. The pathogenesis of GO involves inflammation of orbital connective tissue that leads to the enlargement of extracellular muscles and orbital adipose tissue. Such tissue expansion within the bony orbits is thought to be responsible for the progression and complications of GO.

At the center of this pathogenesis lies inflammation and oxidative stress. Infiltration of inflammatory cells to the orbit triggers and propagates tissue expansion. Oxygen free radicals trigger fibroblast proliferation [1] and the abundance of thyroid hormones in Graves’ disease renders orbital fibroblasts more vulnerable to oxidative damage [2]. Clinically, cigarette smoking is the most important environmental risk factor for the development and progression of GO [3, 4], suggesting that oxidative stress is one of the key effectors in its pathogenesis.

Caffeine (1,3,7-trimethylxanthine) is a plant alkaloid found in coffee, tea, chocolate, cola, and other soft drinks commonly consumed around the world [5]. It is absorbed rapidly and completely from the gastrointestinal tract and is detectable in all body fluids including in umbilical cord blood [6, 7]. Numerous studies have determined that it acts as a free radical scavenger, taking part in antioxidant activities in vitro and in vivo in both animals and humans [8-10]. Furthermore, its anti-adipogenic properties in various cell types in vitro have shown that caffeine inhibited the expression of adipogenesis-related cytokines and transcription factors, resulting in a decrease in adipocyte differentiation and intracellular lipid accumulation [5, 11]. Given the fundamental role of oxidative stress and adipogenesis in the pathogenesis of GO, we investigated the therapeutic effect of caffeine in an in vitro model of GO.
Materials and Methods

Reagents and chemicals

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin, and gentamicin were purchased from Hyclone Laboratories, Inc. (Logan, UT, USA). Caffeine was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay solution was purchased from Promega Corporation (Madison, WI, USA). Cigarette smoke extract (CSE) was freshly prepared within an hour of each experiment from commercially available filtered cigarettes [Marlboro 20 class A cigarettes (8.0 mg tar; 0.7 mg nicotine); Philip Morris Korea, Inc., Seoul, Korea] as described in our previous study [12]. Recombinant human IL-1β was purchased from R&D Systems (Minneapolis, MN, USA) and Oil Red O was purchased from Sigma-Aldrich Corp.

Cell culture and differentiation protocols

Orbital adipose/connective tissue explants were obtained as surgical waste during decompression surgery in five patients with GO (three females, two males; 29–51 years of age). Normal control tissues were harvested during upper-lid blepharoplasty from the post-septal area of five individuals with no history or clinical evidence of thyroid disease or GO (three females, two males; 31–58 years of age). All five patients with GO achieved stable euthyroidism at the time of surgery, at which point their clinical activity scores were less than three. Furthermore, none of the patients with GO received steroid treatment or radiotherapy for at least three months before surgery. The Institutional Review Board of Severance Hospital, Yonsei University College of Medicine (Seoul, Korea) approved the study and written informed consent was obtained from all participants after explanation of the nature and possible consequences of the study. This study followed the tenets of the Declaration of Helsinki.

Primary cultures of orbital fibroblasts were established as described in our previous study [13]. Briefly, minced tissue was placed directly in 1:1 DMEM:F12 medium with 20% FBS and antibiotics. When the growth of fibroblasts was observed, monolayers were passaged serially with trypsin/ethylenediaminetetraacetic acid solution and cultures were maintained in DMEM with 10% FBS and antibiotics. The strains were stored in liquid nitrogen until further analysis; cells between the second and fifth passages were used.

The anti-adipogenic effect of caffeine was evaluated using our previously reported adipocyte differentiation protocol for GO orbital fibroblasts involving dexamethasone, rosiglitazone (10 μM; Cayman Chemical, Ann Arbor, MI, USA), and IL-1β (10 ng/mL) treatment [13, 14]. Briefly, after 10 days of adipogenic differentiation co-treated with serial concentrations of caffeine (0.1–5 mM), differentiated adipocytes were visualized with Oil Red O staining and expression of adipogenic transcription factors were evaluated with western blotting.

