Immature Citrus sunki Peel Extract Exhibits Antiobesity Effects by β-Oxidation and Lipolysis in High-Fat Diet-Induced Obese Mice

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The peel of Citrus sunki Hort. ex Tanaka has been widely used in traditional Asian medicine for the treatment of many diseases, including indigestion and bronchial asthma. In this study, we investigated the antiobesity activity of immature C. sunki peel extract (designated CSE) using high-fat diet (HFD)-induced obese C57BL/6 mice and mature 3T3-L1 adipocytes. In the animal study, body weight gain, adipose tissue weight, serum total cholesterol, and triglyceride in the CSE-administered group decreased significantly compared to the HFD group. Also, CSE supplementation reduced serum levels of glutamic pyruvic transaminase, glutamic oxaloacetic transaminase, and lactate dehydrogenase. Moreover, it significantly decreased the accumulation of fatty droplets in liver tissue, suggesting a protective effect against HFD-induced hepatic steatosis. Dietary supplementation with CSE reversed the HFD-induced decrease in the phosphorylation levels of AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC), which are related to fatty acid β-oxidation, in the epididymal adipose tissue. Also, CSE increased AMPK and ACC phosphorylation in mature 3T3-L1 adipocytes. CSE also enhanced lipolysis by phosphorylation of cAMP-dependent protein kinase (PKA) and hormone-sensitive lipase (HSL) in mature 3T3-L1 adipocytes. These results suggest that CSE had an antiobesity effect via elevated β-oxidation and lipolysis in adipose tissue.

Key words Citrus sunki; high-fat diet; antiobesity; 3T3-L1 adipocyte; β-oxidation; lipolysis

Obesity is a major public health problem in both industrialized and developing countries. The condition is characterized by an excess of adipose tissue, which increases one’s risk of developing insulin resistance and metabolic syndromes. Metabolic deregulation may lead to such complications as diabetes mellitus, coronary heart disease, and hypertension.1) At the cellular level, obesity is caused by an increase in the number and size of adipocytes derived from fibroblastic preadipocytes in adipose tissue.2) Adipose tissue is vital for maintaining metabolic homeostasis, and as an endocrine organ, it secretes various adipokines. Studies on adipose tissue biology have improved our understanding of the mechanisms linking metabolic disorders with altered adipocyte function.3) Experimental evidence suggests that some metabolic disorders may be treatable or preventable through the inhibition of adipogenesis and modulation of adipocyte function.4,5) High-fat feeding has commonly been used to induce visceral obesity in rodent animal models6) because the pathogenesis of obesity is similar to that in humans.7) Also, 3T3-L1 preadipocytes are frequently used to study the function of adipocytes in vitro due to their ability to differentiate into mature adipocytes.8) Currently, however, drugs available for the treatment of obesity have undesirable side effects, and thus a high demand exists for a safe but therapeutically potent antiobesity drug, which has increased interest in the search for antiobesity phytonutrients that effectively reduce visceral fat mass. Citrus fruit peel has been used in Asian medicine for centuries to treat indigestion and to improve bronchial and asthmatic conditions. Citrus flavonoids are generally categorized into two groups, flavones glycosides (e.g., naringin, hesperidin, nephespidin) and polymethoxylated flavones (PMFs, e.g., nobiletin, sinensetin, tangeretin). These flavonoids have various biological activities, such as anti-atherogenic effects, anti-inflammatory effects, and anticancer activity.8,9) The peel of Citrus sunki Hort. ex Tanaka is a rich source of flavonones, as well as many PMFs, which are very rare in other plants.10,11) It has been reported that Citrus phytochemicals, such as synephrine and auraptene, exhibited the anti-obesity properties by enhancing the lipolysis in vivo and in vitro.12,13) However, the effects of immature fruit peel extract on lipid metabolism have not been fully evaluated. In this study, we investigated the antiobesity potential of immature C. sunki peel extracts in murine 3T3-L1 adipocytes and mice fed a high-fat diet (HFD) by focusing on signaling pathway of lipolysis and fatty acid β-oxidation.

