Esculetin Inhibits Adipogenesis and Increases Antioxidant Activity during Adipocyte Differentiation in 3T3-L1 Cells.

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ABSTRACT: This study was conducted to investigate the anti-adipogenic activity of esculetin (ECT) which is reported to be attributable to the modulation of antioxidant enzymes during adipogenesis. After six days of ECT treatment of 3T3-L1 cells, lipid accumulation was determined by Oil red O staining. The levels of glutathione (GSH) and reactive oxygen species (ROS), and the activities of antioxidant enzymes including glutathione reductase, glutathione peroxidase (GPx), and catalase were examined. In addition, the protein expression of glutamate-cysteine ligase catalytic subunit (GCLC) and heme oxygenase-1 (HO-1) was measured by Western blot. ECT significantly inhibited lipid accumulation by approximately 80% and ROS production in a concentration-dependent manner. GSH level and GPx activity were increased by ECT by approximately 1.3-fold and 1.7-fold compared to the control group, respectively. GCLC and HO-1 expression were elevated by ECT. These results showed that ECT treatments strongly inhibit adipogenesis, increase GSH level, and upregulate the expression of GCLC and HO-1, possibly by decreasing ROS production in 3T3-L1 cells during adipogenesis.

Keywords: esculetin, glutathione, antioxidant enzymes, anti-adipogenesis, 3T3-L1 cells

INTRODUCTION

Oxidative stress evoked by reactive oxygen species (ROS) is generally recognized as a contributing factor in the onset of chronic and metabolic diseases (1). ROS overproduction leads to oxidative stress and impairment of cell function (2). Recent studies reported that fat accumulation is accompanied with systemic oxidative stress in animal models (3,4). For example, increased production of ROS in adipose tissue decreases expression of various antioxidant enzymes (3). To prevent ROS overproduction, mammalian cells have several defense mechanisms. The enzymatic defense system includes glutathione reductase (GR), glutathione peroxidase (GPx), glutathione S-transferase, superoxide dismutase (SOD), and catalase (CAT), while the non-enzymatic defense system includes vitamin C and E, β-carotene, glutathione (GSH), and flavonoids (5). Several antioxidants increase GSH levels and the activities of antioxidant enzymes (6,7). Therefore, natural antioxidants could be proposed as therapeutic agents to counteract excessive production of ROS.

Antioxidant and phase II detoxification enzymes, including heme oxygenase-1 (HO-1), nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH):quinone oxidoreductase 1 (NQO1), and the catalytic and modifier subunits of glutamate-cysteine ligase (GCLC and GCLM, respectively), protect against the harmful effects of ROS (8). Nuclear factor erythroid 2-related factor 2 (Nrf2) mediates the induction of these enzymes. Under basal conditions, Nrf2-dependent transcription is repressed by its negative regulator, Kelch-like ECH-associated protein 1 (Keap1). As cells are exposed to oxidative stress, Nrf2 is released from Keap1-mediated repression and translocated into the nucleus (9). Then, Nrf2 binds to the antioxidant response element (ARE), which is the regulatory element sequence found in the promoters of a number of antioxidant and phase II detoxification enzymes (10). This event is associated with a cytoprotective response through upregulation of antioxidant enzymes, thereby decreasing sensitivity to oxidative damage (11).

Esculetin (6,7-dihydroxycoumarin; ECT) is found in various plants, such as Aesculus hippocastanum, Artemisia capillaris, Euphorbia lathyris, Citrus limonia, and Fraxinus rhynchophylla (12,13). ECT has multiple beneficial effects, including antioxidant, anticancer, and hepatoprotective activities (14,15). Some of the preliminary work showed the radical scavenging activity and the cell protective ef-
fect of esculetin against oxidative stress (16,17). Recently, we have reported the anti-adipogenic effect of esculetin in 3T3-L1 cells (18). Recent studies suggest that the polyphenolic compounds, Pycnogenol®️, genistein, and resveratrol, inhibit lipid accumulation by modulating ROS production associated with antioxidant enzyme responses (4,19). However, the effects of ECT on cellular mechanisms associated with oxidative stress and lipid accumulation in adipocytes remain unclear. In this study, we investigated the anti-adipogenic activity of ECT through the modulation of antioxidants and phase II detoxification enzymes during adipogenesis.