Intracellular ROS measurement

ROS release was determined with 5-(and 6)-carboxy-20,70-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen/Thermo Fisher Scientific, Carlsbad, CA, USA), an oxidant-sensitive fluorescent probe, as we described previously [12]. Briefly, primary cultured orbital fibroblasts were pretreated with various concentrations of caffeine for 24 h. The culture medium was then removed, and the cells were washed with PBS, incubated with 10 mM H2DCFDA at 37°C for 30 min, then stimulated with CSE (2%) or H2O2 (10 μM) for 30 min [12, 15, 16]. The fluorescently stained cells were examined microscopically at 40× and were quantified via flow-cytometric analysis.

Western blotting

Western blot analysis was performed as described previously [17]. To assess the induction of oxidative stress by CSE and H2O2 and its inhibition by caffeine, confluent orbital fibroblasts were pre-exposed to serial concentrations of caffeine for 24 h, followed by stimulation with CSE or H2O2 for 24 h. Expression levels of manganese superoxide dismutase (Mn-SOD), copper-zinc superoxide dismutase (Cu/Zn-SOD), thioredoxin (Trx), and heme oxygenase-1 (HO-1) were assessed to evaluate oxidative stress levels. To evaluate the expression of adipogenic transcription factors after adipogenic differentiation, the expression levels of peroxisome proliferator activator gamma (PPARγ), C/EBPα, and C/EBPβ were evaluated in GO cells after ten days of adipocyte differentiation. Activation of intracellular signalling proteins was assayed via western blot analysis of phosphorylated and total AKT, extracellular signal-regulated kinase
(ERK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), c-Jun NH(2)-terminal kinase (JNK), and p38 protein. The relative amount of protein in each immunoreactive band was quantified by densitometry and normalised to the concentration of β-actin in the same sample.

Anti-Mn-SOD, anti-Zn/Cu-SOD, anti-Trx, anti-HO-1, anti-peroxisome proliferator activator gamma (PPARγ), anti-C/EBPα, anti-C/EBPβ, anti-NF-κB, p38, and anti–β-actin antibodies were all obtained from Santa Cruz Biotechnology (Dallas, TX, USA) and antibodies against AKT, ERK, and JNK were produced by Cell Signaling Technology (Danvers, MA, USA).

**Oil Red O staining**

After ten days of adipocyte differentiation of orbital fibroblasts, cells were stained with an Oil Red O working solution as described previously [13, 14]. Stained cells were visualised and photographed at 40×, then quantified using a spectrophotometer at 490 nm according to the manufacturer’s instructions. Experiments for the quantitative assessment of adipogenic differentiation were performed in duplicate using cells from different donors; the results were normalised to the absorbance of untreated differentiated control cells.

**Statistical analysis**

All experiments were performed using cells from at least three different samples and the samples were assayed in duplicate each time. Differences in parameter estimates between the experimental and control groups were assessed by the Student’s t-test or Wilcoxon rank-sum test using R version 3.1.2 (R Foundation, Vienna, Austria). Values of p < 0.05 were considered significant.

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**Results**

**Cell viability using MTS analysis**

To determine non-toxic concentrations of caffeine in orbital fibroblasts, an MTS assay was performed. Orbital fibroblasts from patients with and without GO were treated with caffeine at concentrations ≤5 mM for 48 h and 72 h. The 0.01 to 5 mM range of caffeine did not decrease cell viability below 95% in both normal and GO orbital fibroblasts in the 48 h treatment (Fig. 1, data for non-GO orbital fibroblasts are not shown).

**Caffeine decreased ROS levels induced by H₂O₂ or CSE**

To demonstrate the anti-oxidative effect of caffeine in GO orbital fibroblasts, oxidative stress was induced by 10 μM H₂O₂ or 2% CSE with or without 0.01–5 mM caffeine pretreatment for 24 h. Treatment with 10 μM H₂O₂ or 2% CSE for 30 min significantly increased ROS production relative to that in untreated control cells, which was reduced by caffeine pretreatment in a dose-dependent manner (Fig. 2A). Similarly, upon fluorescent staining of cellular ROS, a significant reduction in fluorescently stained cells was observed after treatment with caffeine (40×; Fig. 2B).

