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7,8-Dihydroxyflavone inhibits adipocyte differentiation *via* antioxidant activity and induces apoptosis in 3T3-L1 preadipocyte cells

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ABSTRACT

Aims: Anti-obesity effects of a natural plant flavonoid 7,8-dihydroxyflavone (7,8-DHF) were evaluated using 3T3-L1 preadipocyte cells.
Main methods: The cell viability was determined using MTT assay. Effects of 7,8-DHF on intracellular lipid droplets and intracellular reactive oxygen species (ROS) were measured using a 2,7-dichlorofluorescein diacetate (DCF-DA) assay and Oil Red O staining method, respectively. Apoptotic cell death was monitored by annexin V-FITC/PI double staining and by a TUNEL assay. Antioxidant enzyme mRNA levels and protein expression of adipogenic transcription factors were determined by real-time PCR and Western blotting, respectively.
Key findings: Whereas the cell viability of 3T3-L1 preadipocytes was not affected by lower concentrations of 7,8-DHF (<20 μM), higher concentrations of 7,8-DHF (>20 μM) induced apoptotic cell death. 7,8-DHF (<20 μM) significantly reduced the intracellular lipid droplets and the expression of major adipogenic transcription factors, such as CCAAT/enhancer-binding protein-α (C/EBP-α), C/EBP-β, and peroxisome proliferator activated receptor-γ (PPAR-γ). 7,8-DHF treatment also dose-dependently reduced the intracellular ROS level, attenuated MAPK pathway activation, and increased the expression of antioxidant enzymes, such as Mn-superoxide dismutase (Mn-SOD), catalase (CAT), and heme oxygenase-1 (HO-1).
Significance: The results of this study indicated that 7,8-DHF inhibits the adipogenesis of 3T3-L1 preadipocyte cells by down-regulating the expression of adipogenic transcription factors, reduces lipid accumulation, and attenuates ROS accumulation by inducing antioxidant enzymes in differentiated 3T3-L1 cells, suggesting for the first time that 7,8-DHF has an anti-obesity effect *in vitro via* its anti-oxidant activity.

1. Introduction

Obesity induces serious health problems independently or in association with other diseases, including coronary heart disease, certain forms of cancer and osteoarthritis of large and small joints [1]. The development of obesity is characterized by an increased number of fat cells and increased lipids in those fat cells as a result of the mitogenesis and differentiation processes of adipocytes [2]. Adipocyte differentiation requires the activation of several adipogenic transcription factors such as CCAAT/enhancer-binding protein-α, β (C/EBP-α, β) and peroxisome proliferator activated receptor-γ (PPAR-γ). At the terminal phase of differentiation, adipocytes synthesize adipose tissue-specific products including an adipocyte-specific fatty acid binding protein (aP2) [3]. The increase in fat-derived reactive oxygen species (fat ROS) during the development of obesity has been reported to be a main causative factor of the aberrant regulation of adipocytokines and the decreased expression of antioxidative enzymes in adipose tissue [4,5]. The increased ROS level is also known to trigger the activation of the mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), p38, and Jun N-terminal kinase (JNK), which are involved in the initiation of the differentiation of preadipocytes, and activated MAPKs trigger the proliferation of adipocytes [6,7]. Indeed, oxidative stress induces lipid accumulation in HepG2 cells and facilitates adipose differentiation by accelerating mitotic clonal expansion [8,9]. Several reports have described that antioxidant compounds could suppress adipogenesis [10,11], and thus any compound eliminating ROS is a potential target for attenuating obesity. Many antioxidants have been reported to exhibit potent anti-obesity effects [12–14].

Recently, flavonoids, which are abundant in plant foods, have attracted significant public attention due to their various biological activities [15,16]. 7,8-Dihydroxyflavone (7,8-DHF, 7,8-dihydroxy-2-phenyl-4H-chromen-4-one) is a naturally occurring flavone found in plants including primula tree [17]. 7,8-DHF has been reported to exert beneficial health effects, such as anti-inflammatory, vasorelaxing and antihypertensive effects [18,19]. Several studies have demonstrated the antioxidant activity of 7,8-DHF, including a protective effect against hydrogen peroxide (H_2O_2)-induced DNA damage, a neuroprotective

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effect against glutamate-induced toxicity, and protective effects against 6-hydroxydopamine-induced cytotoxicity on PC12 cells and oxidative stress-induced cell damage on human keratinocytes [20–23]. Interestingly, several agonists of TrkB such as BDNF, neurotrophon-3,4, and 7,8-DHF have been reported to increase brown adipose tissue which dissipates energy directly, unlike white adipose tissue which accumulates excess energy in *in vivo* models [24].

Therefore, 7,8-DHF, which is a well-known antioxidant against several stimuli, may exhibit a direct anti-obesity effect. However, no specific studies have addressed the direct association between 7,8-DHF and obesity. We hypothesized that 7,8-DHF could regulate the adipogenic differentiation of preadipocytes and lipid metabolism during differentiation. To test this hypothesis, the effects of 7,8-DHF on the proliferation of preadipocytes, lipid accumulation during cell differentiation, and the expression of adipogenesis-specific transcription factor proteins were investigated *in vitro* using the 3T3-L1 preadipocyte cell line [3,25]. Considering that oxidative stress induces lipid accumulation and facilitates adipose differentiation [8,9], the effects of 7,8-DHF on intracellular ROS generation during cell differentiation and the underlying mechanisms of its anti-obesity effect *via* antioxidant activity were also examined.

