Callophyllis japonica extract improves high-fat diet-induced obesity and inhibits adipogenesis in 3T3-L1 cells

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The anti-obesity potential of an ethanolic extract of the edible red alga Callophyllis japonica extract (CJE) was investigated in mice fed a high-fat diet (HFD). CJE administration into HFD mice revealed suppression of body weight, adipose tissue weight, serum total cholesterol, triglyceride, and glucose levels in a dose-dependent manner. Also, it reduced serum levels of glutamic pyruvic transaminase, glutamic oxaloacetic transaminase, and lactate dehydrogenase, as well as the accumulation of fatty droplets in liver tissue. CJE and its ethyl acetate fraction inhibited adipogenesis in 3T3-L1 adipocytes by down-regulating the adipocyte-specific transcriptional regulators. Taken together, these results suggest that CJE reduces obesity in mice fed an HFD by inhibiting lipid accumulation and adipogenesis in the adipose tissues.

Keywords: Callophyllis japonica; high-fat diet-induced obesity; anti-obesity; 3T3-L1 cells

Introduction
As a major risk factor for many chronic diseases, including type 2 diabetes, hypertension, and atherosclerosis, obesity is a major obstacle to efforts aimed at improving human health and quality of life (Kopelman 2000). Obesity is characterized by excessive fat deposition associated with morphological and functional changes in adipocytes (Fruhbeck et al. 2001). Lipid accumulation is caused not only by adipose tissue hypertrophy, but also by adipose tissue hyperplasia (Spiegelman and Flier 1996). Although the molecular basis for these associations remains unclear, experimental evidence suggests that some metabolic disorders might be treatable or preventable through the inhibition of adipogenesis and the modulation of adipocyte function (Trayhurn and Beattie 2001; Lee et al. 2010).

Adipogenesis involves morphological changes, growth arrest, and clonal expansion of adipose cells, followed by a complex sequence of changes in gene expression and lipid storage (Gregoire 2001). The master adipogenic transcriptional regulators are members of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors and peroxisome proliferator-activated receptor γ (PPARγ). These factors regulate adipocyte differentiation by modulating the expression of their target genes in a coordinated fashion (Rangwala and Lazar 2000; Rosen and Spiegelman 2000; Rosen et al. 2000; MacDougald and Lane 2005). They act synergistically to induce the expression of C/EBPz and PPARγ (Darlington et al. 1998; Farmer 2006). C/EBPz and PPARγ in turn promote terminal differentiation by activating the transcription of the genes encoding the fatty acid-binding protein aP2 and

the fatty acid transporter CD36, which are involved in creating and maintaining the adipocyte phenotype. Loss-of-function studies have shown that PPARγ is necessary and sufficient to promote adipogenesis (Barak et al. 1999; Koutnikova et al. 2003), and that C/EBPz is influential in maintaining the expression of PPARγ (Wu et al. 1999).

Currently available drugs for the treatment of obesity cause undesirable side effects. Therefore, there is a strong demand for safe but therapeutically potent anti-obesity drugs. As a result, there has been increasing interest in identifying beneficial plants with anti-obesity properties and/or their direct effects on adipose tissue (Rayalam et al. 2008; Hwang et al. 2009; Kang et al. 2010). Since ancient times, people living in fishing areas have believed that the ingestion of edible algae could be beneficial to human health. The edible red alga Callophyllis japonica is consumed as a traditional food and used as a garnish in fishing areas of Northeast Asia. C. japonica extract (CJE) exhibits antioxidant properties, such as intracellular reactive oxygen species and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, lipid peroxidation inhibitory activity (Kang et al. 2005). Furthermore, hexane and ethyl acetate fractions of CJE reduce the mice’s radiation-induced mortality by protecting the blood progenitor cells from the effects of irradiation (Kim et al. 2008). However, other potentially beneficial properties of C. japonica have not been studied. In the present study, we investigated the anti-obesity potential of CJE in mice fed a high-fat diet (HFD), and the effect of its ethyl acetate fraction (CJE-E) on adipogenesis in murine 3T3-L1 cells.

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Materials and methods

Reagents
Dulbecco’s modified Eagle’s medium (DMEM), bovine calf serum (BCS), fetal bovine serum (FBS), and penicillin–streptomycin (PS) were obtained from Gibco (Grand Island, NY, USA). Phosphate-buffered saline (PBS; pH 7.4), 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, and insulin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies to PPARγ, aP2, C/EBPα, C/EBPβ, and phospho-Thr204-extracellular signal-regulated kinase 1 and 2 (ERK1/2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An antibody to ERK1/2 was obtained from Cell Signaling Technology (Beverly, MA, USA). All other reagents were purchased from Sigma Chemical Co. unless otherwise stated.

