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The Metabolic Benefits of *Polygonum hypoleucum* Ohwi in HepG2 Cells and Wistar Rats under Lipogenic Stress

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Inhibition of acetyl-CoA carboxylase (ACC) is one approach used for treating metabolic syndrome. Using partially purified ACC to screen herbs commonly used in Taiwanese folk medicine, we previously showed that an ethanol extract of *Polygonum hypoleucum* Ohwi (EP) had potent ACC inhibitory activity and partially alleviated metabolic disorders induced by a high fat diet. Since ACC plays a crucial role in *de novo* lipogenesis, the favorable effects of EP on metabolism were tested under lipogenic conditions in the present study. On incubating high glucose (30 mM)-stimulated HepG2 cells with EP (72.5 or 145 μg/mL), ACC and fatty acid synthase activity, triacylglycerol content, and microsomal triacylglycerol transfer protein mRNA levels were all significantly reduced ($P < 0.05$, vs vehicle). When EP was given at low, medium, and high dosages (94, 188, and 470 mg/kg) to sucrose water-treated Wistar rats for four weeks, alleviation of symptoms associated with metabolic syndrome, including obesity, insulin resistance, hypertriglyceridemia, and hypertension, accompanied by hepatic ACC inactivation, was seen in the low dosage group. Four compounds (emodin, emodin-8-*O*-β-D-glucopyranoside, (+)-catechin, and (−)-epicatechin) isolated from EP were identified as ACC inhibitors. These results confirm that *P. hypoleucum* Ohwi, acting partly through ACC inhibition, has favorable effects in alleviating metabolic disturbances occurring under lipogenic conditions.

KEYWORDS: Acetyl-CoA carboxylase; *P. hypoleucum* Ohwi; lipogenic condition; metabolic syndrome

INTRODUCTION

Metabolic syndrome, characterized by the clustering of certain risk factors, including insulin resistance, central obesity, hypertension, and dyslipidemia, which dramatically increase the risk of developing cardiovascular disease and type 2 diabetes mellitus, has become a major global public health problem (*1*, *2*). To discover and develop new agents that can alleviate these metabolic perturbations, we previously established a screening platform based on acetyl-CoA carboxylase (ACC) inhibitory activity and tested extracts from twenty herbs commonly used in Taiwanese folk medicine and found that an ethanol extract of *Polygonum hypoleucum* Ohwi (EP) had marked ACC inhibitory activity (*3*).

ACC (EC 6.4.1.2), an enzyme that plays a crucial role in fatty acid metabolism, is an attractive target for the development of drugs active against obesity, diabetes, and other symptoms associated with metabolic disease (*4*, *5*). Two isoforms, ACC1 and ACC2, encoded by separate genes, play different roles in lipid metabolism. ACC1 is a cytosolic enzyme expressed mainly in lipogenic tissues (liver, adipose tissue, and mammary gland) and catalyzes the rate-limiting step in the biosynthesis of long-chain fatty acids. In contrast, ACC2 is associated with the mitochondrial membrane and is mainly expressed in the heart and skeletal muscle, and its product, malonyl-CoA, is a potent inhibitor of fatty acid oxidation. By reducing malonyl-CoA levels, inhibition of ACC should be effective in reducing fatty acid synthesis and increasing fatty acid oxidation by, respectively, removing the substrate and relieving malonyl-CoA inhibition of carnitine palmitoyltransferase-I (*6*). In animal studies, reducing ACC expression by genetic manipulation, e.g. ACC2 gene knockout (*7*) or ACC1 and/or ACC2 antisense oligonucleotides (*8*), and reducing ACC activity pharmacologically by isozyme-nonselective inhibition (*9*, *10*) have been shown to be effective ways of treating and ameliorating metabolic syndrome.

Polygonum hypoleucum Ohwi is a Chinese herb used in Taiwanese folk medicine for the treatment of arthritis, rheumatoid arthritis, cough, influenza, and nephritis (*11*). Since EP was found to have potent ACC inhibitory activity, we have previously verified the metabolic benefits of EP in a high fat diet-induced obesity model (*3*). Incorporation of EP at a dosage of 3% (approximate 1000 mg/kg BW) of the high fat diet is effective in reducing hyperlipidemia and insulin resistance in C57BL/6J mice, but fails to reduce adiposity (*3*). Since ACC plays a crucial

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role in *de novo* lipogenesis, we expected that the metabolic benefits of *P. hypoleucum* Ohwi might best be seen under lipogenic conditions, i.e. on feeding a fat-free diet or a high sucrose diet, rather than a high fat diet. In the present study, two lipogenic models (high glucose-stimulated lipogenesis of a hepatoma cell line and sucrose-containing drinking water-induced metabolic syndrome in Wistar rats) were used to test the effects of EP. In the feeding study, three dosages (94, 188, and 470 mg/kg) of EP, lower than that previously used in the high fat diet model, were tested. In addition, we tried to identify the ACC inhibitors present in the EP.