MATERIALS AND METHODS

Materials
Dexamethasone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, dimethyl sulfoxide, insulin, isobutylmethylxanthine (IBMX), Oil red O (ORO), thiobarbituric acid, 2’,7’-dichlorofluorescin diacetate (DCFH-DA), β-NADPH, GR, GPx, ethylenediaminetetraacetic acid (EDTA), 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB), GSH, oxidized glutathione (GSSG), xanthine, xanthine oxidase, and hydrogen peroxide (H₂O₂) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), bovine serum (BS), trypsin-EDTA, and penicillin-streptomycin were purchased from Gibco BRL (Gaithersburg, MD, USA). ECT, antibodies to HO-1, GCLC, β-actin, and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ECL™️ detection reagents were purchased from GE Healthcare (Buckinghamshire, UK).

Adipocyte differentiation and ORO staining
The 3T3-L1 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The 3T3-L1 cells were cultured as previously described (20). Briefly, cells were maintained at 37°C in DMEM containing 10% FBS until confluent. At two days post-confluence (day 0), the cell differentiation was induced by a mixture of dexamethasone (1 μM), IBMX (0.5 mM), and insulin (1 μg/mL) in DMEM containing 10% FBS. On day 2, this medium was changed with DMEM containing 10% FBS and insulin only. On day 4, the medium was replaced with DMEM containing 10% FBS only. To measure the levels of intracellular lipids in differentiated adipocytes, ORO staining was performed on the differentiated 3T3-L1 adipocytes on day 6 as previously described (21). Briefly, the cells were washed with phosphate buffered saline (PBS) and fixed with 10% formaldehyde for 10 min. The fixed cells were then washed three times with distilled water. ORO in isopropanol (5 mg/mL) was subsequently added to each well, and the cells were incubated at room temperature for 20 min. Next, the plates were rinsed three or four times with distilled water. Photomicrographs were taken after the cells were air-dried. The dye retained in the cells was extracted with isopropanol and quantified by measuring the absorbance at 500 nm.

Intracellular ROS analysis
ROS were quantified using a DCFH-DA fluorescent probe (22). Briefly, the 3T3-L1 cells were seeded in 96-well black plates, and adipocyte differentiation was induced with a mixture of dexamethasone (1 μM), IBMX (0.5 mM), and insulin (1 μg/mL) in DMEM containing 10% FBS at two days post-confluence, as described above. On day 6, the culture medium was changed with 25 μM DCFH-DA in serum-free medium, and the cells were incubated for 1 h at 37°C. Then, the cells were incubated in Hank’s balanced salt solution. Fluorescence intensity was measured with a spectrofluorometer (PerkinElmer Inc., Shelton, CT, USA) after 3 h at excitation and emission wavelengths of 485 and 530 nm, respectively.

Determination of GSH levels and antioxidant enzyme activities
For the measurement of GSH levels and antioxidant enzyme activities, 3T3-L1 cells were harvested on day 6. The cells were lysed using a sonicator. The lysates were centrifuged at 10,000 g for 10 min at 4°C, and used for protein, GSH, and antioxidant enzyme assays. The amount of GSH in 3T3-L1 cells were determined by a DTNB-GSSG reductase recycling assay (23). GR and GPx activities were measured as previously described (24,25). CAT activity was determined according to the method described by Fossati et al. (26).

