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A water-soluble extract of *Petalonia binghamiae* inhibits the expression of adipogenic regulators in 3T3-L1 preadipocytes and reduces adiposity and weight gain in rats fed a high-fat diet☆

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Abstract

We previously showed that an ethanolic extract of the edible brown algae *Petalonia binghamiae* promotes the differentiation of 3T3-L1 preadipocytes and decreases hyperglycemia in streptozotocin-induced diabetic mice. Here, we report that a water-soluble extract of *P. binghamiae* thalli, prepared by enzymatic digestion, inhibits preadipocyte differentiation and adipogenesis in a dose-dependent manner. In differentiating 3T3-L1 preadipocytes, the extract (designated PBEE) decreased the expression of peroxisome proliferator-activated receptor γ, CCAAT/enhancer-binding proteins α and β, and fatty acid-binding protein aP2. It also inhibited the mitotic clonal expansion process of adipocyte differentiation, and it inhibited insulin-stimulated uptake of glucose into mature 3T3-L1 adipocytes by reducing phosphorylation of insulin receptor substrate-1. In rats with high-fat diet (HFD)-induced obesity, PBEE exhibited potent anti-obesity effects. In this animal model, increases in body weight and fat storage were suppressed by the addition of PBEE to the drinking water at 500 mg/L for 30 days. PBEE supplementation reduced serum levels of glutamic pyruvic and glutamic oxaloacetic transaminases and increased the serum level of high-density lipoprotein cholesterol. Moreover, it significantly decreased the accumulation of lipid droplets in liver tissue, suggesting a protective effect against HFD-induced hepatic steatosis. Taken together, these data demonstrate that PBEE inhibits preadipocyte differentiation and adipogenesis in cultured cells and in rodent models of obesity.

Keywords: 3T3-L1 Preadipocyte; *Petalonia binghamiae*; High-fat diet (HFD)-induced obesity; Adipogenesis; Anti-obesity

1. Introduction

As a major risk factor for many chronic diseases, including type 2 diabetes, hypertension and atherosclerosis [1], obesity is a major obstacle to efforts aimed at improving human health and quality of life. Obesity is characterized by excessive fat deposition associated with morphological and functional changes in adipocytes [2]. Studies of adipose tissue biology have led to an improved understanding of the mechanisms that link metabolic disorders with altered adipocyte functions [3]. Lipid accumulation is caused not only by adipose tissue hypertrophy but also by adipose tissue hyperplasia [4]. Although the molecular basis for these associations remains unclear, the experimental evidence suggests that some metabolic disorders might be treatable or preventable through the inhibition of adipogenesis and the modulation of adipocyte function [5].

Adipogenesis includes morphological changes, growth arrest and clonal expansion of adipose cells, followed by a complex sequence of changes in gene expression and lipid storage [6]. The master adipogenic transcriptional regulators are the members of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors and peroxidase proliferator-activated receptor γ (PPARγ). These factors regulate adipocyte differentiation by modulating the expression of their target genes in a coordinated fashion [7-10]. They act synergistically to induce the expression of C/EBPα, PPARγ and sterol-regulatory element-binding protein 1c (SREBP1c) [11-13]. C/EBPα and PPARγ, in turn, promote terminal differentiation by activating the transcription of the genes for fatty acid-binding protein aP2 and fatty acid transporter CD36, which are involved in creating and maintaining the adipocyte phenotype. SREBP1c increases the expression of many lipogenic genes, including fatty acid synthase.

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Loss-of-function studies have shown that PPARγ is necessary and sufficient to promote adipogenesis [14,15] and that C/EBPα is influential in maintaining the expression of PPARγ [16].

Because the currently available drugs for the treatment of obesity cause undesirable side effects, there is high demand for a safe but therapeutically potent anti-obesity drug. A number of plants, plant extracts and phytochemicals have anti-obesity properties or have direct effects on adipose tissue and have therefore been used as dietary supplements [17]. As a result, interest in exploring the applications of medicinally beneficial plants has increased [18].

The edible brown alga *Petalonia binghamiae* (J. Agaradh) Vinogradova is consumed as a traditional food in fishery areas of Northeast Asia. The shape of *P. binghamiae* is an aggregate of several leaves that are 15–50 mm in width and 100–250 mm in length (Fig. 1). Extracts of *P. binghamiae* have anti-oxidant properties [19]. Galactosyldiacylglycerol [20] and fucoxanthin-related compounds [21] isolated from *P. binghamiae* inhibit mammalian DNA polymerase α and exert suppressive properties on adipocyte differentiation in 3T3-L1 cells, respectively. Moreover, we previously demonstrated that an ethanolic extract of *P. binghamiae* exerts an adipogenic effect in 3T3-L1 cells by acting as a mimetic for insulin, and that it exerts an antidiabetic effect in a streptozotocin-induced diabetic mice model [22]. However, the other potentially beneficial properties of *P. binghamiae* have not been studied. In the present study, we evaluated the anti-obesity potential of a water-soluble extract of enzymatically digested *P. binghamiae* thalli in murine 3T3-L1 cells and in rats fed a high-fat diet (HFD). In these model systems, this extract, designated PBEE, demonstrates multiple anti-adipogenetic properties.