**Effect of caffeine on SOD, Trx, and HO-1**

The relative expression levels of Mn-SOD, Cu/Zn-SOD, and HO-1 increased upon treatment with 10 μM H₂O₂ or 2% CSE in GO orbital fibroblasts for 24 h, while the expression of Trx increased only upon treatment with CSE, not H₂O₂. Upon pretreatment with a serial concentration of caffeine, we found that caffeine concentrations >1 mM decreased the expression of Mn-SOD, Cu/Zn-SOD, Trx, and HO-1 (Fig. 3).
Caffeine reduced adipogenesis in GO orbital fibroblasts

To determine the effect of caffeine on adipogenesis in orbital fibroblasts, GO cells were treated with caffeine during adipocyte differentiation. When caffeine (0.5–5 mM) was added to the adipogenic medium, it reduced the number of adipocytes and suppressed the accumulation of lipid droplets. The optical density of Oil Red O-stained cell lysates showed that caffeine-treated cells exhibited decreased absorbance at 490 nm (Fig. 4A). Western blot analysis was then performed to investigate whether caffeine affects the production of the adipogenic transcription factors PPARγ, C/EBPα, and C/EBPβ during adipogenesis. Caffeine had dose-dependent inhibitory effects on the expression of adipogenic transcription factors (Fig. 4B). Each experiment was performed in duplicate with cells from three different GO samples and samples were assayed in duplicate.

Effect of caffeine on intracellular signalling pathways

To investigate the signalling pathways through which caffeine mediates its effects in GO orbital fibroblasts, the expression levels of multiple transcription factors were assessed after caffeine treatment. Both GO and non-GO cells were treated with 1 mM caffeine for various durations. Western blot analysis (Fig. 5) showed that caffeine inhibited the phosphorylation of ERK and p38 protein in a time-dependent manner. The phosphorylation of AKT, JNK, and NF-κB remained unchanged with 1 mM caffeine treatment (data not shown).

Discussion

In this study, we found that caffeine curtailed oxidative stress and adipogenesis, the main pathogenic mechanisms of GO. Based on our results, caffeine appears to have an inhibitory effect on ROS generation in response to oxidative stress, as well as an anti-adipogenic effect.

A state of oxidative stress has been described in Graves’ disease and GO [18-20]. In general, an increase in ROS or the reduced elimination of radicals by antioxidative enzymes will result in oxidative damage, inflammation, and loss of function. In the in vitro pathogenic mechanism of GO, superoxide radicals stimulate orbital fibroblasts to proliferate and differentiate into mature adipocytes [20]. Based on clinical reports, cigarette smoking—likely the most important environmental factor associated with GO occurrence and progression—may act, among other mechanisms, by stimulating the generation of ROS and reducing antioxidant production [3, 20, 21]. In this regard, several in vitro studies demonstrate the therapeutic effectiveness of drugs with antioxidant potential in primary cultured orbital fibroblasts from GO patients [12, 15-17]. Clinical studies on the use of antioxidants for the management of GO have also shown them to be clinically effective in patients experiencing mild severity [22-24]. Although caffeine is known to act as a free radical scavenger—the basis for its antioxidant activities—it should be noted that it exhibits both anti-oxidant and pro-oxidant properties depending on dose: at low concentrations, caffeine shows antioxidant effects, while at high concentrations, it may increase cytotoxicity, leading...
Fig. 3  Effect of caffeine on H$_2$O$_2$- and CSE-induced expression of SOD, Trx, and HO-1 in orbital fibroblasts from GO patients
Orbital fibroblasts from GO patients (n = 3) were treated with 10 μM H$_2$O$_2$ (A) or 2% CSE (B) for 24 h with or without pretreatment with 0.1–5 mM caffeine for 24 h. The cell lysates were subjected to western blot analysis. Experiments were performed three times using different strains and samples were assayed in duplicate. Results are expressed as the relative density of each protein normalised to the level of β-actin and presented as means ± standard deviation. GO cells treated with neither CSE nor H$_2$O$_2$ were used as controls (*p < 0.05 and **p < 0.01 versus H$_2$O$_2$- or CSE- treated cells without pretreatment).
to the subsequent generation of reactive oxygen species (ROS) [5, 25, 26]. In this study, however, orbital fibroblasts were resistant to the generation of ROS at high concentrations of caffeine, which acted as an antioxidant in $H_2O_2$- or CSE-stimulated orbital fibroblasts even at the highest concentration (5 mM) without causing cell damage.