MATERIALS AND METHODS

Identification of *P. hypoleucum* Ohwi and Preparation of the Ethanol Extract. *P. hypoleucum* Ohwi was collected from a local herbal store, and its authenticity was confirmed by the Department of Life Sciences, National Chung-Hsing University. The voucher specimen (Hsu 3156) was deposited in the herbarium of the same university. The air-dried stem and roots of *P. hypoleucum* Ohwi were chopped up and immersed in 95% ethanol (8 L/kg) for 48 h at room temperature. The extract was then filtered through Whatman No. 1 filter paper, the filtrate evaporated under reduced pressure, and the residue collected. The yield of EP was 9% (g/g). The EP used in the present study (including cell culture and animal experiments) is the same batch as that used in the previous study (*3*).

Cell Culture and Treatment. A model of high glucose-induced ACC activation in hepatocytes was used (*12*). Human hepatoma HepG2 cells (BCRC no. 60025), obtained from the Bioresource Collection and Research Center (Taipei, Taiwan), were grown in Dulbecco's modified Eagle medium containing 5.5 mM D-glucose, 10% fetal bovine serum, 100 μg/mL of streptomycin, and 100 units/mL of penicillin in a humidified atmosphere of 5% CO_2 at 37 °C and passaged every 3 days by trypsinization. For experiments, HepG2 cells were incubated in low glucose (5.5 mM) medium containing 10% fetal bovine serum. When 70% confluence was reached, the cells were maintained overnight in serum-free low glucose medium and then were switched to serum-free high glucose (30 mM) for 6 h; then EP was added at final concentrations of 72.5 or 145 μg/mL [2.5- and 5-fold higher than the IC_{50} of EP on ACC activity (*3*)]. EP was prepared as a 100 mg/mL stock solution in absolute ethanol and diluted with medium, and the same amount of absolute ethanol was used in the vehicle control. Cells were collected at the time points indicated in the figure.

Intracellular Enzyme Activity and Triacylglycerol Content. The harvested cells were homogenized at 4 °C in 10 mM potassium phosphate buffer containing 0.25 M sucrose, 5 mM 2-mercaptoethanol, 1 mM EDTA, and 1% protease inhibitor cocktail (Sigma, St. Louis, MO). After centrifugation at 13000*g* for 45 min at 4 °C, the supernatant was collected for enzyme activity assays. ACC activity was measured using the $^{14}CO_2$ fixation method (*13*), and fatty acid synthase (FAS) activity was measured using a spectrophotometric method (*14*). The details of these assays have been described in Chen et al. (*3*). The protein content of the homogenate was determined by the Bradford method. For intracellular triacylglycerol (TG) measurement, the cells were broken by sonication and freezing/thawing in distilled water, and then TG was measured using an enzymatic assay kit from Randox Laboratories (Crumlin, Northland, U.K.).

RNA Isolation and mRNA Detection. Total RNA was extracted from HepG2 cells using TRIZOL reagent according to the manufacturer's instructions (Invitrogen, New York, NY). The quality of the extracted RNA was confirmed by a value of 2 for the 28S rRNA/18S rRNA ratio after ethidium bromide staining. Levels of mRNAs for a housekeeping gene (β-actin) and microsomal triacylglycerol transfer protein (MTTP) were measured by real-time PCR. A TaqMan One-Step RT-PCR Master Mix reagent kit and inventory primer and probe (Applied Biosystems, Foster, CA) were used for quantitative real-time PCR following a TaqMan gene expression assay. Amplification using 40 cycles of two steps (95 °C for 15 s and 60 °C for 1 min) was performed on an ABI Prism TM model 7900HT sequence detection system.

Animals and Feed. Forty male Wistar rats were purchased from the National Applied Research Laboratories (Taipei, Taiwan) at 7 weeks of age. After acclimation to a standard rodent chow diet (6 g of water, 51 g of crude carbohydrate, 23.5 g of crude protein, 4.5 g of crude lipid, 6 g of crude fiber, and 9 g of ash/100 g diet; Fwusow Industry, Taichung, Taiwan) for 1 week, the rats were divided into two groups which were given plain water (water without sucrose addition) (group C; $n = 8$) or 30% sucrose in water as drinking water ($n = 32$). After 30 weeks, the rats given sucrose water were split into groups SW, EPL, EPM, and EPH to receive, respectively, 0, 94, 188, or 470 mg/kg of EP by gavage. The animals were kept in a room maintained at 23 ± 2 °C on a controlled 12 h light:dark cycle with free access to food and drinking water. Body weight was recorded weekly. The protocols for animal care and handling were approved by the Institutional Animal Care and Use Committee of the China Medical University.

