Biochemical and Biophysical Research Communications 411 (2011) 219–225

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Piperidine alkaloids from *Piper retrofractum* Vahl. protect against high-fat diet-induced obesity by regulating lipid metabolism and activating AMP-activated protein kinase

Kyung Jin Kim [a], Myoung-Su Lee [b], Keunae Jo [b], Jae-Kwan Hwang [a,b,c,*]

[a] Department of Biomaterials Science and Engineering, Yonsei University, Seoul 120-749, Republic of Korea
[b] Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea
[c] Translational Research Center for Protein Functional Control, Yonsei University, Seoul 120-749, Republic of Korea

ARTICLE INFO

Article history:
Received 20 June 2011
Available online 29 June 2011

Keywords:
Piper retrofractum Vahl.
Piperidine alkaloids
Obesity
Lipid metabolism

ABSTRACT

The fruits of *Piper retrofractum* Vahl. have been used for their anti-flatulent, expectorant, antitussive, antifungal, and appetizing properties in traditional medicine, and they are reported to possess gastroprotective and cholesterol-lowering properties. However, their anti-obesity activity remains unexplored. The present study was conducted to isolate the anti-obesity constituents from *P. retrofractum* Vahl. and evaluate their effects in high-fat diet (HFD)-induced obese mice. Piperidine alkaloids from *P. retrofractum* Vahl. (PRPAs), including piperine, pipernonaline, and dehydropipernonaline, were isolated as the anti-obesity constituents through a peroxisome proliferator-activated receptor δ (PPARδ) transactivation assay. The molecular mechanism was investigated in 3T3-L1 adipocytes and L6 myocytes. PRPA treatment activated AMP-activated protein kinase (AMPK) signaling and PPARδ protein and also regulated the expression of lipid metabolism-related proteins. In the animal model, oral PRPA administration (50, 100, or 300 mg/kg/day for 8 weeks) significantly reduced HFD-induced body weight gain without altering the amount of food intake. Fat pad mass was reduced in the PRPA treatment groups, as evidenced by reduced adipocyte size. In addition, elevated serum levels of total cholesterol, low-density lipoprotein cholesterol, total lipid, leptin, and lipase were suppressed by PRPA treatment. PRPA also protected against the development of nonalcoholic fatty liver by decreasing hepatic triglyceride accumulation. Consistent with the *in vitro* results, PRPA activated AMPK signaling and altered the expression of lipid metabolism-related proteins in liver and skeletal muscle. Taken together, these findings demonstrate that PRPAs attenuate HFD-induced obesity by activating AMPK and PPARδ, and regulate lipid metabolism, suggesting their potential anti-obesity effects.

1. Introduction

Obesity is a chronic metabolic disorder caused by energy imbalance that is characterized by increased fat mass and dysregulated lipid metabolism [1]. Hyperlipidemia caused by the dysregulation of lipid metabolism is a critical feature of obesity, and it is strongly related to metabolic disorders [2]. Circulating free fatty acids (FFAs) are taken up by adipose, liver, and muscle tissues, where they are metabolized via mitochondrial fatty acid oxidation (FAO) or stored as triglycerides (TGs) via the lipid synthesis pathway [3]. In mice, the downregulation of lipogenic proteins and upregulation of FAO proteins attenuate obesity and dyslipidemia in the high-fat diet (HFD)-induced obese rodent model [4]. Therefore, improving lipid metabolism has become one of the most important strategies for obesity prevention and treatment [5].

Peroxisome proliferator-activated receptors (PPARs) control systemic fatty acid metabolism via the transcriptional activation of target genes and act as fatty acid sensors to alter metabolic pathways in response to changes in fuel [6]. PPARδ is predominantly expressed in liver and skeletal muscle, where they regulate lipid metabolism, and is well known as a fat burning-transcription factor exhibiting an anti-obesity function [7].

AMP-activated protein kinase (AMPK) functions as a key energy sensor, and it is activated by energy depletion [8]. Once activated, AMPK inactivates acetyl-CoA carboxylase (ACC) and increases FAO

Abbreviations: AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; SREBPs, sterol regulatory element-binding proteins; FAS, fatty acid synthase; CPT, carnitine palmitoyltransferase; UCP, uncoupling protein; PPAR, peroxisome proliferator-activated receptor; HFD, high-fat diet; FAO, fatty acid oxidation; TG, triglyceride; PP, piperine; PN, pipernonaline; DPN, dehydropipernonaline.