MATERIALS AND METHODS

Reagents Dulbecco’s modified Eagle’s medium (DMEM), bovine calf serum (BCS), fetal bovine serum (FBS), and penicillin–streptomycin (PS) were purchased from Gibco-BRL (Grand Island, NY, U.S.A.). Phosphate-buffered saline (PBS; pH 7.4), 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, insulin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), as well as the standards for flavonoid analysis, were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The lactate dehydrogenase (LDH) Cytotoxicity Detection Kit was purchased from Takara Shuzo Co. (Otsu, Shiga, Japan). An antibody to phosphor-Ser431-LKB1 was acquired from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), and antibodies to adenosine AMP-activated protein kinase (AMPK) α, phospho-Thr172-AMPKα (p-AMPK), acetyl-CoA carboxylase (ACC), phospho-Ser79-ACC (p-ACC), phospho-Ser660-hormone-sensitive lipase (p-HSL), and phospho-Ser/Thr-cAMP-dependent protein kinase (p-PKA) substrate were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). Sinensetin, nobiletin, and tangeretin were purchased from Wako Pure Chemical Industries, Ltd.

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Preparation of Immature Citrus sunki Peel Extract (CSE) Immature C. sunki peel was collected from Jeju Island, Republic of Korea. One kilogram of dried peel powder was extracted with 80% ethanol (10L) twice at room temperature for 48h. The combined CSE was concentrated on a rotary evaporator under reduced pressure, freeze-dried to a powder, and stored at −20°C until use. To determine the flavonoid content of CSE, HPLC analysis was performed using a Waters 2695 Alliance HPLC system (Waters Corp., Milford, MA, U.S.A.) consisting of two pumps, an autosampler, a column oven, and a PDA detector (model 2998 photodiode array detector; Waters Corp.). The flavonoid compounds were monitored at 320 nm using a Sunfire® C18 Column (250×4.6 mm, i.d.; 5 μm; Waters Corp.). The column was operated at 40°C, with a sample injection volume of 10 μL and a flow rate of 1 mL/min. The mobile phase consisted of acetonitrile (MeCN) containing 0.5% acetic acid (A) and water containing 0.5% acetic acid (B). The gradient elution program was as follows: a 25 min gradient was started using 20% A, held for 10 min, linearly increased to 45% A over 12 min, held for 5 min, linearly increased to 75% A over 19 min, and held for 2 min, then finally returned to the initial conditions and held for 3 min. It was confirmed that CSE contained abundant polymethoxylated flavones (PMFs) such as tangeretin and nobiletin (Table 1).

Animals The animal study protocol was approved by the Institutional Animal Care and Use Committee of Jeju National University. After purchase, 30 male 4-week-old C57BL/6 mice (Nara Biotech Co., Ltd., Seoul, Republic of Korea) were adapted for 1 week to a specific temperature (22±2°C), humidity (50±5%), and lighting (light from 08:00 to 20:00). The animals were housed in plastic cages (two/cage) and given free access to drinking water and food. After adaptation, the C57BL/6 mice (now 5 weeks old; 22.6±1.2 g) were randomly divided into three groups of 10 mice each. One group (normal diet, ND) was fed a 10% kcal fat diet (D12450B; Research Diets, New Brunswick, NJ, U.S.A.; protein: 19.2%, carbohydrate: 67.3%, fat: 4.3%; and other; 3.85 kcal/g), and two groups (high-fat diet, HFD; HFD+CSE) were fed a 60% kcal fat diet (D12492; Research Diets; protein: 26.2%, carbohydrate: 26.3%, fat: 34.9%; and other: 5.24 kcal/g). CSE was dissolved in 0.1% carboxymethyl cellulose (CMC) and administrated orally to the animals at a dosage of 150 mg/kg/d for 70d. The oral administration volume was approximately 100 μL per 10g weight. Mice in the ND and the HFD groups were given 0.1% CMC.