Western blot analysis
3T3-L1 cells were collected on day 6 by centrifugation and washed once with PBS. Washed cell pellets were lysed in cell lysis buffer, and protein concentration was measured using a bicinchoninic acid assay kit (Thermo Scientific, Rockford, IL, USA). Proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred onto nitrocellulose membranes. After incubation for 1 h in blocking solution (5% skim milk), the membranes were incubated for 1 h at room temperature in primary antibodies (1:1,000 dilution). The membranes were then washed with Tris-buffered saline with Tween-20 (TBST), and incubated in horseradish peroxidase-conjugated secondary antibody (1:1,000 dilution) for 1 h at room temperature. The membranes were washed with TBST and then developed using ECL™️ detection re-
agents. The autoradiograms were quantified by optical densitometry with Image J software (NIH, Bethesda, MD, USA).

Statistical analyses
Experimental results are presented as mean with standard error (SE) or standard deviation (SD), and are representative of at least three independent experiments. Statistical significance was assessed by Duncan’s multiple range test, and $P<0.05$ were considered significant.

RESULTS AND DISCUSSION

Lipid accumulation in ECT-treated 3T3-L1 cells
None of the experimental concentrations of ECT used in the present study showed cytotoxicity in our prior study (18). ECT significantly reduced lipid accumulation during the adipocyte differentiation period compared to that in the control cells on day 6 (Fig. 1). At the highest concentration of ECT (100 μM), lipid accumulation was inhibited up to 70%. In our previous study, ECT inhibited adipogenesis through the activation of AMP-activated protein kinase (AMPK) (18). It has also been reported previously that ECT can induce apoptosis and inhibit adipogenesis in 3T3-L1 cells (27). The present results confirm those findings by showing that ECT inhibited adipogenesis of 3T3-L1 cells.

ROS generation after ECT treatment
To investigate the effects of ECT on ROS generation in 3T3-L1 cells, intracellular ROS production was measured using DCFH-DA staining. As shown in Fig. 2, ECT significantly decreased the generation of ROS on day 6. At the highest concentration (100 μM), ECT lowered ROS generation to approximately 40% compared to that in the control cells. The increase in intracellular ROS has been shown to occur during the first phase of adipogenesis (4). In fat accumulation, increased ROS levels induce NADPH oxidase activation via the high expression levels of NADPH oxidase 4 (Nox4) (28). Elevated ROS production from accumulated fat also leads to increased oxidative stress in blood, which can negatively affect the skeletal muscle, aorta, and liver (3). Therefore, inhibition of ROS production in adipocytes is a potential target for improving obesity-related metabolic syndrome. These results suggest that ECT inhibits ROS generation during adipogenic differentiation of 3T3-L1 cells. The antioxidant property of ECT seems to be effective in inhibiting adipogenesis. Therefore, the levels of GSH and antioxidative property of ECT in adipocytes.
GSH production and antioxidant enzyme activity

The changes in intracellular GSH levels were monitored in 3T3-L1 cells on day 6. As shown in Fig. 3, treatment with ECT increased GSH levels compared to that in the control cells in a dose-dependent manner. GSH is a major cellular antioxidant molecule against oxidative damage. Redox imbalance has been related to obesity-associated metabolic syndrome and insulin resistance (29). In the study using adipocytes from obese Zucker rats, lowered levels of GSH have been found (30). In addition, it has been reported that accelerating adipogenesis led to decreased GSH levels in 3T3-L1 cells, while treatment with resveratrol showed anti-adipogenic effects by upregulating GCLC and increasing GSH content (31). From these results, the anti-adipogenic effects conferred by ECT seem to be associated with its ability to increase GSH levels. Thus, the use of phytochemicals that are able to maintain GSH redox balance in adipose tissue could be promising in reducing obesity.