* Corresponding author. Address: Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, 262 Seongsanno, Seodaemun-gu, Seoul 120-749, Republic of Korea. Fax: +82 2 362 7265.
E-mail address: jkhwang@yonsei.ac.kr (J.-K. Hwang).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.06.153

regulation genes such as carnitine palmitoyltransferase-1 (CPT-1), which transfers long-chain fatty acids into the mitochondria, and uncoupling proteins (UCPs), which are involved in energy expenditure through fat burning [7,9]. Moreover, AMPK decreases fatty acid synthesis by reducing the protein expression of mature sterol regulatory element-binding proteins (SREBPs) [10]. SREBPs are transcription factors that regulate the expression of lipogenic enzymes, such as ACC and fatty acid synthase (FAS) [11]. Therefore, AMPK also has been suggested as a therapeutic target for dyslipidemia as well as obesity.

Piper retrofractum Vahl. (syn. *Piper chaba* Hunter), belonging to the Piperaceae family, is widely distributed in tropical and subtropical regions of the world [12]. The fruits of *P. retrofractum* have been used for their anti-flatulent, expectorant, antitussive, antifungal, uterus-contractile, sedative-hypnotic, appetizing, and counter-irritant properties in traditional medicine [13]. *P. retrofractum* carry a variety of piperidine alkaloids, such as piperine (PP), pipernonaline (PN), dehydropipernonaline (DPN), which possess gastroprotective and hepatoprotective properties; however, their anti-obesity effects remain to be studied [14,15]. The present study was conducted to isolate the anti-obesity constituents from *P. retrofractum* and evaluate their effects in HFD-induced obese mice.

2. Materials and methods

2.1. Plant material and anti-obesity constituent isolation

The dried fruits of *P. retrofractum* were collected in Jakarta, Indonesia. A voucher specimen is deposited in the Department of Biotechnology, Yonsei University (Seoul, Korea). The ground *P. retrofractum* (100 g) was extracted with 95% ethanol (500 ml), and the extract (5.75 g) was further fractionated with ethyl acetate (4.53 g). Then, it was subjected to silica gel open column chromatography (70–230 mesh, Merck & Co., Whitehouse Station, NJ, USA) and eluted with *n*-hexane and ethyl acetate solution (2:1, v/v) to obtain five fractions (Fractions I to V). Fraction V (Fr. V, 0.24 g) was eluted with 80% methanol using reversed-phase column chromatography (LiChroprep®, RP-18, 25–40 μm, Merck & Co.), yielding fraction V-D (Fr. V-D, 0.077 g). Fr. V-D was eluted with 80% methanol using a prep HPLC (column: W-252, 20.0 mm ID × 500 mm L, Japan Analytical Industry Co., Ltd., Tokyo, Japan), and alkaloid compound 1 (0.022 g), compound 2 (0.022 g), and compound 3 (0.027 g) were finally obtained separately as a single compound. Careful comparison of several spectral data of compounds 1, 2, and 3 including EI-MS, ^{13}C-NMR, and ^{1}H-NMR with those in the literature [14,16,17] suggested the chemical structures to be PP, DPN, and PN, respectively (Fig. 1A).

2.2. Instrumentation

NMR spectra were recorded on a Bruker Avance-500 spectrometer (Rheinstein, Germany) at 500 MHz for ^{1}H-NMR and ^{13}C-NMR in $CDCl_3$ with TMS as an internal standard. Complete proton and carbon assignments were based on one-dimensional (^{1}H and ^{13}C) NMR. Mass spectra (EI-MS) were measured with a JMS-700 mass spectrometer (Jeol Ltd., Tokyo, Japan). All instrumental data are available upon request.