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and penicillin–streptomycin (PS) were obtained from Gibco BRL (Grand Island, NY, USA). Antibodies to PPARγ, fatty acid-binding protein aP2, C/EBPα, C/EBPβ, phospho-Ser473 Akt1 and phospho-Thr204-extracellular signal-related kinase 1 and 2 (ERK 1/2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to SREBP1c and phospho-Ser636/639-insulin receptor substrate (IRS)-1 were obtained from BD Biosciences (San Jose, CA, USA) and Cell Signaling Technology (Beverly, MA, USA), respectively. 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) were obtained from Sigma (St. Louis, MO, USA). The lactate dehydrogenase (LDH) Cytotoxicity Detection Kit was purchased from Takara Shuzo Co. (Otsu, Shiga, Japan). 2-Deoxy-D-[^{3}H]glucose was obtained from Amersham Biosciences (Piscataway, NJ, USA). All other reagents were purchased from Sigma unless otherwise noted.

Fig. 1. Picture of wild *Petalonia binghamiae* used in this study. The seaweed was collected in the Jeju island, Republic of Korea.

2.2. Preparation of a water-soluble extract of P. binghamiae (PBEE)

Thalli of the brown seaweed *P. binghamiae* were collected from the coast of Jeju Island, Korea. To enhance the water solubility of seaweed, each 250-g sample of dried seaweed was mixed with 10 L of citric acid buffer solution (pH 4.5) and incubated for 30 min. Then, enzymatic hydrolysis reactions were initiated by the addition of 6.25 ml each of Viscozyme and Celluclast (Novozyme Nordisk, Bagsvaerd, Denmark), and the mixture was incubated at 50°C for 5 h on a platform shaker. After heating at 100°C for 10 min, the enzymatic extract was centrifuged at 3000×*g* at 4°C for 20 min to recover the supernatant fraction containing the water-soluble extract, designated PBEE. The PBEE solution was lyophilized and then stored at −20°C until use.

2.3. Cell culture and differentiation

3T3-L1 preadipocyte cells obtained from American Type Culture Collection (Rockville, MD, USA) were cultured in DMEM containing 1% PS and 10% bovine calf serum (Gibco BRL) at 37°C under a 5% CO_2 atmosphere. To induce differentiation, 2-day postconfluent preadipocytes (designated Day 0) were cultured in MDI differentiation medium (DMEM containing 1% PS, 10% FBS, 0.5 mmol/L IBMX, 1 μmol/L dexamethasone and 5 μg/ml insulin) for 2 days. The cells were then cultured for another 2 days in DMEM containing 1% PS, 10% FBS and 5 μg/ml insulin. Thereafter, the cells were maintained in postdifferentiation medium (DMEM containing 1% PS and 10% FBS), with replacement of the medium every 2 days. To examine the effects of PBEE on the differentiation of preadipocytes to adipocytes, the cells were cultured with MDI in the presence of various concentrations of PBEE. Differentiation, as measured by the expression of adipogenic markers and the appearance of lipid droplets, was complete on Day 8. The effect of PBEE on cell viability and cytotoxicity was determined by the MTT and LDH assay. Cells were seeded at a density of 1×10^4 cell/well into a 96-well plate, then treated PBEE after 24 h, and then incubated for 72 h. The MTT (400 μg/ml) was added to each well, and the plates incubated for 4 h at 37°C. The liquid in the plate was removed, and dimethyl sulfoxide was added to dissolve the MTT–formazan complex. Optical density was measured at 540 nm. The effect of PBEE on cell viability was evaluated as the relative absorbance compared with that of control cultures. The cytotoxic effect of PBEE was measured by the LDH Cytotoxicity Detection Kit. The LDH activities in medium and cell lysate were measured for evaluation of cytotoxicity according to the manufacturer's protocol (LDH released into the medium/maximal LDH release×100).

2.4. Oil Red O staining and cell quantification

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 with 3.7% formaldehyde (Sigma) in PBS for 1 h, washed an additional 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. The stained lipid droplets were dissolved in isopropanol containing 4% Nonidet P-40 and then quantified by measuring the absorbance at 520 nm. Results are given as the lipid content of each experimental group relative to that of MDI-differentiated cells (designated as 100%).

2.5. 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 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L $NaVO_4$, 1 mmol/L NaF and 1 μg/ml each of aprotinin, pepstatin and leupeptin] and incubated on ice for 1 h. After the cell debris was removed by centrifugation, protein concentrations in the lysates were determined using Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA, USA). The cell lysates were then subjected to electrophoresis on 8%, 10% and 15% polyacrylamide gels containing SDS and transferred to polyvinylidene difluoride membranes. The membranes were blocked with a solution of 0.1% Tween 20 in Tris-buffered saline containing 5% nonfat dry milk for 1 h at room temperature. After incubation overnight at 4°C with primary antibody, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunodetection was carried out using ECL Western blotting detection reagent (Amersham Biosciences).