Blood Pressure and Insulin Sensitivity. The diastolic and systolic blood pressure and heart rate were measured using a tail-cuff system (BP 2000, Visitech Systems, Apex, NC) that uses a photoelectric sensor to detect blood flow in the tail (*15*); for each rat, at least one set of ten measurements with nine or more successful readings was obtained. For measurement of insulin sensitivity, the oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were performed on rats after 4 weeks of treatment. For the OGTT, the animals were fasted overnight (only plain water was supplied for all animals), then tail blood was collected before (0 min) and at 30, 60, 90, and 120 min after oral administration of a 2.5 M glucose solution (1.5 g/kg body weight). For the ITT, the animals were fed for 3 h after overnight fasting, and then tail blood was collected before (0 min) and at 5, 10, 30, and 60 min after intraperitoneal injection of a 0.1 U/mL solution of insulin (0.75 U/kg body weight). The area under the curve for serum glucose (AUC_{glu}) over 2 h or over 1 h was calculated for the OGTT or ITT, respectively.

Measurement of Biomedical Indices. After treatment for 4 weeks, the rats were killed by carbon dioxide asphyxiation after 10 h of fasting. Blood was collected from the orbital capillary and serum immediately separated and stored at −20 °C until analysis. The visceral (epididymal and retroperitoneal) and subcutaneous (inguinal) fats were excised and weighed. A 0.5 cm^3 cube of retroperitoneal fat was fixed in 10% formaldehyde, embedded in paraffin, cut into 10 μm sections, and examined under a light microscope (OLYMPUS I×71) equipped with a SPOT RT color-2000 digital camera (Diagnostic Instruments Inc., Sterling Heights, MI) to obtain images for cell size determination. Enzyme-linked immunosorbent assays were used to measure serum insulin (Linco, St. Charles, MO) and leptin (R&D, Minneapolis, MN). Lipids in the liver were extracted using the method of Folch et al. (*16*). TG, total cholesterol (TC), and glucose were measured by enzymatic methods using commercial kits (Randox Lab, Crumlin, Northland, U.K.).

Fractionation and Identification of ACC Inhibitors in EP. The EP prepared as above was evaporated under reduced pressure, and then the residue was suspended in water for fractionation based on polarity. The aqueous suspension was extracted sequentially, each for 3 times, with an equal volume of chloroform to obtain the chloroform fraction (FrC), an equal volume of ethyl acetate to obtain fraction FrEA and an equal volume of *n*-butanol to obtain fraction FrB. The remaining aqueous phase was designated as FrW. Starting from 928 g of EP, 100, 231, 460, and 120 g of FrC, FrEA, FrB and FrW were obtained respectively. All fractions were tested on ACC, and two fractions (FrEA and FrB) showing inhibitory activity were subjected to further fractionation. FrEA (231 g) was subjected to normal phase silica gel chromatography with $CHCl_3$/methanol elution to give 15 fractions (Fr1−15). Compounds **3** and **7** (16.2 and 21.3 mg, respectively) from Fr2 (760 mg), compound **8** (36.5 mg) from Fr10 (962 mg) and compounds **4** and **9** (17.4 and 53.2 mg, respectively) from Fr12 (5.75 g) were purified by silica chromatography and Sephadex LH-20. Compounds **5** (12.7 mg) and **6** (7.5 mg) were isolated from Fr13 (1.2 g) by column chromatography over silica gel and reverse phase HPLC (Cosmosil $5C_{18}$ MS-II). Further fractionation of FrB (130 g) by silica gel column chromatography with gradient elution (CH_2Cl_2/methanol = 10:1−1:1) gave 7 fractions (Fr16−22). Compounds **1** and **2** (6.2 and 15.6 mg, respectively) from Fr 18 (832 mg) were isolated by Sephadex LH-20 and preparative TLC. The pure compounds **1**−**9** isolated were tested for ACC inhibitory activity and identified by mass, ^1H and ^{13}C NMR. The NMR spectra were run on a Bruker AVANCE 400 spectrometer (Bruker BioSpin MRI GmbH, Ettlingen, Germany).