Preparation of CJE and its fractions
C. japonica was collected from Jeju Island, South Korea. Dried C. japonica powder was extracted with 80% ethanol (8 L) at room temperature for 48 h. The CJE was then concentrated using a rotary evaporator under reduced pressure and freeze-dried to a powder. The crude CJE was successively extracted with n-hexane, ethyl acetate, and n-butanol, yielding n-hexane (CJE-H), ethyl acetate (CJE-E), n-butanol (CJE-B) fractions, and water-soluble fraction (CJE-W). The fractions were concentrated using a rotary evaporator under reduced pressure and freeze-dried into powders.

Animals
The animal study protocol was approved by the Institutional Animal Care and Use Committee of Jeju National University. After purchase, 50 male, 4-week-old C57BL/6 mice (Nara Biotech Co., Ltd., Seoul, Korea) were allowed 1 week to adapt to the new environment in 8-wk boxes and were given free access to drinking water and food. After adaptation, the C57BL/6 mice were randomly divided into five groups (10 mice per group). One group (normal diet, ND) was fed a diet in which fat provided 10% of the calories (D12450B, Research Diets, New Brunswick, NJ, USA; protein content, 19.2%; carbohydrate, 67.3%; fat, 4.3%; other constituents, 3.85 kcal/g). The other four groups (HFD; HFD + CJE1; HFD + CJE2; HFD + CJE4) were fed a diet in which fat provided 60% of the calories (D12492, Research Diets; protein content, 26.2%; carbohydrate, 26.3%; fat, 34.9%; other constituents, 5.24 kcal/g). CJE was dissolved in 0.1% carboxymethyl cellulose (CMC) and administrated orally to the animals at a dosage of 100 (HFD + CJE1), 200 (HFD + CJE2), and 400 (HFD + CJE4) mg/kg/day for 70 days. The volume administered was approximately 100 μL per 10 g body weight. Mice in the ND and HFD groups were given 0.1% CMC.

Measurement of body weight, food intake, epididymal adipose tissue weight, and perirenal adipose tissue weight
Body weight and food intake were measured once every 5 days for 70 days. At the end of the feeding period, the mice were anesthetized with diethyl ether after an overnight fast. The epididymal adipose tissue and perirenal adipose tissue were weighed.

Biochemical analysis
After 70 days, the mice were sacrificed by ether overdose. Blood was drawn from the abdominal aorta into a vacuum tube and allowed to stand at room temperature for 30 min to allow clotting. Serum samples were then collected by centrifugation at 1000 × g for 15 min. Total cholesterol (T-CHO), triglyceride (TG), glucose (GLU), glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), and lactate dehydrogenase (LDH) concentrations in sera were assayed using a commercially available kit (ASANPHARM, Seoul, Korea) and an automatic blood analyzer (Kuadro, BPC BioSed, Rome, Italy).

Histology
After blood was drained from the livers, the livers and epididymal adipose tissue were fixed through incubation in 10% neutral formalin solution for 48 h. The tissues were subsequently dehydrated in a graded ethanol series (75–100%) and embedded in paraffin wax. The embedded tissue was sectioned to a thickness of 8 μm, stained with hematoxylin and eosin (H&E), and examined using an Olympus BX51 light microscope (Olympus Optical, Tokyo, Japan).

Cell culture and differentiation
3T3-L1 preadipocytes obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured in DMEM containing 1% PS and 10% BCS at 37°C under 5% CO2 atmosphere. At day 0 (2 days after they reached confluence), preadipocytes were cultured in MDI differentiation medium (DMEM containing 1% PS, 10% FBS, 0.5 mM IBMX, 1 μM dexamethasone, and 5 μg/mL insulin) for 2 days. The cells were then cultured for a further 2 days in DMEM containing 1% PS, 10% FBS, and 5 μg/mL insulin. Thereafter,
the cells were maintained in post-differentiation medium (DMEM containing 1% PS and 10% FBS), which was replaced with a fresh medium every 2 days. To examine the effects of CJE and its fractions on the differentiation of preadipocytes to adipocytes, cells were cultured with MDI in the presence of CJE or its fractions. Differentiation, as measured by the expression of adipogenic markers, was complete on day 6, and the formation of lipid droplets was complete on day 8.