2.6. Flow cytometric analysis of the cell cycle

Post-confluent 3T3-L1 preadipocytes were treated with MDI in the presence or absence of PBEE for 24 h, washed twice with PBS, harvested and fixed in 70% ethanol at 4°C for 1 h. After the ethanol was removed, the cells were washed twice with PBS and treated with RNase (2 μg/ml) for 30 min. The cells were stained with propidium iodide (5 μg/ml; Sigma) for 30 min, and the cell-cycle distribution was determined by fluorescence-activated cell sorting (FACS) on a flow cytometer (FACSCalibur; BD Biosciences, Heidelberg, Germany).

2.7. Glucose uptake activity assay

Glucose uptake activity was analyzed by measuring the uptake of radiolabeled glucose. Briefly, differentiated 3T3-L1 adipocytes grown in 12-well plates were washed twice with serum-free DMEM and incubated with 1 ml of the same medium at 37°C for 6 h. The cells were then washed three times with Krebs-Ringer-Heps (KRH) buffer [20 mmol/L HEPES (pH 7.4), 136 mmol/L NaCl, 4.7 mmol/L KCL, 1.25 mmol/L $MgSO_4$, 1.25 mmol/L $CaCl_2$, 2 mg/ml bovine serum albumin] and incubated with 0.9 ml of KRH buffer at 37°C for 30 min. They were then incubated with or without PBEE at 37°C for 30 min. Insulin was added, and the adipocytes were incubated for another 20 min at 37°C. Glucose uptake was initiated by the addition of 0.1 ml of KRH buffer containing 2-deoxy-D-[^{3}H]glucose (37 MBq/L; Amersham Bioscience) and glucose (1 mmol/L). After 15 min, glucose uptake was terminated by washing the cells three times with cold PBS. The cells were lysed in 0.7 ml of 1% Triton X-100 at 37°C for 20 min. The radioactivity retained by the cell lysates was determined by scintillation counting (Tri-Carb 2700TR; Packard, Meriden, CT, USA).