2.3. Animal studies

Twenty-six 4-week-old male C57BL/6J mice (DooYeol Biotech, Seoul, Korea) were housed in a controlled environment (25 ± 2 °C, 55 ± 5% relative humidity, 12-h light/dark cycle). After acclimation for 1 week, they were fed a HFD (rodent diet D12451, 45% fat/35% carbohydrates/20% protein; Research Diet, New Brunswick, NJ, USA). After 7 weeks of dietary manipulation to induce obesity, the 26 mice were divided into four experimental groups: group 1 received the HFD with vehicle (n = 6); group 2 received the HFD with PRPAs (Fr. V-D) 50 mg/kg/day (PRPA 50, n = 7); group 3 received the HFD with PRPAs 100 mg/kg/day (PRPA 100, n = 7); group 4 received the HFD with PRPAs 300 mg/kg/day (PRPA 300, n = 7). Animals were fed via oral feeding needles for 8 weeks. Water and food intake and body weight were measured twice per week throughout the experiment.

At the end of the 8-week oral administration period, all mice were sacrificed via diethyl ether inhalation after an overnight fast. Their fat pads and liver and skeletal muscle tissues were removed, weighed, and frozen in liquid nitrogen. Micro-computed tomography (micro-CT) experiments were performed using an animal positron emission tomography (PET)/CT/single photon emission CT system (INVEON, Siemens Medical Solutions, Knoxville, TN, USA) at the Korea Basis Science Institute in Ochang. This study adhered to the Guide for the Care and Use of Laboratory Animals developed by the Institute of Laboratory Animal Resources of the National Research Council, and it was approved by the Institutional Animal Card and Use Committee (IACUC) of Yonsei University in Seoul, Korea.

2.4. Antibodies and reagents

Antibodies against phosphorylated AMPK (Thr172), ACC, phosphorylated ACC (Ser79), and FAS were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against SREBP-1, UCP2, UCP3, CPT-1 liver form (CPT-1L), CPT-1 muscle form (CPT-1M), PPARδ, and α-Tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An electrochemiluminescence (ECL) solution for Western blot analysis was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

2.5. Galactosidase 4 (GAL4)/PPAR chimera transactivation assay and reporter gene assay

PPARδ activation was examined using plasmids encoding the PPARδ binding domain as described previously [18]. The human PPARδ domain was subcloned in-frame into the pFA-CMV vector (Stratagene, La Jolla, CA, USA) with the DNA-binding domain from the yeast GAL4 transcription factor to generate pFA-PPARδ. PPARδ activation was assessed through luciferase assay by cotransfection of pFA-PPARδ with pFR-luc (UAS-GAL4-luciferase). COS-7 cells were transfected with plasmids containing three PPAR response elements (PPREs), PPRE-tk-luc (Addgene plasmid 1015, Cambridge, MA, USA), and the cells were incubated with PRPA or PPARδ activator GW501516 (Alexis Biochemicals, Lausen, Switzerland) for 16 h. Luciferase activity was determined with luciferase assay substrates (Promega, Madison, WI, USA) with a MicroLumatPlus LB 96V luminometer (Berthold Technologies (GmbH & Co., Bad Wildbad, Germany).

2.6. Cell culture

COS-7 cells and L6 rat skeletal myoblast cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM supplemented with antibiotics (100 U/ml penicillin A and 100 U/ml streptomycin) and 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA). Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. To investigate the regulation of lipid metabolism-related proteins, the cells were treated with PRPAs for 24 h.