**Oil Red O Staining**

After the induction of differentiation, cells were stained with Oil Red O (six parts saturated Oil Red O dye [0.6%] in isopropanol plus four parts water). Briefly, the cells were washed twice with PBS, fixed through incubation with 3.7% formaldehyde (Sigma Chemical Co.) in PBS for 1 h, washed a further three times with water, dried, and stained with Oil Red O for 1 h. Excess stain was removed by washing with water, and the stained cells were dried.

**Western blot analysis**

Cells were washed with ice-cold PBS, collected, and centrifuged. The cell pellets were resuspended in lysis buffer (1 × RIPA [Upstate Biotechnology, Temecula, CA, USA], 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 1 mM NaF, 1 μg/mL aprotinin, 1 μg/mL peptatin, and 1 μg/mL leupeptin) and incubated on ice for 1 h. After the cell debris was removed by centrifugation, lysate protein concentrations were determined using Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA, USA). The cell lysates were then subjected to electrophoresis on 10% polyacrylamide gels containing sodium dodecyl sulfate (SDS) and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 0.1% Tween 20 in Tris-buffered saline containing 5% nonfat dry milk or 5% bovine serum albumin (BSA) at room temperature for 1 h. After incubation overnight at 4°C with primary antibody, the membranes were incubated with horseradish peroxygenase-conjugated secondary antibody at room temperature for 1 h. Immunodetection was carried out using ECL Western blotting detection reagent (Amersham Biosciences, Piscataway, NJ, USA).

**Statistical analysis**

Values are expressed as the mean ± standard deviation (SD) or standard error (SE). One-way analysis of variance (ANOVA) was used for multiple comparisons. Treatment effects were analyzed by paired t-test or
Duncan's multiple range test using SPSS software (ver. 12.0; SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant at \( P < 0.05 \).

Table 1. Effects of *Callophyllis japonica*'s extract (CJE) on body weight gain, and food intake in high-fat diet-induced obese mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>ND</th>
<th>HFD</th>
<th>HFD + CJE1</th>
<th>HFD + CJE2</th>
<th>HFD + CJE4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>22.6±0.5</td>
<td>22.6±0.4</td>
<td>22.7±0.4</td>
<td>22.8±0.3</td>
<td>22.5±0.3</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>28.3±1.0( ^a )</td>
<td>38.2±1.1( ^b )</td>
<td>37.0±1.3( ^{bc} )</td>
<td>35.9±1.3( ^{bc} )</td>
<td>34.4±1.1( ^b )</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>5.7±0.6( ^a )</td>
<td>15.6±0.8( ^b )</td>
<td>14.4±1.0( ^{bc} )</td>
<td>13.1±1.1( ^{bc} )</td>
<td>11.9±1.0( ^b )</td>
</tr>
<tr>
<td>Intake of CJE (mg/kg of body weight/day)</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>200</td>
<td>400</td>
</tr>
<tr>
<td>Food intake (g/cage/5 days)</td>
<td>26.6±0.4( ^a )</td>
<td>21.2±0.3( ^b )</td>
<td>21.4±0.4( ^b )</td>
<td>21.7±0.4( ^b )</td>
<td>21.2±0.3( ^b )</td>
</tr>
</tbody>
</table>

Note: Values are expressed as means ± SE (n = 10). Values with different superscript letters indicate statistically significant differences (\( P < 0.05 \)).

Results

**CJE improves HFD-induced obesity**

After 70 days on the HFD, mean body weight and body weight gain in the HFD group were higher than those in the ND group, indicating that the HFD induced obesity (Figure 1, Table 1). Administration of CJE reduced body weight and body weight gain in a dose-dependent manner. Food intake did not differ significantly among the HFD groups, although there was the difference in food intake between ND and HFD groups. Notably, the HFD + CJE4 group (treated with CJE at a dose of 400 mg/kg/day) showed significantly reduced body weight gain compared to the non-CJE-treated control HFD group (23.7% lower).

Histological analysis of epididymal adipose tissue confirmed that adipocyte size was markedly increased in the HFD group compared to the ND group. CJE reduced adipocyte size in a dose-dependent manner compared to the HFD group (Figure 2). Epididymal and perirenal adipose tissue weights were also significantly higher in the HFD group than in the ND group. Epididymal and perirenal adipose tissue weights were significantly lower in the HFD + CJE4 group (23.8 and 23.6%, respectively) than in the HFD group (Table 2). Moreover, the serum levels of T-CHO, TG, and GLU were significantly lower in the HFD + CJE4 group (12.8 and 32.8%, respectively) than in the HFD group (Table 2).