To test for ACC inhibitory activity, partially purified ACC (10 μU/μL) from rat liver was used (*3*). All test fractions and compounds were

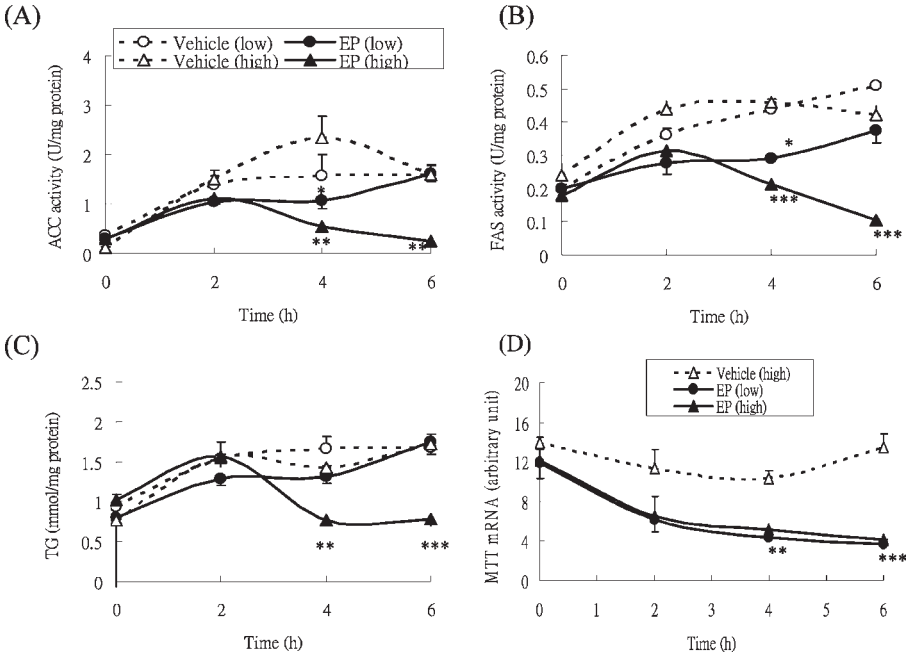


Figure 1. Acetyl-CoA carboxylase (ACC) activity (A), fatty acid synthase (FAS) activity (**B**), intracellular triacylglycerol (TG) content (**C**), and microsomal triacylglycerol transfer protein (MTTP) mRNA levels (**D**) in high glucose-stimulated HepG2 cells treated with vehicle or ethanol extract of *P. hypoleucum* Ohwi (EP). Cells were stimulated with high glucose for 6 h, then two concentrations of EP (72.5 or 145 μg/mL; low and high respectively) or vehicle were added during a further 6 h of high glucose stimulation, and ACC and FAS activity, TG content, and MTTP mRNA levels were measured every 2 h. One unit of ACC activity is defined as the amount which catalyzes the formation of 1 μmol of malonyl-CoA per minute, while one unit of FAS activity is defined as the amount required to synthesize 1 nmol of palmitic acid (equivalent to the oxidation of 14 nmol of NADPH) per minute. The values are the mean ± SEM ($n = 4-6$). The significance of differences between groups (EP vs the corresponding vehicle control; for MTTP, only one vehicle control) was analyzed using Student's *t* test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$.

evaporated to dryness to prepare a 0.5 mg/mL stock solution dissolved in DMSO. ACC inhibitory activity was assayed by incubating 7 μL of enzyme solution (70 μU) with 10 μL of the test sample (final concentration 25 μg/mL) or vehicle for 15 min at 37 °C and then measuring the ACC activity as described previously (*3*).

Statistical Analysis. The data are expressed as the mean ± SEM. The significance of differences between groups was analyzed statistically by one-way ANOVA and Duncan's multiple range test. In the cell culture study, the significance of differences between the experimental group and the corresponding vehicle control group was analyzed using Student's *t* test. The data were transformed to log values for the statistical analysis if the variances were not homogeneous. The General Linear Model of the SAS package (SAS Institute, Cary, NC) was used for both statistical analyses, and differences were considered significant at $P < 0.05$.

RESULTS

Effect of EP on High Glucose-Stimulated Lipogenesis in a Hepatoma Cell Line. A model of high glucose-induced ACC activation in hepatocytes (*12*) was used to characterize the effects of EP in a cell culture system. When HepG2 cells were stimulated with high glucose for 0−24 h, the ACC and FAS activities and the TG content were significantly increased at the 12 h time-point compared to the low glucose control (data not shown). To test if EP could suppress lipogenesis and reduce intracellular lipid levels, cells were preincubated with high glucose for 6 h, then two doses of EP (72.5 or 145 μg/mL) or vehicle were added to the medium and the cells were incubated for another 6 h, enzyme activity and TG content being measured every 2 h. Significant differences in ACC and FAS activity and TG content between EP-treated cells and the corresponding vehicle control were seen at 4 h for both doses of EP and at 6 h with the high dose (**Figure 1A**−**C**). Thus, EP dose-dependently suppressed the induction of lipogenic enzyme activities and the lipid accumulation caused by high glucose in HepG2 cells.

MTTP is a protein responsible for the assembly and secretion of apolipoprotein B (apoB)-containing lipoproteins in the presence of lipids (*17*). Both doses of EP significantly reduced MTTP mRNA levels in high glucose-stimulated HepG2 at 4 and 6 h of treatment, with no difference between the high and low dose-treated cells (**Figure 1D**).