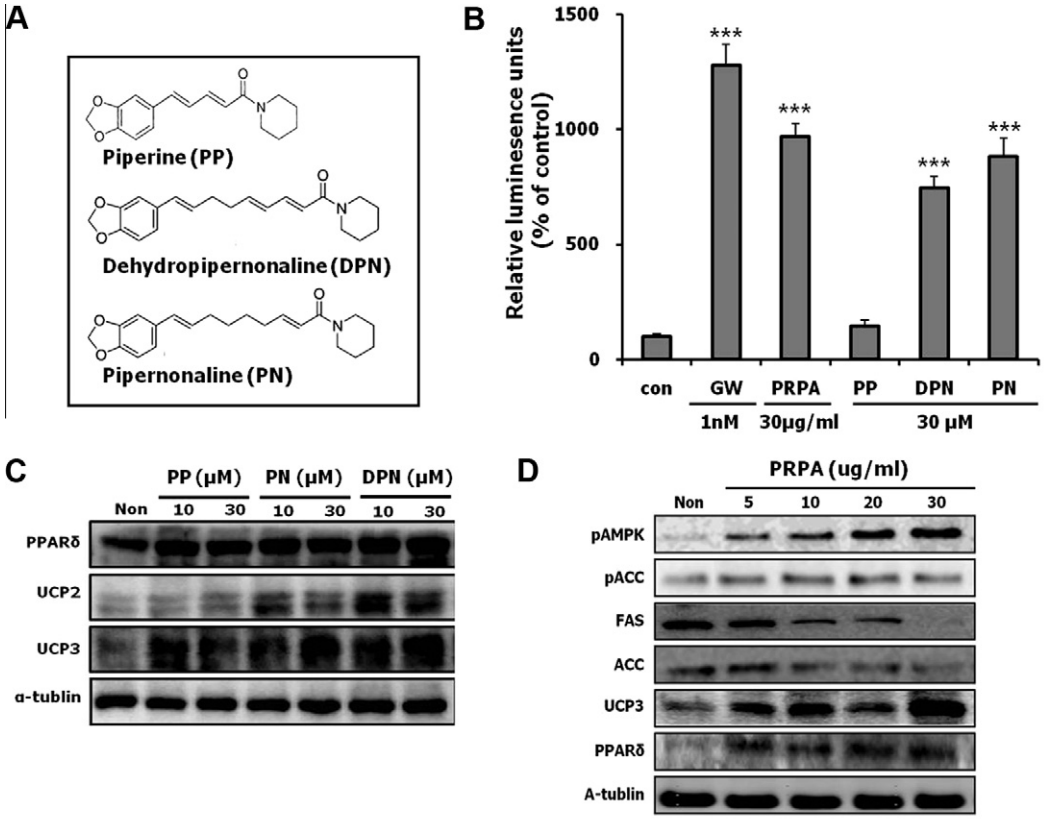

Fig. 1. The anti-obesity constituents were isolated from *P. retrofractum* and found to increase fat-burning protein expression in 3T3-L1 adipocytes and L6 myocytes. (A) Chemical structures of anti-obesity constituents of PP, PN, and DPN. (B) COS-7 cells were transfected with pFA-PPARδ and pFR-luc and then treated with the PPARδ activator GW501516 (1 nM) or the anti-obesity constituents in *P. retrofractum* such as piperidine alkaloids (PRPAs, 30 μg/ml), PP (30 μM), PN (30 μM), or DPN (30 μM). (C) L6 myocytes were incubated with PP, PN, and DPN for 12 h, and then whole cell lysates were subjected to Western blot analysis of PPARδ, UCP2, and UCP3 expression. The Western blots are representative of the results for six mice in each group. (D) 3T3-L1 adipocytes were incubated with PRPAs (5, 10, 20, or 30 μg/ml) for 24 h, and then whole cell lysates were subjected to Western blot analysis of pAMPK, pACC, FAS, ACC, UCP3, and PPARδ expression. α-Tubulin was used as a protein loading control.

2.7. Histological and blood analysis

White adipose tissue, livers, and skeletal muscle obtained from all mice were embedded in a tissue-freezing medium (Leica, Wetzlar, Germany) and fixed as previously described [19]. After fixation, they were stained with hematoxylin and eosin and analyzed for lipid accumulation and adipocyte size with an Eclipse TE2000U Inverted Microscope with twin CCD cameras (magnification, ×200; Nikon, Tokyo, Japan).

Blood was collected from all mice by heart puncture and held at room temperature for 1 h; serum was then prepared by centrifugation at 4000 rpm for 15 min and stored at −70 °C until analysis. Serum lipid profiles and serum levels of hepatotoxicity markers were determined using a commercial enzyme-linked immunosorbent assay kit from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's instructions.

2.8. Western blot analysis

Homogenized tissues were lysed with lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.25% deoxycholate, 1 mM EDTA, and 1 mM PMSF) and incubated on ice for 10 min. The mixture was then centrifuged, and the supernatant was used to determine protein concentrations. Total protein (30 μg) was subjected to SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Blotted membranes were blocked with 5% skim milk in tris-buffered saline containing 0.1% Tween 20 for 1 h and then incubated with primary antibodies for 16 h at 4 °C. After three washes in tris-buffered saline containing 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-linked secondary antibodies for 2 h. Proteins were detected with an enhanced ECL detection system (Amersham Biosciences, Little Chalfont, UK) and visualized with a LuminoImager (LAS-3000 Bio Imaging Analysis System; Fuji Film Co., Tokyo, Japan).