2. Methods and materials

2.1. Materials and reagents

Dulbecco's modified eagle's media (DMEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA), and antibiotics and PBS were obtained from GIBCO-BRL (Grand Island, NY, USA). The murine 3T3-L1 pre-adipocyte cell line (ATCC® CL-173™) was purchased from the American Type Culture Collection (Manassas, VA, USA), and 7,8-dihydroxyflavone (7,8-DHF), Oil-Red O stock solution, IBMX (3-isobutyl-1-methylxanthine), dexamethasone, insulin, and a triglyceride (TG) assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies specific to C/EBP-α, C/EBP-β, PPAR-γ, aP2, total extracellular signal-regulated kinases (t-ERK), phospho-ERK (p-ERK), total-p38 MAPK (t-p38), phospho-p38 MAPK (p-p38), manganese superoxide dismutase (Mn-SOD), catalase (CAT), heme oxygenase-1 (HO-1), cleaved-caspase 8, cleaved-caspase 9, cleaved-caspase 3 and goat anti-rabbit IgG-HRP were obtained from Cell Signaling Technology (Danvers, MA, USA). 3-(4,5-dimethylthiazol-yl)-diphenyl tetrazoliumbromide (MTT) was provided by DUCHEPA Biochemie (Haarlem, Netherlands) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Wako Pure Chemical Industries (Chuo-Ku, Osaka, JPN).

2.2. Cell culture

3T3-L1 cells (Mouse embryonic fibroblast-adipose like cell line) were grown in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin and maintained at 37 °C in a 5% CO_2 incubator. Upon reaching confluence (Day 0), cells were stimulated with 0.5 μM IBMX, 1 μM dexamethasone and 10 μg/ml insulin in DMEM containing 10% FBS (MDI differentiation medium) for 2 days (Day 2). Cells were then maintained in DMEM containing 10% FBS and 10 μg/ml insulin for another 2 days (Day 4), followed by DMEM containing 10% FBS for 4 days (Day 8), at which point over 90% of cells became mature adipocytes with lipid-filled droplets.

2.3. Measurement of cell viability

The cytotoxicity of 7′8′-DHF to 3T3-L1 cells was determined by measuring the cell viability using the MTT assay. Precultured cells (2×10^4 cells/well) in DMEM were seeded on a 96-well microplate and exposed to different concentrations (0, 1, 10, and 20 μM) of 7′8′-DHF for 24 h. MTT solution (100 μg/well) was then added and the plates were incubated for 4 h. After removal of the media, 200 μl DMSO was added into each well to solubilize the formazan crystals, and the absorbance was read at 570 nm on a microplate reader (Molecular Devices, CA, USA).

2.4. Oil Red O staining and measurement of triglyceride content

To determine the lipid content, 3T3-L1 preadipocytes (7×10^4 cells/ml) were incubated in MDI differentiation medium with 7,8-DHF (0–20 μM) for 8 days in 6-well plates and stained with Oil Red O dye. After incubation, cells were washed gently with PBS, fixed with 4% paraformaldehyde at room temperature for 1 h and then stained with Oil Red O staining solution for 30 min. After staining, excess dye was removed and the plates were rinsed with PBS and dried. The stained lipid droplets were first visualized under the microscope (×100, Olympus, Tokyo, Japan), and then the stained lipid droplets were solubilized by adding isopropanol (Duksan, Korea) to each well to enable quantitative measurement of the lipid content. The absorbance was read at 500 nm using a microplate reader. Cellular triglyceride (TG) contents were measured using a commercial triglyceride assay kit (Sigma-Aldrich, MO, USA) according to the manufacturer's instructions. Differentiated adipocytes (Day 8) were treated with different concentrations of 7,8-DHF (0–20 μM) in 6-well plates. Cells were washed with PBS, scraped into 200 μL PBS, and homogenized by sonication for 1 min. The lysates were assayed for total TG.

2.5. Western blot analysis

The cells grown for 8 days in differentiation medium with 7,8-DHF (0–20 μM) were collected by scraping in lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, pH 7.8) containing protease and phosphatase inhibitor cocktails (Roche, Germany). After 1 h on ice, the cell lysates were centrifuged at 14,000 rpm for 30 min at 4 °C. The supernatant was used to determine the protein concentration by the Bradford assay. After SDS-PAGE on 10 and 12% gels, proteins were transferred to nitrocellulose membranes and incubated with the indicated primary antibodies overnight at 4 °C, and washed three times. The protein bands were then detected using horseradish peroxidase (HRP)-conjugated antibodies for 2 h at room temperature and viewed using the enhanced chemiluminescence method (AbClon, Seoul, Korea). The relative protein levels were quantified using Image J software from the NIH (Bethesda, MD, USA).

2.6. DPPH radical-scavenging assay

The effect of 7,8-DHF on the scavenging of DPPH radicals was examined by a modified method described earlier by Yamaguchi et al. [26]. Briefly, 1.8 ml of DPPH solution (0.1 mM, in methanol) was incubated with varying concentrations of 7,8-DHF (0.2 ml). The reaction mixture was incubated for 10 min at room temperature, and the absorbance of the resulting solution was read at 518 nm on a microplate reader. The radical scavenging activity was determined as a decrease in the absorbance of DPPH and calculated using the following equation:

$$\text{Scavenging effect } (\%) = [(1 - A_{518}\,(\text{sample})/A_{518}\,(\text{control})] \times 100.$$

2.7. Measurement of intracellular ROS

The fluorescent dye DCF-DA was used to detect the level of intracellular ROS. 3T3-L1 preadipocytes (7×10^4 cells/ml) were incubated in MDI differentiation medium with 7,8-DHF (0–20 μM) for 8 days in 6-well plates. Cells were harvested and stained with 10 μM DCF-DA (Thermo Fisher Scientific, MA, USA) for 45 min at 37 °C. The fluorescence intensity was monitored using a fluorescence spectrophotometer with excitation and emission wavelengths of 485 nm and 530 nm,