**CJE reduces signs of liver pathology**

We next examined the effects of CJE on the serum levels of GPT, GOT, and LDH in HFD mice. Administration of CJE significantly reduced the levels of these markers in a dose-dependent manner. Serum levels of GPT and GOT were significantly lower in the HFD + CJE4 group (50.3 and 20.0%, respectively) than in the HFD group (Figure 3A and B). Serum LDH levels were significantly lower in the HFD + CJE4 group (53.1%) than in the HFD group (Figure 3C). Figure 3D presents photomicrographs of liver tissue samples stained with H&E. H&E analysis of the liver revealed greater accumulation of fat in the HFD group than in the ND group. No fat accumulation was observed in the livers of mice in the HFD + CJE4 group.

**CJE and its organic solvent fractions inhibit lipid accumulation in 3T3-L1 cells**

Next, we tested whether CJE and its organic fractions (CJE-H, CJE-E, CJE-B, and CJE-W) inhibit
MDI-induced differentiation of 3T3-L1 preadipocytes.
On day 0, CJE and its fractions were added to the MDI differentiation medium; on day 8, the adipocytes were stained using Oil Red O. CJE, CJE-H, and CJE-E inhibited 3T3-L1 adipocyte differentiation in a dose-dependent manner (Figure 4B,C). Among the organic solvent fractions assayed, CJE-E most effectively inhibited 3T3-L1 adipocyte differentiation in a dose-dependent manner (Figure 4C). Importantly, at their maximal treatment concentrations, CJE (200 μg/ml) and its fractions (100 μg/ml) did not affect the viability of 3T3-L1 preadipocytes, as assessed by MTT assay (data not shown).

CJE-E inhibits 3T3-L1 adipocyte differentiation by modulating the expression of key transcriptional regulators

To determine whether CJE-E inhibits adipocyte differentiation by down-regulating key transcriptional regulators, we examined the expression of C/EBPα, C/EBPβ, and PPARγ during adipocyte differentiation in the presence and absence of CJE-E. Levels of

<table>
<thead>
<tr>
<th>Group</th>
<th>ND</th>
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<th>HFD + CJE1</th>
<th>HFD + CJE2</th>
<th>HFD + CJE4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epididymal adipose tissue (g)</td>
<td>0.86 ± 0.05a</td>
<td>2.02 ± 0.12b</td>
<td>1.86 ± 0.11c</td>
<td>1.72 ± 0.12bc</td>
<td>1.54 ± 0.10b</td>
</tr>
<tr>
<td>Perirenal adipose tissue (g)</td>
<td>0.50 ± 0.06a</td>
<td>1.23 ± 0.07bc</td>
<td>1.15 ± 0.08bc</td>
<td>0.98 ± 0.10b</td>
<td>0.94 ± 0.07b</td>
</tr>
<tr>
<td>T-CHO (mg/dL)</td>
<td>119.71 ± 4.09a</td>
<td>179.14 ± 3.90c</td>
<td>171.43 ± 3.91bc</td>
<td>162.86 ± 7.77bc</td>
<td>156.14 ± 5.03b</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>92.29 ± 4.86a</td>
<td>138.43 ± 9.15b</td>
<td>95.57 ± 3.07a</td>
<td>94.43 ± 6.31a</td>
<td>93.00 ± 4.13a</td>
</tr>
<tr>
<td>GLU (mg/dL)</td>
<td>219.80 ± 6.07a</td>
<td>250.60 ± 7.07bc</td>
<td>243.10 ± 6.00bc</td>
<td>235.70 ± 4.50bc</td>
<td>225.60 ± 3.82abc</td>
</tr>
</tbody>
</table>

Note: Values are expressed as means ± SE (n = 10). Values with different superscript letters indicate statistically significant differences (P < 0.05).
expression of all three of these transcription factors were reduced in CJE-E-treated cells, as was that of aP2 (Figure 5A and B). In contrast, CJE-E did not affect the level of phosphorylation of ERK1/2 (Figure 5C).

Discussion

Adipose tissue is a dynamic tissue that plays an important role in energy balance and changes in mass according to the metabolic requirements of the organism (Harp 2004). We examined the effects of CJE on HFD-induced fat accumulation in the adipose tissue of C57BL/6 mice, because HFDs are commonly used to induce visceral obesity in rodent models (Hansen et al. 1997). Body weight gain, adipose tissue weight, and serum levels of T-CHO, TG, and GLU were significantly lower in CJE-treated mice than in the HFD group, although there was no difference in food intake. Histological analysis revealed a greater number of large cells in the epididymal adipose tissue of the HFD group, a typical sign of obese adipose tissue. However, the epididymal adipose tissue of the HFD + CJE groups exhibited a small number of large cells and fewer pathological signs. These results indicate that CJE may have anti-obesity activity in vivo, but that it does not affect food intake.