2.9. Statistics

Results are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using SPSS 9.0 (SPSS Inc., Chicago, IL, USA). Significance was assessed by analysis of variance followed by Tukey's test. Statistical significance was set at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

3. Results and discussion

3.1. The anti-obesity constituents in P. retrofractum increase fat-burning protein levels in adipocytes and myocytes

PPARδ regulates lipid metabolism and is an established fat burning-transcription factor exerting anti-obesity effects [7]. The GAL4/PPARδ chimera transactivation assay was conducted to isolate the anti-obesity constituents of *P. retrofractum*. PRPAs, including PP, PN, and DPN, were identified as the PPARδ activators. GW501516 (PPARδ activator), PRPAs, DPN, and PN significantly activated PPARδ via PPRE activation (Fig 1B). In addition, PP,

DPN, and PN exerted effects on fat-burning regulatory proteins such as PPARδ, UCP2, and UCP3 in L6 myocytes (Fig. 1C). This results indicated that piperidine alkaloids such as PP, PN, and DPN exert anti-obesity effects through upregulating fat-burning proteins.

AMPK is a key regulator of proteins involved in lipogenesis and FAO in metabolic tissues [8]. It was reported that AMPK interacted with PPARδ [20], and we assessed whether PRPAs activated AMPK and regulated proteins involved in lipid metabolism. PRPA treatment increased AMPK and ACC phosphorylation compared to the levels in the HFD control group (Fig. 1D). The expression of proteins involved in fat burning (PPARδ and UCP3) was increased, whereas the expression of proteins involved in fat storage (ACC and FAS) was decreased in 3T3 adipocytes (Fig. 1D). This result indicates that PRPAs activate AMPK signaling and selectively regulate the expression of lipid metabolism-related proteins. Therefore, the activation of AMPK and regulation of lipid regulatory proteins may mediate the fat-burning effects of the anti-obesity constituents in *P. retrofractum.*

3.2. PRPAs attenuate HFD-induced adiposity and weight gain by reducing fat fad and white adipocyte size

To determine whether PRPAs improve obesity in the animal model, C57BL/6J mice with HFD-induced obesity received oral doses of PRPAs (50, 100, or 300 mg/kg/day) for 8 weeks. Micro-CT data revealed that whole body fat accumulation was lower in the PRPA treatment groups than in the HFD control group (Fig. 2A). In addition, PRPA 50, 100, and 300 treatment groups gained 19.5, 22.0, and 29.2% less weight, respectively, than mice in the HFD control group, (Fig. 2B) without reducing food intake (data not shown). These results suggest that PRPAs reduced HFD-induced body weight gain, which was not attributed to the amount of food intake. The PRPA 100 and 300 treatment groups exhibited significantly reduced perirenal, epididymal, and subcutaneous fat pad mass (Fig. 2C) without reductions in the weight of the heart and spleen (data not shown). Histological analysis of epididymal fat indicated that the PRPA-induced reduction of fat mass was due to a dose-dependent decrease in white adipocyte size (Fig. 2D and E). In conclusion, the long-term weight loss in the PRPA treatment groups was accompanied by a smaller fat pad mass and adipocyte size compared to the HFD control group without changes in energy intake change, indicating the possibility to alter energy expenditure by increasing the expression of fat-burning proteins.

3.3. PRPAs improve the HFD-induced dysregulation of lipid metabolism

An elevated plasma concentration of low-density lipoprotein cholesterol (LDL-C) increases the risk of coronary heart disease, and the clinical complications of atherosclerosis could be diminished when plasma lipids are lowered by hypocholesterolemic agents [21]. In this study, total cholesterol (Total-C) levels were reduced by 28.4% and 44.3% in the PRPA 100 and 300 treatment groups, respectively. In addition, LDL-C concentrations were reduced by 39.8%, 37.9%, and 57.6% in the PRPA 50, 100, and 300 treatment groups, respectively; however, no significant changes were observed in the levels of high-density lipoprotein cholesterol (HDL-C). The HFD-induced atherogenic index (AI) was significantly improved by 33.2% and 25.3% in the PRPA 50 and 100 treatment groups, respectively (Fig. 3A). Furthermore, the serum total lipid concentration was significantly decreased by 49.1% in the PRPA 300 treatment group, and PRPA treatment group decreased serum