Effect of EP on Sucrose-Containing Drinking Water-Induced Metabolic Syndrome in Wistar Rats. Giving rats sucrose water to drink is a successful way of inducing metabolic syndrome by upregulating *de novo* lipogenesis (*18*, *19*). As expected, the rats in group SW were obese, with hypertriglyceridemia, hyperinsulinemia, and glucose intolerance, and were hypertensive (group SW vs C; **Table 1**, **2**). Serum levels of glucose and uric acid and the AUC_{glu} for the ITT tended to be higher than in group C (**Tables 1**, **2**).

When EP was administered to the sucrose water-treated rats, all adiposity indexes measured (body weight, body fat mass, serum leptin levels, adipocyte size, and TNF-α protein levels in adipose tissue) were significantly reduced compared to in group SW, the best results being seen in the low dosage group (group EPL) (**Table 1**).

As shown in **Table 2**, although EP had no significant effect on serum glucose levels, significant reductions were seen in insulin levels in groups EPL and EPH, in the AUC_{glu} for the OGTT in group EPL, and in the AUC_{glu} for the ITT in groups EPL and EPM ($P < 0.05$, vs group SW). TG and uric acid levels were significantly reduced by an equal extent in all three EP groups ($P < 0.05$, vs group SW). A significant reduction in diastolic blood pressure and a tendency to a reduction in systolic blood pressure were seen in group EPL, while blood pressures in groups EPM and EPH were not different from those in group SW.

In accordance with the greater improvement in metabolic derangements seen in group EPL, a significant reduction in liver

Table 1. Indices Associated with Obesity in the Treated Rats[a]

	C	SW	EPL	EPM	EPH
final body weight (g)	496 ± 11 c	714 ± 29 a	619 ± 23 b	643 ± 16 ab	668 ± 29 ab
body fat (g)					
retroperitoneal	7.33 ± 1.24 c	35.79 ± 4.24 a	23.84 ± 3.97 b	29.16 ± 3.01 ab	31.10 ± 4.59 ab
epididymal	7.39 ± 0.66 c	20.52 ± 1.53 a	14.28 ± 1.21 b	18.43 ± 1.66 ab	22.51 ± 3.14 a
inguinal	3.72 ± 0.42 c	16.58 ± 2.89 a	8.89 ± 1.41 b	18.27 ± 4.88 ab	10.11 ± 1.89 ab
serum leptin (μg/L)	1.16 ± 0.19 c	6.03 ± 0.83 a	1.96 ± 0.25 bc	2.90 ± 0.49 b	3.46 ± 0.96 b
adipocyte diameter (μm)	94 ± 14 c	152 ± 6 a	116 ± 7 b	120 ± 5 b	129 ± 7 b
TNF-α in adipose tissue (ng/mg protein)	4.63 ± 2.35 b	8.77 ± 4.16 a	5.02 ± 1.57 b	7.13 ± 1.27 ab	6.43 ± 2.35 ab

[a] The values are the mean ± SEM (n = 8). The significance of differences among groups was analyzed by one-way ANOVA and Duncan's multiple range test. Values for which the groups do not share a letter are significantly different (P < 0.05). C, rats given plain water which served as controls; SW, rats given sucrose water to induce metabolic syndrome; EPL, EPM, and EPH, rats given sucrose water plus ethanol extract of *P. hypoleucum* Ohwi at 94, 188, and 470 mg/kg, respectively.

Table 2. Metabolic Profiles of the Treated Rats[a]

	C	SW	EPL	EPM	EPH
serum levels					
glucose (mmol/L)	2.67 ± 0.16 b	3.70 ± 0.24 ab	3.70 ± 0.36 ab	3.44 ± 0.4 ab	4.18 ± 0.23 a
TG (mmol/L)	1.31 ± 0.10 b	2.06 ± 0.26 a	1.31 ± 0.13 b	1.31 ± 0.07 b	1.44 ± 0.22 b
TC (mmol/L)	2.41 ± 0.18 a	2.32 ± 0.17 a	1.77 ± 0.10 b	2.03 ± 0.12 ab	1.98 ± 0.07 ab
insulin (pmol/L)	0.54 ± 0.05 b	1.28 ± 0.23 a	0.70 ± 0.04 b	1.03 ± 0.21 ab	0.62 ± 0.04 b
uric acid (mmol/L)	0.38 ± 0.03 ab	0.48 ± 0.05 a	0.34 ± 0.03 b	0.33 ± 0.02 b	0.31 ± 0.03 b
AUC_{glu} (mmol × min/L)					
OGTT	686 ± 22 b	807 ± 30 a	704 ± 9 b	754 ± 34 ab	722 ± 33 ab
ITT	206 ± 6 ab	228 ± 9 a	195 ± 11 b	196 ± 9 b	225 ± 5 ab
blood pressure (mmHg)					
systolic	91 ± 15 b	128 ± 9 a	102 ± 7 ab	128 ± 8 a	121 ± 7 a
diastolic	83 ± 33 b	103 ± 8 a	74 ± 18 b	108 ± 7 a	111 ± 3 a