Cellular antioxidant enzymes, including GPx, GR, and CAT, play an important role in the defense system against oxidative stress. GPx catalyzes the reduction of lipid hydroperoxides by oxidizing GSH and, in turn, GR reduces oxidized glutathione back to GSH. CAT catalyzes the dismutation of two molecules of H₂O₂ into two molecules of water and one molecule of oxygen. To understand the antioxidative effects of ECT, changes in the activities of intracellular antioxidant enzymes were examined (Table 1). Treatment with ECT induced significant and dose-dependent increases of GPx activity compared to that in the control cells. In contrast, GR and CAT activities decreased following treatment with ECT. Antioxidant enzyme activities may play an important role in the recovery of steady-state concentrations of GSH (32). The expression of antioxidant enzymes, including Cu/Zn-SOD, GPx, and CAT, decreased in white adipose tissue of obese mice (3). In addition, the expression levels of antioxidant enzymes increased when adipocytes were treated with procyanidin-enriched extract (19). However, several studies reported that antioxidant enzyme activities were significantly decreased by treatment with natural antioxidants (6,7). From these results, the changes in GPx, GR, and CAT activities by ECT treatment indicate the antioxidant defense system of cells to modulate the adipogenic differentiation response.

Induction of HO-1 and GCLC by ECT treatment of 3T3-L1 cells

Since ECT can increase GSH levels, it is assumed that ECT affects adipogenesis by modulating the expression of key enzymes in GSH metabolism. The GCLC expression is dependent on Nrf2, which is a transcription factor for the induction of phase II enzymes, including HO-1 (33). Therefore, we examined whether ECT upregulates the antioxidant enzymes, HO-1 and GCLC. The protein expressions of HO-1 and GCLC were analyzed using Western blotting. Treatment with ECT induced an increase in HO-1 (Fig. 4A) and GCLC (Fig. 4B) protein expressions in a dose-dependent manner. Induction of phase II and antioxidant enzymes is known to be essential for protecting cells from oxidative insults (34). Nrf2 is a transcription factor that responds to oxidative stress by activating antioxidant genes, including HO-1 and GCLC. In adipocytes, omega-3 polyunsaturated fatty acids showed antioxidant effects via enhanced, Nrf2-mediated expression of HO-1 (35). Several antioxidants, such as carnosic acid and carnosol, also showed inhibition of adipocyte differentiation through ARE activation and induction of GCLC in 3T3-L1 cells (36). Moreover, ECT treatment enhanced HO-1 expression in 3T3-L1 adipocytes in a previous study (37). These results confirm that ECT induces antioxidant activity by increasing HO-1 and GCLC expression and decreasing ROS production during adipogenesis.

Table 1. Effects of esculetin (ECT) on antioxidant enzyme activities (GR, GPx, and CAT) in 3T3-L1 cells

| Treatment | GR  | GPx  | CAT  |
|-----------|-----|------|------|
| Control   | 4.18±0.31 | 8.87±0.75 | 5.37±0.38 |
| ECT 12.5 µM | 3.59±0.16 | 8.42±1.15 | 4.09±1.10 |
| ECT 25 µM | 3.59±0.07 | 11.70±0.73 | 4.27±0.16 |
| ECT 50 µM | 3.31±0.15 | 12.20±1.44 | 4.17±0.18 |
| ECT 100 µM | 3.03±0.17 | 15.11±1.76 | 2.32±0.26 |

Antioxidant enzyme activities of glutathione reductase (GR, µmol/min/mg protein), glutathione peroxidase (GPx, nmol/min/mg protein), and catalase (CAT, mmol/min/mg protein) were measured in 3T3-L1 cells on day 6. Values are mean±SD (n=3). Different letters (a-c) in the same column indicate significant differences by Duncan’s multiple range test at P<0.05.
In conclusion, these results show that treatment with ECT strongly inhibits adipogenesis, increases GSH levels, increases expression of antioxidant enzymes including HO-1 and GCLC, and decreases ROS production in 3T3-L1 cells during adipocyte differentiation. The molecular signaling mechanisms involved in cellular protection against oxidative stress during adipocyte differentiation are currently being investigated in our laboratory. This antioxidant defense of ECT may have therapeutic value for clinical conditions associated with systemic oxidative stress.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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