Adipogenesis, the process by which orbital fibroblasts develop into mature adipocytes, is one of the key pathogenic mechanisms in GO. We found that drugs with antioxidant effects significantly suppressed adipocyte differentiation in primary Graves’ orbital fibroblasts [12, 15-17]. Recently, groups studying obesity and metabolism have reported that caffeine inhibits adipogenic differentiation and reduces lipid accumulation [5, 11]. They showed that caffeine may prohibit the activation of C/EBPβ, the first response to adipogenic inducers, blocking the adipogenic cascade involving PPARγ, aP2, or leptin protein. In this study, we showed that ≥0.5 mM caffeine inhibited adipogenic differentiation and lipid accumulation, as well as the expression of adipogenic transcription factors (PPARγ, C/EBPa, and C/EBPβ).

The differentiation of preadipocytes is triggered by hormonal agents and proceeds by the coordination and precise control of transcriptional cascades. The first stage of adipocyte differentiation is growth arrest at a confluent state; proliferating cells will not accumulate lipid droplets in their cytoplasm until they become confluent. Upon induction of differentiation, growth-arrested cells undergo additional rounds of cell division, known as mitotic clonal expansion [27]. A recent study showed that ROS are important in regulating mitotic clonal expansion during adipogenesis and that they facilitate adipocyte differentiation by accelerating mitotic clonal expansion [28]. Moreover, it has been demonstrated that caffeine inhibits adipogenesis through the modulation of mitotic clonal expansion in 3T3-L1 adipocytes [11].

Mitogen-activated protein kinases (MAPKs) constitute a family of protein kinases (ERK, JNK, and the p38-MAPKs in mammals) that play an essential role in relaying extracellular signals from the cell membrane to the nucleus via a cascade of phosphorylation events [29]. MAPKs are key participants in signal transduction pathways activated by mitogenic stimuli, environmental stress, and inflammatory agents [30]. Various studies have documented the involvement of the MAPK signalling pathways in redox-stressed cells and tissues [31]. Moreover, it has been shown that the pathogenesis of GO involves activation of the MAPK signalling pathways, especially the p38 and ERK pathways [32, 33]. Our results showed that the levels of phosphorylated p38 and ERK decreased when they were cotreated at increasing
The results of the present study show that caffeine has anti-oxidative and anti-adipogenic properties; however, it should be noted that the concentration of caffeine used in this *in vitro* study is somewhat higher than the normal physiological range. A caffeine concentration of 0.1 mM (19.4 μg/mL) is approximately equivalent to that in a cup of coffee containing 150 mg caffeine (Starbucks® latte) [5]; it has been reported that caffeine causes adverse effects at excessively high blood concentrations (>80 μg/mL) in humans [34]. In this study, caffeine exhibited anti-adipogenic effects beyond concentrations of 0.5 mM and anti-oxidative effects at 0.1 mM. As GO is a heterogeneous autoimmune disease affected by multiple genetic and environmental factors, *in vitro* results may not correlate precisely with *in vivo* conditions. Additionally, we induced pathologic conditions in cells by stimulation using concentrations of chemicals, hormones, and growth factors beyond physiological ranges—for example, our use of oxidants such as H$_2$O$_2$ or CSE.

In conclusion, to the best of our knowledge, this is the first study to investigate the effects of caffeine on oxidative stress and adipogenesis in orbital fibroblasts from GO patients. Until now, no clinical or epidemiologic studies have emerged that assess the relationship between caffeine and GD or GO. Our *in vitro* results provide evidence to support further investigation regarding the influence of caffeine on GO progression in clinical settings.

**Acknowledgements**

Funding: This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (NRF-2017R1A2B4009565).
Disclosure

The authors have no conflicts of interest to declare.

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