[a] The values are the mean ± SEM (n = 8). The significance of differences among groups was analyzed by one-way ANOVA and Duncan's multiple range test. Values for which the groups do not share a letter are significantly different (P < 0.05). C, rats given plain water which served as controls; SW, rats given sucrose water to induce metabolic syndrome; EPL, EPM and EPH, rats given sucrose water plus ethanol extract of *P. hypoleucum* Ohwi at 94, 188, and 470 mg/kg, respectively.

ACC activity was seen in group EPL, but not groups EPM and EPH (**Figure 2**).

ACC Inhibitors in EP. To identify ACC inhibitors, EP was fractionated and the ACC inhibitory activity of the fractions evaluated using partially purified enzyme from rat liver. Based on the IC_{50} of EP on ACC [30 μg/mL from ref *3*], the ACC inhibitory activities of the four fractions, FrC, FrEA, FrB, and FrW, were tested at a concentration of 25 μg/mL, and caused 9, 73, 46, or 18% inhibition, respectively. FrEA and FrB with potent ACC inhibitory activity was further fractionated to obtain 9 compounds (compounds **3**−**9** from FrEA and compounds **1** and **2** from FrB), which were also tested at a concentration of 25 μg/mL for ACC inhibitory activity. In contrast to no inhibition being found for compounds **7**−**9**, compounds **1**−**6** showed ACC inhibitory activity (66 ± 4, 88 ± 6, 49 ± 1, 49 ± 0.2, 46 ± 3, 42 ± 1% inhibition), and their identification was attempted based on mass as well as ^{1}H and ^{13}C NMR spectra. Compounds **1** and **2** were not identified, but compounds **3**−**6** were identified as emodin, emodin-8-*O*-β-glucopyranoside, (+)-catechin, and (−)-epicatechin, respectively (**Table 3**).

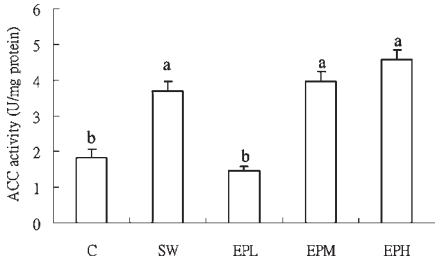


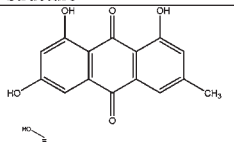
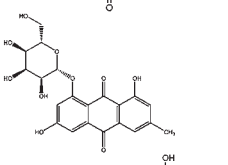
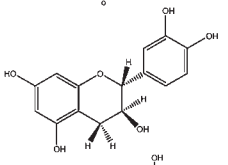
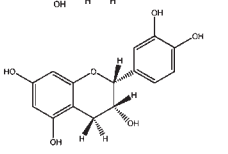
Figure 2. Liver acetyl-CoA carboxylase (ACC) activity of rats fed the experimental diets. C, rats given plain water which served as controls; SW, rats given sucrose water to induce metabolic syndrome; EPL, EPM, and EPH, rats given sucrose water plus ethanol extract of *P. hypoleucum* Ohwi at 94, 188, and 470 mg/kg, respectively. One unit of ACC activity is defined as the amount which catalyzes the formation of 1 μmol of malonyl-CoA per minute. The values are the mean ± SEM (n = 8). The significance of differences among groups was analyzed by one-way ANOVA and Duncan's multiple range test. Values for which the groups do not share a letter are significantly different (P < 0.05).

DISCUSSION

Using ACC activity as a screening platform, an ethanol extract of *P. hypoleucum* Ohwi (EP) was previously found to have potent ACC inhibitory activity and shown to lower serum lipid and insulin levels, but not body fat mass, in high fat diet-fed mice (*3*). Since *de novo* lipogenesis is repressed rather than increased by the high-fat diet regimen (*20*), inhibition of ACC might not be a good strategy to reverse the metabolic aberrations in these mice. In the present study, we used lipogenic models of upregulated ACC and lipogenesis to test whether EP reduced lipid synthesis/secretion in hepatocytes and ameliorated symptoms associated with metabolic syndrome in rats. The high glucose-induced lipogenesis and lipid accumulation in HepG2 cells was reversed by EP in a dose-dependent manner. Moreover, the EP-mediated downregulation of MTTP expression observed in the present study might contribute to a hypolipidemia effect. In the sucrose water-induced metabolic syndrome model in rats, a much smaller dosage of EP than that used previously in the high fat diet model (1/10 of the dose) was enough to demonstrate the benefits of EP in attenuating the obesity, hypertriglyceridemia, hyperinsulinemia, and hypertension caused by drinking sucrose water.