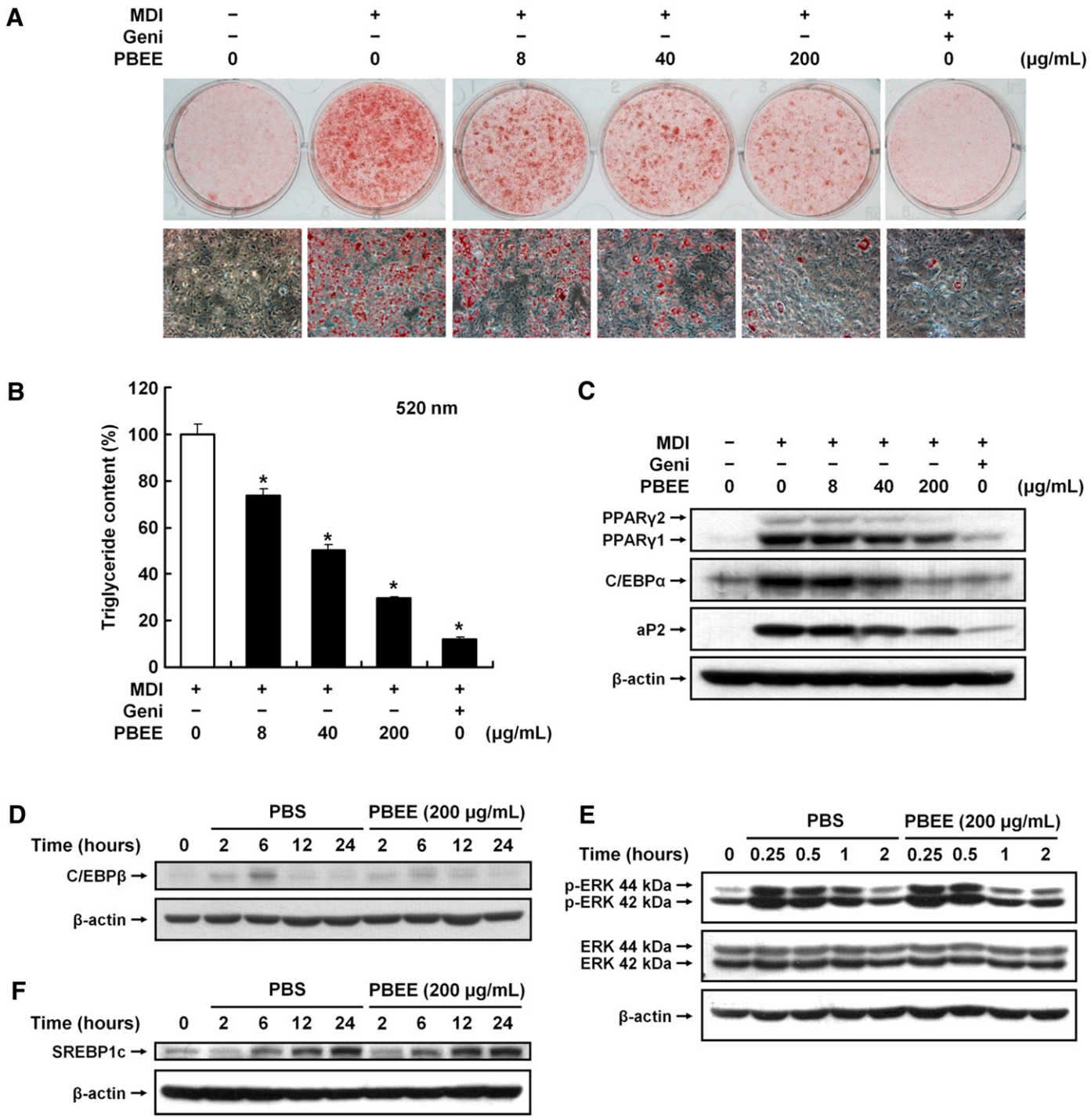

Fig. 2. PBEE inhibits the differentiation of adipocytes in 3T3-L1 preadipocytes. Cells were cultured in MDI differentiation medium in the presence or absence of PBEE (Geni: genistein 100 μmol/L). (A) Differentiated adipocytes were stained with Oil Red O on Day 8 (after 4 days of PBEE treatment). (B) Lipid accumulation was assessed by the quantification of OD_{520} as described in Materials and Methods. Results are shown as means±S.D. (n=3; *P<.05 compared with no PBEE). (C) Western blot analysis of PPARγ, C/EBPα and aP2 expression. Proteins were prepared from 3T3-L1 cells on Day 6. (D–F) Western blot analysis of C/EBPβ (D), phospho-ERK (E) and SREBP1c (F) expression in post-confluent differentiated 3T3-L1 cells. Proteins were harvested at the indicated times.

2.8. Animals

The animal study protocol was approved by the Institutional Animal Care and Use Committee of Jeju National University. After purchase, 40 male 4-week-old Sprague-Dawley (SD) rats (Orient Bio Inc-QC, Seoul, Korea) were adapted for 1 week to specific conditions of temperature (22±2°C), humidity (50±5%) and lighting (light from 0800 to 2000). The animals were housed in plastic cages and given free access to drinking water and food as described below. After adaptation, the SD rats (now 5 weeks old; 196.5±1.9 g) were randomly divided into four groups of 10 rats each. One group (ND) was fed a normal basal diet (Harlan, Bemis, Vancouver, Canada; protein: 18.9%, carbohydrate: 57.33%, fat: 5% and others: 3.3 kcal/g), and three groups (HFD, HFD+$PBEE_{100}$ and HFD+$PBEE_{500}$) were fed a HFD consisting of 40% beef tallow-modified AIN-76A (Jung-Ang Laboratory Animal, Inc., Ansung, Korea; protein: 17.7%, carbohydrate: 31.4%, fat: 40% and others: 5.542 kcal/g). The ND group and the HFD group were provided with tap water. The other two HFD groups were provided with tap water containing PBEE at 100 mg/L (HFD+$PBEE_{100}$) or 500 mg/L (HFD+$PBEE_{500}$).

2.9. Measurement of total body weight, parametrial white adipose tissue weight, and food and water intake

Body weight was measured at the beginning of the experiment and at 5-day intervals for 30 days. The amount of food and water intake by each group was recorded every week. The parametrial white adipose tissue was weighed after quick removal from sacrificed rats.

2.10. Biochemical analysis

After 30 days, the rats were sacrificed by ether anesthesia overdose. Blood samples were collected from the heart and were allowed to stand for 30 min at room temperature for clotting. Serum samples were then collected by centrifugation at 1000×*g* for 15 min. High-density lipoprotein (HDL) cholesterol, glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) concentrations in serum were assayed using an automatic blood analyzer (Kuadro, BPC Biosed, Rome, Italy).

2.11. Histology

After blood was drained from the livers, the livers were fixed in 10% neutral formalin solution for 48 h. The hepatic 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 eosin, and examined by light microscopy (Olympus BX51; Olympus Optical, Tokyo, Japan), and then photographed at a final magnification of 100×.

2.12. Statistical analysis

Values are expressed as means±S.D. or S.E. One-way analysis of variance was used for multiple comparisons. Treatment effects were analyzed by the paired *t* test or Duncan's multiple range test using SPSS software. Differences were considered statistically significant at $P<.05$.

3. Results

3.1. PBEE inhibits 3T3-L1 adipocyte differentiation by modulating the expression of key transcriptional regulators

First of all, the effect of PBEE on cell viability and cytotoxicity of 3T3-L1 cells was evaluated by the MTT and LDH assay. PBEE at a