Measurements of Body Weight, Food Intake, Liver Weight, Epididymal Adipose Tissue Weight, and Perirenal Adipose Tissue Weight Body weight and food intake were measured every 5d for 70d. At the end of the feeding period, mice were anesthetized with diethyl ether after an overnight fast. The liver, epididymal adipose tissue, and perirenal adipose tissue were weighed after rapid removal from dead mice.

Biochemical Analysis After 70d, the mice were killed by ether anesthesia overdose. Blood samples were drawn from the abdominal aorta into a vacuum tube and allowed to clot at room temperature for 30min. Serum samples were then collected by centrifugation at 1000×g for 15min. Total cholesterol (T-CHO), triglyceride (TG), glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), and lactate dehydrogenase (LDH) concentrations in serum were assayed using a commercial kit (Asanpharm, Seoul, Republic of Korea) and an automatic blood analyzer (Kuadro; BPC Biossed, Rome, Italy).

Histology After blood had been drained, the livers and epididymal adipose tissue were fixed in 10% neutral formalin solution for 48h. The tissue was subsequently dehydrated in a graded ethanol series (75—100%) and embedded in paraffin wax. The embedded tissue was sectioned (8-μm-thick sections), stained with hematoxylin and cosin (H&E), and examined by light microscopy (Olympus BX51; Olympus Optical, Tokyo, Japan), then photographed at final magnifications of 50×, 100×, or 200×.

Cell Culture and Differentiation 3T3-L1 Preadipocytes obtained from the American Type Culture Collection (ATTC, Rockville, MD, U.S.A.) were cultured in DMEM containing 1% PS and 10% BCS at 37°C under a 5% CO2 atmophere. To induce differentiation, 2d post-confluence preadipocytes (designated day 0) 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 2d. Cells were then cultured for another 2d in DMEM containing 1% PS, 10% FBS, and 5 μg/mL insulin. Thereafter, cells were maintained in post-differentiation medium (DMEM containing 1% PS and 10% FBS), with replacement of the medium every 2d.

The effect of CSE on cell viability and cytotoxicity was determined using MTT and LDH assays. Mature 3T3-L1 adipocytes were cultured in DMEM containing 1% PS, 10% FBS, and CSE for 24h. MTT (40 μg/mL) was added to each well, and plates incubated at 37°C for 4h. The liquid in the plate was removed, and dimethyl sulfoxide (DMSO) was added to dissolve MTT—formazan complexes. Optical density was measured at 540 nm. The effect of CSE on cell viability was evaluated by comparing the relative absorbance with that of control cultures. The cytotoxic effect of CSE was measured using the LDH Cytotoxicity Detection Kit. The LDH activities in medium and cell lysate were measured to evaluate cytotoxicity, according to the manufacturer’s protocol (LDH released into the medium/maximal LDH release ×100).

Western Blot Analysis Adipose tissue was homogenized in ice-cold buffer containing lysis buffer [1×RIPA (Upstate Biotechnology, Temecula, CA, U.S.A.), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na3VO4, 1 mM NaF, and 1 μg/mL

Table 1. Flavonoid Content (mg/g) in CSE

<table>
<thead>
<tr>
<th>Group</th>
<th>CSE</th>
<th>R.S.D</th>
</tr>
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<tbody>
<tr>
<td>Rutin</td>
<td>17.02</td>
<td>0.09</td>
</tr>
<tr>
<td>Naringin</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Hesperidin</td>
<td>17.11</td>
<td>0.08</td>
</tr>
<tr>
<td>Quercetin</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Naringenin</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Hesperitin</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Sinensetin</td>
<td>4.23</td>
<td>0.09</td>
</tr>
<tr>
<td>Nobiletin</td>
<td>38.83</td>
<td>0.89</td>
</tr>
<tr>
<td>Tangeretin</td>
<td>55.13</td>
<td>1.30</td>
</tr>
</tbody>
</table>