High glucose-stimulated HepG2 cells have been used as a hepatic model of steatosis and insulin resistance (*12*, *21*). In this model system, intracellular ACC is increased at the transcriptional

Table 3. Compounds with Acetyl-CoA Carboxylase Inhibitory Activity in the EP

Name	Structure	NMR
Emodin (compound **3**)		^{1}H NMR (400 MHz, acetone-d_6): δ 2.43 (3H, s, Ar-CH$_3$), 6.61 (1H, d, J=2.4 Hz, H-2), 7.07 (1H, br s, H-7), 7.18 (1H, d, J=2.4 Hz, H-4), 7.48 (1H, br s, H-5), 12.00 (1H, s, OH), 12.12 (1H, s, OH). ^{13}C NMR (400 MHz, acetone-d_6): δ 191.6, 182.1, 166.4, 166.2, 163.2, 149.5, 136.5, 134.1, 124.9, 121.4, 114.4, 110.3, 109.7, 108.5, 22.0
Emodin-8-O-β-D-glucopyranoside (compound **4**)		^{1}H NMR (400 MHz, DMSO-d_6): δ 2.43 (3H, s, Ar-CH$_3$), 3.20-3.80 (6H, m, sugar protons), 5.15 (1H, d, J=7.6 Hz, H-1'), 6.97 (1H, d, J=2.4 Hz, H-2), 7.14 (1H, d, J=1.2 Hz, H-7), 7.26 (1H, d, J=2.4 Hz, H-4), 7.44 (1H, d, J=1.2 Hz, H-5), 13.15 (1H, s, OH). ^{13}C NMR (400 MHz, DMSO-d_6): δ 186.6, 182.3, 164.5, 161.8, 161.2, 147.2, 136.7, 132.2, 124.4, 119.5, 114.6, 113.4, 108.5, 108.6, 100.9, 77.4, 76.5, 73.4, 69.6, 60.7, 21.6
(+)-catechin (compound **5**)		^{1}H NMR (200 MHz, acetone-d_6): δ 2.54 (1H, dd, J=16.1, 8.4 Hz, H-4β), 2.90 (1H, dd, J=16.1, 5.4 Hz, H-4α), 4.00 (1H, m, H-3), 4.55 (1H, d, J=7.6 Hz, H-2), 5.86 (1H, d, J=2.2 Hz, H-6), 6.01 (1H, d, J=2.2 Hz, H-8), 6.74 (1H, d, J=8.0 Hz, H-5'), 6.79 (1H, dd, J=8.0, 1.6 Hz, H-6'), 6.89 (1H, br d, J=1.6 Hz, H-2'). ^{13}C NMR (200 MHz, acetone-d_6): δ 156.6, 156.1, 155.7, 144.6, 144.5, 130.9, 118.8, 114.5, 114.1, 99.4, 94.9, 94.2, 81.5, 67.2, 48.6
(-)-epicatechin (compound **6**)		^{1}H NMR (400 MHz, methanol-d_4): δ 2.75 (1H, dd, J=16.4, 2.8 Hz), 2.87 (1H, dd, J=16.4, 4.4 Hz), 4.16 (1H, m, H-3eq), 4.80 (1H, br s, H-2ax), 5.92 (1H, d, J=2.0 Hz, H-6), 5.95 (1H, d, J=2.0 Hz, H-8), 6.76 (1H, d, J=8.0 Hz, H-5'), 6.80 (1H, br d, J=8.0 Hz, H-6'), 6.96 (1H, br s, H-2'). ^{13}C NMR (400 MHz, methanol-d_4): δ 157.9, 157.6, 157.3, 145.8, 145.7, 132.2, 119.4, 115.9, 115.3, 100.1, 96.4,. 95.9, 79.8, 67.4, 48.7

level through transactivation of sterol regulatory element binding protein-1c (SREBP-1c) and carbohydrate response element binding protein (ChREBP) (*22*). Covalent modulation by dephosphorylation of AMP-activated protein kinase and ACC is also involved in ACC activation (*12*). High concentrations of glucose also selectively attenuate insulin signaling in HepG2 cells (*21*). Whether EP can rescue insulin signaling of high glucose-treated hepatocytes, accompanied by reductions in the lipid content as observed in the present study, remains to be elucidated.