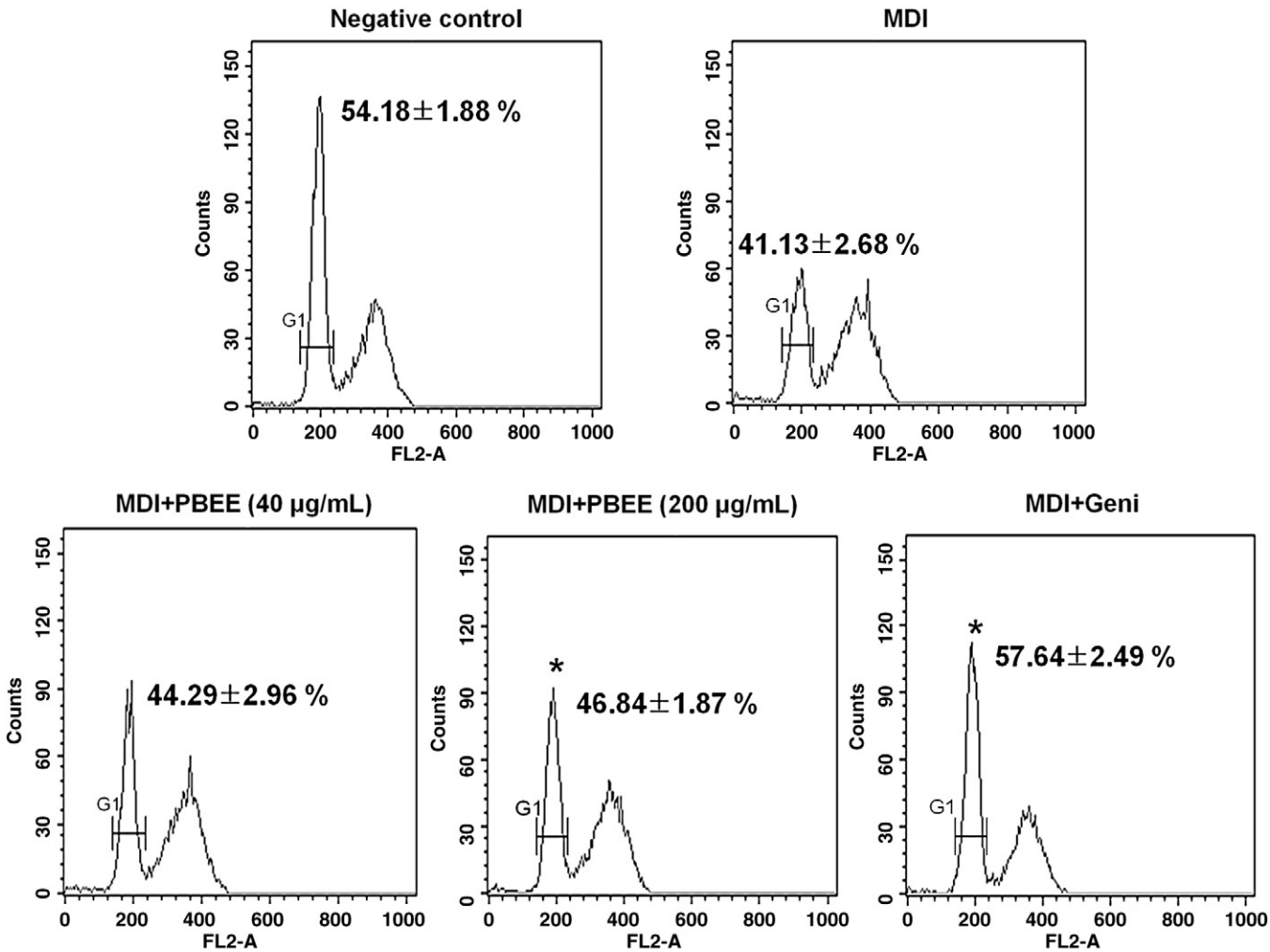

Fig. 3. PBEE causes arrest of differentiation-induced 3T3-L1 preadipocytes at G_1. The preadipocytes were induced to differentiate in the presence or absence of PBEE. Twenty-four hours after induction, cells were harvested, stained with propidium iodide and analyzed by FACS. The mean values of the results are shown with the S.D. ($n=3$; *$P<.05$ compared with no PBEE) (Geni: genistein 100 μmol/L).

concentration of 500 mg/ml did not affect the viability (2.43±3.30% compared to control) as well as the cytotoxicity (−1.59±0.95% compared to control) of the 3T3-L1 cells, as determined by MTT and LDH assays. Then, we tested whether PBEE inhibits MDI-induced differentiation of 3T3-L1 preadipocytes. On Day 0, PBEE was added to the MDI differentiation medium (which contains IBMX, dexamethasone and insulin); on Day 8, the adipocytes were stained using Oil Red O. The Oil Red O staining results demonstrated that PBEE treatment at 8, 40 and 200 μg/ml inhibited 3T3-L1 adipocyte differentiation in a dose-dependent manner. The positive-control cells, which had been treated with 100 μmol/L genistein, exhibited dramatic inhibition of lipid accumulation (Fig. 2A and B).

To determine whether PBEE inhibits adipocyte differentiation by negatively regulating the expression of key transcriptional regulators, we examined the expression levels of C/EBPα, C/EBPβ and PPARγ during adipocyte differentiation in the presence and absence of PBEE. Expression levels of all three factors were reduced in PBEE-treated cells, as was the expression level of aP2 (Fig. 2C and D). However, PBEE did not affect the expression levels of phospho-ERK or SREBP1c (Fig. 2E and F).