Numerical values are the means of three experiments. R.S.D., relative standard deviation (%); ND, not detected.
each of aprotinin, pepstatin, and leupeptin. 3T3-L1 Cells were washed with ice-cold PBS, collected, and centrifuged. The cell pellets were resuspended in lysis buffer and incubated on ice for 1 h. The adipose tissue and 3T3-L1 cells debris were then removed by centrifugation and protein concentrations in the lysates were determined using the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The adipose tissue and 3T3-L1 cell lysates were then subjected to electrophoresis on 10% polyacrylamide gels containing sodium dodecyl sulfate (SDS) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with a solution of 0.1% Tween 20 in Tris-buffered saline containing 5% bovine serum albumin (BSA) at room temperature for 1 h. After incubation overnight at 4°C with the primary antibody, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Immunodetection was carried out using the ECL Western blotting detection reagent (Amershams Biosciences, Piscataway, NJ, U.S.A.).

RNA Preparation and Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction (Real-Time RT-PCR) Analyses Total RNA was extracted from adipose tissue and 3T3-L1 adipocytes using the TRizol reagent according to the manufacturer’s instructions, and then treated with DNase (Wako Pure Chemical Industries, Ltd., Osaka, Japan). cDNA was synthesized from 1µg of total RNA in a 20µL reaction using a Maxime RT PreMix Kit (iNtRON Biotechnology, Seongnam, Kyunggi, Republic of Korea). The following primers were used in real-time RT-PCR analyses: adiponectin, 5'-GAC CTG GCC ACT TTC TCC TC-3' and 5'-GTC ATC TTC GGC ATG ACT GG-3'; carnitine palmitoyltransferase-1a (CPT-1a), 5'-ACC CTG AGG CAT CTA TTC TCC TC-3' and 5'-GTC ATC TTC GGC ATG ACT GG-3'; β-actin, 5'-AGG CTG TGC TGT CCC TGT AT-3' and 5'-ACC CAA GAA GGA AGG CTG GA-3'. Samples were prepared using iQ SYBR Green Supermix (Bio-Rad Laboratories) according to the manufacturer’s instructions. Adiponectin, CPT-1a, and β-actin mRNA expression was measured by quantitative real-time RT-PCR using the Chromo4 Real-Time PCR System (Bio-Rad Laboratories). The formation of a single product was verified by melting curve analysis. The expression levels of adiponectin and CPT-1a were normalized to that of β-actin. Data were analyzed using Opticon Monitor software (ver. 3.1; Bio-Rad Laboratories).

Lipolysis Assay To obtain fully differentiated 3T3-L1 cells, confluent cells were induced in MDI differentiation medium for 2d. The cells were then cultured for a further 2 d in DMEM containing 1% PS, 10% FBS, and 5µg/mL insulin. Then, they were maintained in post-differentiation medium, which was replaced every 2 d. Fully differentiated 3T3-L1 cells were next incubated for 16 h with serum-free DMEM containing 0.2% BSA. The cells were then treated with post-differentiation medium containing various concentrations of CSE. Culture supernatants were assayed for glycerol levels at 24 h post-treatment using a free glycerol reagent kit (Sigma-Aldrich).

**Statistical Analysis** Values are expressed as means± standard deviations (S.D.) or standard errors (S.E.). A one-way analysis of variance (ANOVA) was used for multiple comparisons. Treatment effects were analyzed using the paired t-test or Duncan’s multiple range test using the SPSS software package (ver. 12.0; SPSS Inc., Chicago, IL, U.S.A.). Differences were considered statistically significant at p<0.05.

**RESULTS**

**CSE Improved High-Fat Diet-Induced Obesity** After 70 d on the HFD, the mean body weight and body weight gain in the HFD group were 35.2% and 176.1% higher, respectively, than those in the ND group, indicating that the HFD induced obesity (Table 2). CSE administration (150 mg/kg/d) significantly decreased both body weight and body weight gain in the HFD+CSE group relative to those in the non-CSE-treated HFD group (16.9, 40.7% lower, respectively). Histological analysis of epididymal adipose tissue confirmed that adipocyte size was markedly elevated in the HFD group compared to the ND group after 70 d, whereas adipocyte size markedly decreased in the HFD+CSE group compared to the HFD group (Fig. 1A). The weights of epididymal and perirenal adipose tissue were also significantly higher in the HFD group (134.2, 146.9%, respectively) than in the ND group. Epididymal and perirenal adipose tissue weights were significantly lower in the HFD+CSE group (35.6, 35.9%, respectively) than in the HFD group (Figs. 1B,C).