Figure 4. *C. japonica* extract (CJE) and its ethyl acetate fraction (CJE-E) inhibit lipid accumulation in 3T3-L1 cells. Cells were cultured in MDI differentiation medium in the presence or absence of CJE or its fractions. (A) Flow diagram of the fractionation of the crude CJE. Differentiated adipocytes were stained with Oil Red O on day 8. (B) Cells treated with CJE for 2 days. (C) Cells treated with *n*-hexane (CJE-H), ethyl acetate (CJE-E), *n*-butanol (CJE-B), or water-soluble (CJE-W) fraction for 2 days (Con, concentration).

Figure 5. *C. japonica* ethyl acetate fraction (CJE-E) inhibit the differentiation of 3T3-L1 preadipocytes. Cells were cultured in MDI differentiation medium in the presence or absence of CJE or CJE-E. (A) Western blot analysis of the PPARγ, C/EBPα, and aP2. Proteins were prepared from 3T3-L1 cells on day 6. Western blot analysis of (B) C/EBPβ and (C) phospho-ERK in postconfluent differentiating 3T3-L1 cells. Proteins were harvested at the indicated times.
We also analyzed the effects of CJE on the development of fatty liver, which is strongly associated with obesity (James and Day 1999). Histological analysis showed the accumulation of numerous fatty droplets in the livers of mice in the HFD group, a typical sign of fatty liver. However, the livers of the HFD + CJE groups exhibited a much lower degree of lipid accumulation and fewer pathological signs. Moreover, liver weight was significantly lower in the HFD + CJE groups than in the HFD group. Serum GPT, GOT, and LDH levels are clinically and toxicologically important indicators, and increase as a result of tissue damage caused by toxicants or disease conditions. Levels of liver function markers, including serum GPT and GOT, and LDH were significantly higher in the HFD group than in the ND group, and were reduced by CJE treatment. These results indicate that administration of CJE can dramatically suppress the development of HFD-induced fatty liver.

The regulation of adipogenesis involves a number of complex, interconnected cell signaling pathways. Thus various natural products, such as edible seaweed extracts, may potentially be used in therapies designed to protect against metabolic disorder. In this study, we found that CJE and CJE-E dose-dependently reduced lipid accumulation in differentiating 3T3-L1 preadipocytes. At the molecular level, CJE-E dose-dependently reduced levels of PPARγ, C/EBPβ, αP2 in differentiating 3T3-L1 preadipocytes. Down-regulation of PPARγ and C/EBPβ by CJE-E suggests the existence of a CJE-E target molecule upstream of PPARγ and C/EBPβ. To elucidate the signaling pathway by which CJE-E modulates adipocyte differentiation, we examined the possible involvement of ERK1/2, which have been linked to adipocyte differentiation (Hung et al. 2005; Kim et al. 2007). CJE-E treatment did not affect ERK phosphorylation. C/EBPβ plays a critical role in adipocyte differentiation (Tang et al. 2003). It is expressed early in the differentiation program and drives the subsequent expression of C/EBPα (Christy et al. 1991). Our data show that CJE-E inhibited C/EBPβ expression in 3T3-L1 cells. Given the importance of C/EBPβ during adipocyte differentiation, the inhibition of C/EBPβ expression by CJE-E may be sufficient to block terminal differentiation. Thus, our results suggest that the inhibition of adipocyte differentiation by CJE-E correlates with the inhibition of C/EBPβ expression. However, the bioactive compounds responsible for the anti-obesity effects of CJE were not identified. Thus, further research is necessary to clarify the active compounds responsible for the anti-obesity effects of CJE-E.

In conclusion, CJE reduced body weight gain, adipose tissue weight, adipose tissue cell size, and liver accumulation of fatty droplets, as well as serum T-CHO and TG levels, in obese (HFD-fed) mice. Moreover, CJE-E inhibited adipogenesis in 3T3-L1 adipocytes by down-regulating the adipocyte-specific transcriptional regulators C/EBPα, C/EBPβ, and PPARγ. These results suggest that C. japonica may potentially be used to prevent and treat obesity.

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References