3.2. PBEE blocks cell-cycle progression in 3T3-L1 cells

To confirm the effect of PBEE on cell mitosis after adipogenic induction, the effect of PBEE on the cell cycle was analyzed by FACS. In cells with MDI and PBEE, the G_1/S transition of the cell cycle was blocked 24 h after induction in a PBEE concentration-dependent manner, as shown in Fig. 3.

3.3. PBEE inhibits glucose uptake by 3T3-L1 adipocytes

To investigate the effect of PBEE on glucose uptake, differentiated 3T3-L1 adipocytes were incubated in the presence of radiolabeled glucose and various concentrations of PBEE. As shown in Fig. 4A, PBEE dramatically inhibited glucose uptake in a concentration-dependent manner; at the highest PBEE concentration (200 μg/ml), glucose uptake decreased by 42%. We then examined whether the observed decrease in glucose uptake caused by PBEE was associated with an insulin-dependent signaling pathway by evaluating the phosphorylation of IRS-1 and Akt-1 in PBEE-treated 3T3-L1 adipocytes. PBEE treatment was found to inhibit phosphorylation of both IRS-1 and Akt-1, comparable to the effect of genistein used as the positive control (Fig. 4B).

3.4. PBEE prevents HFD-induced obesity

As shown in Table 1, after 30 days on the HFD, the mean body weight and body weight gain of the HFD rats were more than 110% and 119% higher than those of the ND group ($P<.05$), indicating that the HFD did induce obesity. PBEE administration through drinking water (at 100 or 500 mg/L) significantly decreased the body weight and body weight gains of HFD+PBEE rats relative to those of the non-PBEE-treated control HFD group (8% and 12% lower, respectively; $P<.05$). The mass of white adipose tissue was also significantly larger in the HFD group (by 138%) than in the ND group ($P<.05$). Supplementation of the drinking water of the HFD rats with PBEE at 100 or 500 mg/L significantly decreased their white adipose tissue mass (by 16% or 19%, respectively) relative to that of the non-PBEE-treated HFD group ($P<.05$, Table 1).

3.5. PBEE dramatically decreases signs of liver pathology

We next examined the effect of PBEE on the levels of serum GPT and GOT in HFD rats. PBEE administration decreased the levels of these markers of cell damage significantly and in a concentration-dependent manner. The levels of serum GPT and GOT were 30% and 17% lower, respectively, in HFD+$PBEE_{500}$ rats than in the non-PBEE-treated control HFD group ($P<.05$, Table 1). Moreover, serum HDL-cholesterol levels were also significantly higher (by 126%) in the HFD+$PBEE_{500}$ rats ($P<.05$, Table 1).

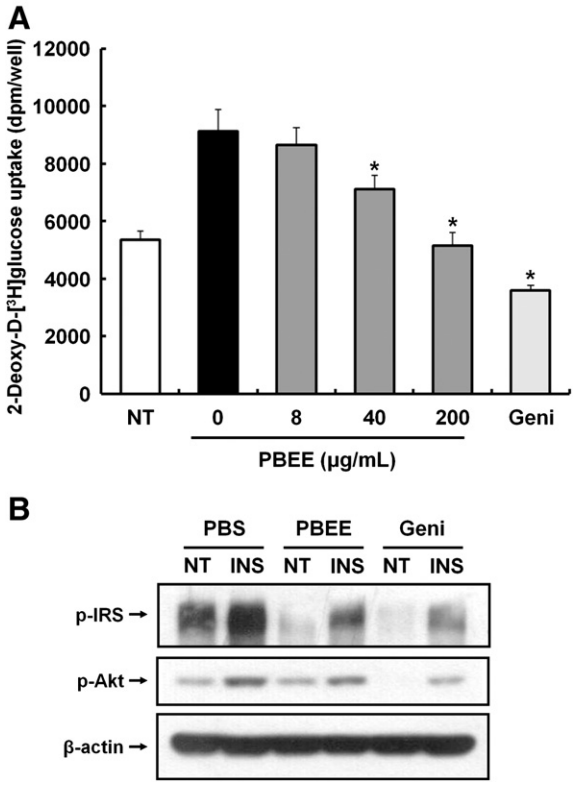

Fig. 4. PBEE inhibits glucose uptake in mature 3T3-L1 adipocytes. (A) Mature adipocytes were incubated in 12-well plates in the presence of insulin, PBEE and genistein (Geni, 100 μmol/L), and then assayed for uptake of 2-deoxy-D-[^{3}H]glucose. Results are shown as means±S.D. ($n=3$; *$P<.05$ compared with untreated control). (B) Western blot analysis of phospho-IRS and phospho-Akt levels. Differentiated 3T3-L1 adipocytes were not treated or were treated with insulin (INS; 100 nmol/L), PBEE (200 μg/ml) or genistein (Geni; 100 μmol/L), as indicated.

Photomicrographs of liver samples stained with hematoxylin and eosin are shown in Fig. 5. Livers from the ND group fed tap water for 30 days were normal (Fig. 5A), and livers from the control group fed a HFD for 30 days exhibited an increased number of fatty droplets (Fig. 5B). However, livers from the HFD+PBEE groups exhibited a decreased number of fatty droplets relative to the control HFD livers, and the decrease was dose dependent (Fig. 5C and D).