Food intake did not significantly differ among the HFD groups. However, serum T-CHO and TG levels were significantly lower in the HFD+CSE group (17.6, 39.3%, respectively) than the HFD group (Table 2).

**CSE Reduced the Signs of Liver Pathology** We next examined the effect of CSE on serum GPT, GOT, and LDH levels in HFD mice. CSE administration significantly reduced the levels of these markers of cell damage. The levels of se-

<table>
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<th>Group</th>
<th>ND</th>
<th>HFD</th>
<th>HFD+CSE</th>
</tr>
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<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>22.61±0.53</td>
<td>22.60±0.42</td>
<td>22.50±0.31</td>
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<tr>
<td>Final body weight (g)</td>
<td>28.26±0.99</td>
<td>38.20±1.12</td>
<td>31.75±1.38</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>5.65±0.60</td>
<td>15.60±0.78</td>
<td>9.25±1.20</td>
</tr>
<tr>
<td>Intake of CSE (mg/kg of body weight/d)</td>
<td>—</td>
<td>—</td>
<td>150</td>
</tr>
<tr>
<td>Food intake (g/cage/5d)</td>
<td>26.57±0.40</td>
<td>21.19±0.28</td>
<td>20.81±0.23</td>
</tr>
<tr>
<td>T-CHO (mg/dL)</td>
<td>191.71±4.09</td>
<td>179.14±3.90</td>
<td>147.57±5.53</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>92.29±4.86</td>
<td>138.43±9.15</td>
<td>84.00±2.66</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.07±0.06</td>
<td>1.30±0.08</td>
<td>0.98±0.04</td>
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</table>

Values are expressed as means±S.E. (n=10). Mean separation was performed using Duncan’s multiple range test. Different letters indicate significant differences (p<0.05).
Fig. 1. Effect of *Citrus sunki* Peel Extract (CSE) on Fatty Droplets in the Epididymal Adipose Tissue of Mice Fed a Normal Diet (ND), High-Fat Diet (HFD), or HFD+CSE (150 mg/kg/d)

(A) Hematoxylin and eosin-stained photomicrographs of epididymal adipose sections are shown at 50× and 100×. (B) Epididymal and (C) perirenal adipose tissue weight were evaluated. Results are shown as means±S.E. (n=10). Mean separation was performed using Duncan’s multiple range test. Different letters indicate significant differences (p<0.05).

Fig. 2. Effect of *Citrus sunki* Peel Extract (CSE) on Fatty Droplets in the Livers of Mice Fed a Normal Diet (ND), High-Fat Diet (HFD), or HFD+CSE (150 mg/kg/d)

The serum levels of (A) glutamic pyruvic transaminase (GPT), (B) glutamic oxaloacetic transaminase (GOT), and (C) lactate dehydrogenase (LDH) were evaluated. Results are shown as means±S.E. (n=10). Mean separation was performed using Duncan’s multiple range test. Different letters indicate significant differences (p<0.05). (D) Hematoxylin and eosin-stained photomicrographs of liver tissue sections are shown at 100× and 200×.
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rum GPT and GOT were significantly lower in the HFD+CSE group (56.2, 19.2%, respectively) than in the HFD group (Figs. 2A, B). Serum LDH level was also significantly lower in the HFD+CSE group (43.6%) than in the HFD group (Fig. 2C). In addition, liver weight was significantly lower in the HFD+CSE group than in the HFD group (Table 2).

Figure 2D shows photomicrographs of H&E-stained liver tissue. H&E analysis of the liver revealed fatty accumulation in the HFD group compared to the ND group; however, no fatty accumulation was observed in the livers of the HFD+CSE group.