MTTP is an essential chaperone for the assembly and secretion of apoB-containing lipoproteins (*17*). The reduction in MTTP mRNA levels seen in high glucose-stimulated hepatocytes incubated with EP implies a favorable effect on the lipoprotein profile. Some plant-derived compounds have been reported to reduce MTTP activity or expression in hepatocytes, accompanied by lowered apoB secretion (*23*−*25*). Insulin downregulates MTTP expression via the mitogen-activated protein kinase pathway (*26*, *27*). Thus, it is highly possible that the EP-mediated reduction in MTTP expression might be due to increased hepatic insulin sensitivity. Furthermore, fructose-fed insulin-resistant hamsters display significantly higher MTTP expression in the liver and intestine and overproduction of apo-B-containing lipoprotein (*28*). Correction of the insulin resistance in this model results in the simultaneous normalization of MTTP expression and apoB secretion (*29*).

We and others have shown that symptoms associated with human metabolic syndrome can be elicited in rodents by giving them sucrose water (10−30%) to drink (*18*, *30*−*32*). An increase in hepatic ACC activity and mRNA levels and upregulation of SREBP-1c and ChREBP associated with sucrose water drinking has been observed (*19*). In contrast to the lack of ACC inhibition, albeit with a mild benefit on metabolism, observed in high fat diet-fed mice supplemented with a high dose of EP (1000 mg/kg BW) (*3*), the present study showed that the best results in attenuating symptoms associated with metabolic syndrome were seen in the low EP group (94 mg/kg BW), the only group that showed significant repression of hepatic ACC activity (**Figure 2**). It is not clear why the ACC inhibitory effect was lost on increasing the dose of EP, but we speculate that some antagonists of ACC inhibitors (or ACC activators) may be present in the crude extract which compromise the metabolic benefits of EP when given at high levels.

The dosages of EP used in the HepG2 study were based on the IC_{50} of EP for purified ACC (i.e., 30 μg/mL) (*3*). Given that the active components in EP have to pass through the cell membrane to reach the cytoplasm where the enzyme is located, EP was added to the medium at concentrations equivalent to 2.5 × IC_{50} and 5 × IC_{50} (i.e., 72.5 or 145 μg/mL). Extrapolation of the effective dosage seen in *in vitro* (72.5 μg/mL) to the *in vivo* study by assuming an intestinal absorption rate of EP of 1−5% in rats weighing approximately 500 g yields a test dose range of 94−470 mg/kg.

Since the EP-mediated ACC inhibition was observed in a cell-free system (*3*), EP must contain ACC inhibitors which might contribute, at least in part, to the favorable effects observed. Using partially purified ACC, four compounds in EP with ACC inhibitory activity were identified as emodin, emodin-8-O-β-glucopyranoside, (+)-catechin, and (−)-epicatechin. Emodin isolated from *P. hypoleucum* Ohwi had been reported to have antitumor (*33*) and immunomodulatory (*11*, *34*, *35*) activities. In terms of its benefits on lipid metabolism, one paper has reported that emodin given to rats at a dosage of 40 mg/kg is effective in treating nonalcoholic fatty liver (*36*). Watanabe et al. (1998) searched for ACC inhibitors from foodstuffs and identified (−)-epigallocatechin gallate (EGCG) and epicatechin-3-O-gallate (ECG) as the most potent ACC inhibitors in green tea. In their study, catechin and epicatechin had 30% of the inhibitory activity of EGCG (*37*). However, using HPLC analysis, we failed to detect EGCG or ECG in EP (data not shown). It is noteworthy that other potential ACC inhibitors in EP (compounds **1** and **2**) have not yet been identified. In fact, the four compounds identified were no more potent than the crude extract in terms of ACC inhibitory activity, so more active constituents may still await discovery.

The ACC activity platform had been used pharmacologically for high throughput screening for compounds with potential to treat metabolic syndrome. We are the first to use this platform to screen for indigenous herbs with potential for treating metabolic syndrome. Although *P. hypoleucum* Ohwi was identified using this screening platform, its favorable effects on metabolism might act through mechanisms other than ACC inhibition. In addition, it is plausible that glucose oxidation and thermogenesis are increased to dispose of surplus carbohydrate when ACC inhibitors are present and lipogenesis is blocked. It had been documented that blocking *de novo* lipogenesis by disrupting the *SCD* (stearoyl-CoA desaturase) or *ACC2* gene is accompanied by an increase in total energy expenditure or expression of genes involved in thermogenesis (*38*, *39*).

In conclusion, our results confirm that *P. hypoleucum* Ohwi, acting partly through ACC inhibition, has favorable effects in alleviating metabolic disturbances occurring under lipogenic conditions. The ACC inhibitory components in EP other than catechin, epicatechin, emodin and emodin-8-*O*-*β*-D-glucopyranoside need to be further explored.

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