4. Discussion

The regulation of adipogenesis involves a number of complex, interconnected cell signaling pathways; thus multiple natural products like edible seaweed extract might have the potential for use in therapy designed to protect metabolic disorder. In this regard, we have evaluated the potential of several extracts of the edible seaweed *P. binghamiae in vitro* using murine 3T3-L1 preadipocytes and *in vivo* using animal models [22]. In this study, we found that a water-soluble *P. binghamiae* extract, designated PBEE, dose dependently decreased the levels of triglycerides, PPARγ1 and 2, C/EBPα and aP2 in differentiating 3T3-L1 preadipocytes. The PBEE-induced decrease in PPARγ and C/EBPα expression suggested that another PBEE target molecule lay upstream of PPARγ and C/EBPα. To elucidate the signaling pathway through which PBEE affected preadipocyte

Table 1
Effects of supplementing PBEE on body weight gain, food and water consumption energy intake, and serum profiles in HFD-induced experimental group for 30 days

Group	ND	HFD	HFD+PBEE$_{100}$	HFD+PBEE$_{500}$
Initial body weight (g)	197.31±2.99	198.59±4.40	195. 76±4.08	194.17±4.32
Final body weight (g)	417.86±9.82^{a}	460.90±8.69^{b}	434.99±9.57^{ab}	423.97±13.65^{a}
Body weight gain (g)	220.54±7.02^{a}	262.31±6.33^{b}	239.23±6.37^{a}	229.80±10.33^{a}
Intake of PBEE (mg/kg of body weight per day)	–	–	8.59±0.97	46.71±1.52
Water intake (ml/rat per day)	29.80±0.59	28.80±1.82	28.92±1.07	29.38±1.27
Food intake (g/rat per day)	26.49±0.55^{a}	17.57±0.30^{b}	17.75±0.58^{b}	17.38±0.63^{b}
Energy intake (kcal/rat per day)	87.41±1.18^{a}	97.37±1.69^{b}	98.35±3.22^{b}	96.30±3.51^{b}
White adipose weight (mg/g of body weight)	19.32±0.29^{a}	26.64±1.29^{b}	22.27±1.11^{a}	21.56±1.34^{a}
GPT (IU/L)	38.14±0.83^{a}	47.71±5.45^{b}	35.29±1.85^{a}	33.29±1.95^{a}
GOT (IU/L)	65.29±2.04^{a}	95.86±5.97^{b}	82.57±12.91^{ab}	79.29±4.17^{ab}
HDL (mg/dl)	14.00±1.23^{a}	8.71±0.81^{b}	12.86±1.68^{a}	11.00±1.23^{ab}

The values were expressed as mean±S.E. (n=10). Mean separation was performed by Duncan's multiple range test.
Different letters indicate significant differences (P<.05).

proliferation, we first examined the possible involvement of ERKs, which have been linked to adipocyte differentiation [23,24]. However, PBEE treatment did not affect ERK phosphorylation. It did not affect the expression of SREBP1c either, which has been reported to induce the production of an endogenous ligand that enhances PPARγ transcriptional activity [25] and can increase the expression of several genes involved in fatty acid metabolism.

C/EBPβ plays a critical role in adipocyte differentiation [26]. It is expressed early in the differentiation program and drives the subsequent expression of C/EBPα [27]. Our data showed that PBEE inhibits C/EBPβ expression in 3T3-L1 cells. Given the importance of C/EBPβ during adipocyte differentiation, the inhibition of C/EBPβ expression by PBEE may be sufficient to block terminal differentiation. Thus, our results suggest that the inhibition of adipocyte differentiation by PBEE correlates with inhibition of C/EBPβ expression. According to Tang et al. [28], mitotic clonal expansion is a prerequisite for differentiation of 3T3-L1 preadipocytes into adipocytes. Also, Harmon and Harp [29] have found that genistein inhibits mitotic clonal expansion and induces lipolysis in adipocytes. Our FACS cell-cycle data suggest that PBEE affects preadipocyte proliferation. Like genistein, PBEE blocks the cell cycle at the G_1/S transition. Thus, PBEE might inhibit the differentiation of preadipocytes by preventing them from traversing the G_1/S checkpoint.

Binding of insulin to its receptor induces tyrosine phosphorylation of the receptor, thereby initiating the insulin signal transduction. Subsequent tyrosine phosphorylation of IRS-1 and -2 leads to activation of phosphatidylinositol 3-kinase [30], which produces phosphatidylinositol phospholipids. These phospholipids in turn activate phosphatidylinositol phosphate-dependent kinase-1. The subsequent activation of Akt stimulates glucose uptake into cells by inducing the translocation of glucose transporter 4 from intracellular storage sites to the plasma membrane [31]. We found that PBEE, like the often-used tyrosine kinase inhibitor genistein [32,33], inhibited insulin-stimulated glucose uptake in mature 3T3-L1 adipocytes by reducing the level of phospho-IRS-1.