**CSE Restored AMPK Phosphorylation and Adiponectin Expression in Epididymal Adipose Tissue**

Next, we investigated the expression of proteins responsible for fatty acid β-oxidation in epididymal adipose tissue. As shown in Fig. 3A, expression of the phosphorylated forms of AMPK and its immediate substrate (phosphorylated forms of ACC) were higher in the HFD+CSE group than in the HFD group after 70 d. After 70 d on the HFD, expression of the adiponectin gene was lower in the HFD group than in the ND group (Fig. 3B) but was restored in the HFD+CSE group.

**CSE Activated the AMPK Pathway in Mature 3T3-L1 Adipocytes**

The effect of CSE on the viability and cytotoxicity of mature 3T3-L1 adipocytes was first evaluated by MTT and LDH assays. A CSE concentration of 250 μg/mL did not affect viability (111.22 ± 1.51% compared to the control) or cytotoxicity (2.97 ± 2.55% compared to the control) of the mature 3T3-L1 adipocytes (Fig. 4A). To characterize the ef-
fects of CSE on the phosphorylation of LKB1, AMPK, and ACC in vitro, we treated mature 3T3-L1 adipocytes with various concentrations of CSE. Consistent with the in vivo data, CSE markedly induced phosphorylation of LKB1, AMPK, and ACC in a dose-dependent manner (Fig. 4B). After mature 3T3-L1 adipocytes were cultured in post-differentiation medium and then exposed to 200 μg/mL CSE for 2, 6, 12, and 24h, CSE markedly induced phosphorylation of LKB1, AMPK, and ACC beginning 2h after treatment (Fig. 4C). Thus, we investigated the effects downstream of AMPK activation by treating mature 3T3-L1 adipocytes with CSE. CSE increased dose-dependently the levels of CPT-1a mRNA, which is involved in fatty acid oxidation (Fig. 4D).

**CSE Activated the PKA Pathway in Mature 3T3-L1 Adipocytes** We next examined whether CSE stimulated lipolysis in mature 3T3-L1 adipocytes by measuring glycerol levels in culture supernatants. CSE significantly increased lipolysis at 24h in a dose-dependent manner (Fig. 5A). Then, we examined whether the increase in lipolysis caused by CSE was associated with a signaling pathway by evaluating the phosphorylated forms of PKA and its immediate substrate (HSL) in CSE-treated mature 3T3-L1 adipocytes. CSE treatment (200 μg/mL) was found to stimulate the phosphorylation of both PKA substrate and HSL (Fig. 5B).

**DISCUSSION**

Adipose tissue is a dynamic organ that plays an important role in energy balance and changes in mass according to the metabolic requirements of the organism. We examined the effects of CSE on HFD-induced fat accumulation in the adipose tissue of C57BL/6 mice. Body weight gain, adipose tissue weight, and T-CHO and TG serum levels were significantly lowered in CSE-treated mice compared to the HFD group, with no change in food intake. Moreover, 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+CSE group exhibited fewer large cells and fewer pathological signs. Adiponectin is a protein hormone that modulates a number of metabolic processes, including glucose regulation and fatty acid catabolism. Obesity, diabetes, and atherosclerosis have been associated with reduced adiponectin levels. Thus, we examined the effects of CSE on the levels of adiponectin mRNA in adipose tissue of the HFD-induced mice. In the present study, CSE recovered the expression of adiponectin mRNA, which had been lowered in C57BL/6 mice fed the HFD. These results indicate that CSE might have antiobesity activities in vivo, without affecting the amount of food intake.

We also analyzed the effects of CSE on the development of fatty liver, which is strongly associated with obesity. Upon histological analysis, the livers of the HFD group exhibited an accumulation of numerous fatty droplets, a typical sign of fatty liver. However, the livers of the HFD+CSE group exhibited a much smaller degree of lipid accumulation and fewer pathological signs. Moreover, liver weight was significantly lower in the HFD+CSE group than in the HFD group. Serum GPT, GOT, and LDH levels are clinically and toxicologically important indicators, and rise as a result of tissue damage caused by toxicants or disease conditions. In the HFD group, the activities of liver function markers, including serum GPT, GOT, and LDH, were significantly elevated relative to those in the ND group and were improved by CSE supplementation. These results indicate that administration of CSE can dramatically suppress the development of HFD-induced fatty liver.