Recently, we demonstrated that an ethanolic extract of *P. binghamiae* (PBE) exerts an adipogenic effect in 3T3-L1 cells and an anti-diabetic effect in streptozotocin-induced diabetic mice [22]. However, this study revealed that the water-soluble extract of *P. binghamiae* (PBEE) exerts an anti-adipogenic effect in 3T3-L1 cells. We suppose that these different biological activities between PBEE and PBE occur due to different chemical compound contents by two different extraction methods. The contents of the total polyphenol and polysaccharides contained in PBEE and PBE were 3.96 and 25.39 mg/g, and 424.3 and 8.3 mg/g, respectively. The contents of total polyphenol compounds were richer in PBE than in PBEE. On the other hand, total polysaccharides were much lower (about 50-fold) in PBE than in PBEE. Accordingly, the present results indicate that the water-soluble components, such as polysaccharides, which were contained as major constituents in PBEE, inhibit the adipogenesis and glucose uptake in 3T3-L1 cells. While the lipid-soluble constituents, such as polyphenol compounds, which were contained as major constituents in PBE, may exert adipogenic effect in 3T3-L1 cells. However, identifications of their active compound(s) from PBEE and PBE remain to be studied in future research.

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 [34]. Consistent with our *in vitro* results, the administration of PBEE (100 or 500 mg/L drinking water) over 30 days to rats fed a HFD decreased the body weight and adipose tissue weight of the rats without changing their energy intake (Table 1).

We also analyzed the effects of PBEE on the development of fatty liver, which is strongly associated with obesity [35]. In HFD rats, livers were enlarged and yellowish, indicating liver steatosis, whereas the livers of the HFD+PBEE rats remained red and healthy looking. Upon

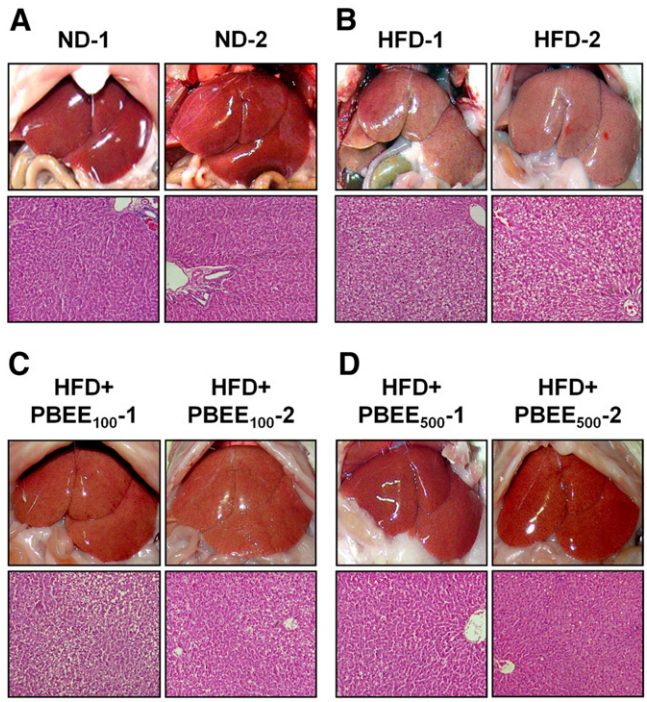

Fig. 5. PBEE decreases fatty droplet accumulation in livers of HFD-fed rats. Hematoxylin and eosin-stained photomicrographs of liver sections are shown at 100×. (A) Livers from ND rats appear normal. (B) Livers from HFD-fed control rats exhibit an increased number of fatty droplets after 30 days. (C, D) Drinking water supplementation with PBEE at 100 mg/L (C, PBEE$_{100}$) or 500 mg/L (D, PBEE$_{500}$) decreased fatty droplet accumulation in livers of HFD-fed rats.

histological analysis, the HFD livers exhibited an accumulation of numerous fat droplets, a typical sign of fatty liver. However, the HFD+PBEE livers exhibited a much smaller degree of lipid accumulation and fewer signs of pathology.

Plasma GOT and GPT levels are clinically and toxicologically important indicators, rising as a result of tissue damage caused by toxicants or disease conditions. In HFD rats, the activities of liver function markers, including serum GOT and GPT, were significantly elevated relative to those in the ND rats and were improved by PBEE supplementation. Taken together, these results indicate that administration of PBEE can partially suppress the development of HFD-induced fatty liver. PBEE supplementation of drinking water at 500 mg/L also significantly increased serum HDL-cholesterol levels in HFD rats.

In conclusion, we have shown that PBEE inhibits adipogenesis by down-regulating the adipocyte-specific transcriptional regulators C/EBPβ, C/EBPα and PPARγ. Furthermore, PBEE attenuates the mitotic clonal expansion process of adipocyte differentiation and significantly inhibits glucose uptake in mature 3T3-L1 adipocytes. Finally, the administration of PBEE to rats with HFD-induced obesity reduces body weight gain, adiposity and the accumulation of fatty droplets in the liver. Taken together, PBEE (or a biologically active component) may protect against HFD-induced obesity through inhibiting adipocyte differentiation and glucose uptake in mature adipocytes.

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