AMPK is known to play a major role in glucose and lipid metabolism and to control metabolic disorders such as diabetes, obesity, and cancer. To detect other specific molecular targets through which CSE exerted inhibitory effects on obesity, we examined the effects on AMPK signaling in epididymal adipose tissue and mature 3T3-L1 adipocytes. AMPK is known to be a metabolic master switch that is activated by LKB1 under intracellular stress conditions, including glucose deficiency, hypoxia, and reactive oxygen species (ROS) activity. AMPK activation is associated with metabolic organs including the liver, skeletal muscle, pancreas, and adipose tissue; thus, AMPK has been targeted in the development of drugs to treat metabolic diseases. In the present study, CSE recovered the expression of phosphorylated forms of AMPK, which had been reduced in C57BL/6 mice fed the HFD. Furthermore, treatment with CSE induced AMPK phosphorylation by stimulating LKB1 phosphorylation in a dose-dependent manner, and the activation of this kinase led to the phosphorylation of its substrate, ACC, in mature 3T3-L1 adipocytes. These results suggest that CSE influenced metabolic processes related to the AMPK signaling pathway. AMPK activation increases fatty acid oxidation by reducing malonyl-CoA through the inhibition of ACC, and this process upregulates CPT-1a expression. CPT-1a regulates long-chain fatty acid transport across the mitochondrial membrane. CSE enhanced the expression of CPT-1a in CSE-treated mature 3T3-L1 adipocytes.
of CPT-1α mRNA in a dose-dependent manner in mature 3T3-L1 adipocytes. These results suggest that CSE promotes fatty acid β-oxidation by activating AMPK in HFD-induced obese mice and mature 3T3-L1 adipocytes.

The major physiological role of white adipose tissue fat stores is to supply lipid energy when it is needed by other tissues. This is achieved by a highly regulated pathway whereby the triglycerides stored in adipocytes are hydrolyzed, and fatty acids are delivered to the plasma. Lipolysis plays a pivotal role in controlling the quantity of triglycerides stored in fat depots and in determining plasma free fatty acid levels. Hence, modulators of lipolysis may exert beneficial properties if the same molecule or another compound stimulates the oxidation of fatty acids and energy expenditure. The effects of the CSE on lipolysis were examined in mature 3T3-L1 adipocytes. Compared to control adipocytes, medium glycerol concentration was dose-dependently increased by the CSE. Lipolysis is triggered by an increase in the intracellular cAMP level, which in turn activates PKA and HSL. The CSE stimulated the activation of PKA and HSL. These results suggest that CSE promotes lipolysis by activating PKA in mature 3T3-L1 adipocytes.

In this study, we did not determine the active components exerting antiobesity effects. However, we confirmed that CSE contained abundant PMFs as compared to other Citrus species. PMFs decrease plasma cholesterol and TG levels at lower doses than hesperidin and naringenin in hamsters with diet-induced hypercholesterolemia. Supplementation with PMFs and palm tocotrienols decrease plasma TG and cholesterol levels in humans. Recently, Lee et al. reported that PMF-rich Citrus depressa extract has antiobesity effects in HFD-induced obese mice. Thus, PMFs might be major components that mediate the antiobesity effect of CSE.

In conclusion, we showed that administration of CSE to mice with HFD-induced obesity reduced body weight gain, adipose tissue weight, the cell size of adipose tissue, and the accumulation of fatty droplets in the liver. CSE reduces serum total-cholesterol and triglycerides, thereby regulating lipid metabolism. Also, CSE increased β-oxidation in epididymal adipose tissue and mature 3T3-L1 adipocytes by activating the phosphorylation of AMPK and ACC. CSE markedly enhanced lipolysis in mature 3T3-L1 adipocytes by stimulating the phosphorylation of PKA. Taken together, our findings demonstrate that CSE improves HFD-induced obesity through elevated β-oxidation and lipolysis in adipose tissue.

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