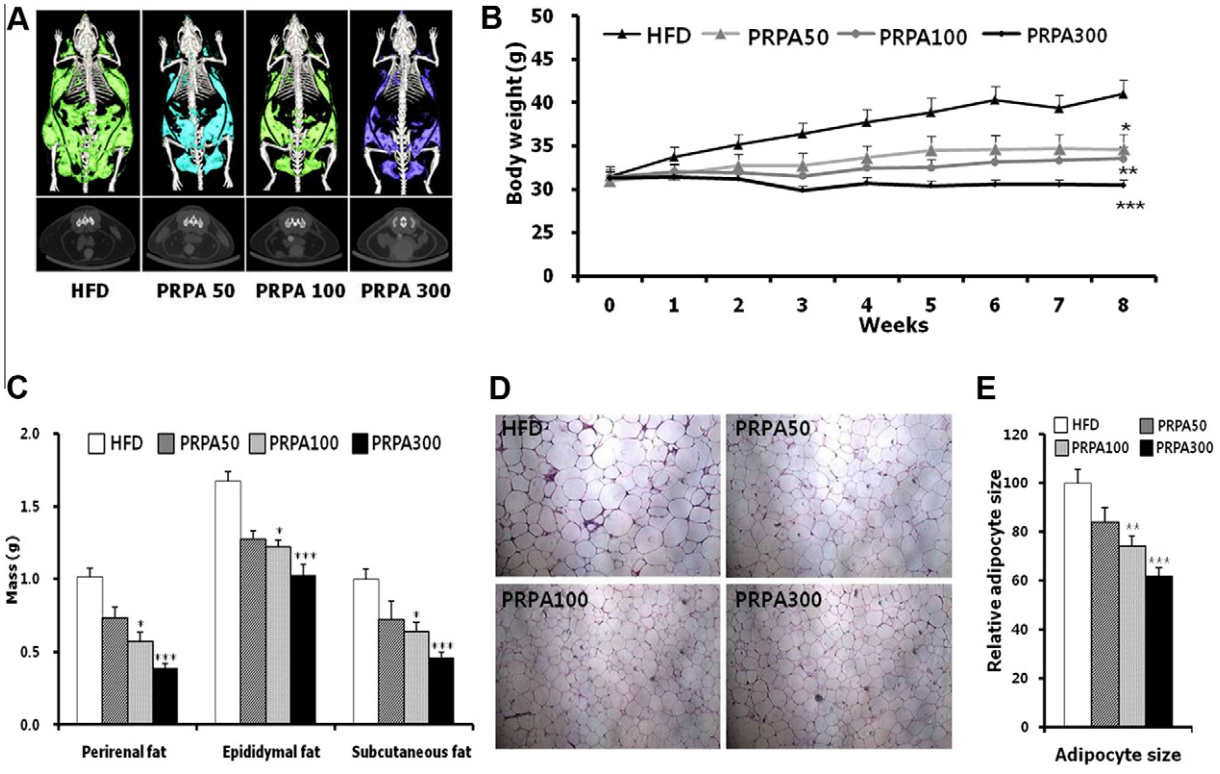

Fig. 2. PRPAs attenuate HFD-induced adiposity and weight gain by reducing fat pad and white adipocyte size. (A) Micro-CT images of the whole body and abdomen. Mice were fed a HFD, HFD with PRPA 50 mg/kg/day (PRPA 50), HFD with 100 mg/kg/day PRPA (PRPA 100), or HFD with 300 mg/kg/day PRPA (PRPA 300). (B) Body weight changes in mice fed a HFD, PRPA 50, PRPA 100, or PRPA 300 for 8 weeks. (C) Fat pad mass in mice fed a HFD, PRPA 50, PRPA 100, or PRPA 300. (D) Histological analysis of adipose tissue in mice fed a HFD, PRPA 50, PRPA 100, or PRPA 300 (hematoxylin and eosin staining, magnification, ×200). (E) Adipocyte size of adipose tissue in mice fed a HFD, PRPA 50, PRPA 100, or PRPA 300. Data are expressed as the mean ± SEM, ($n \geqslant 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$ compared with the HFD control group.

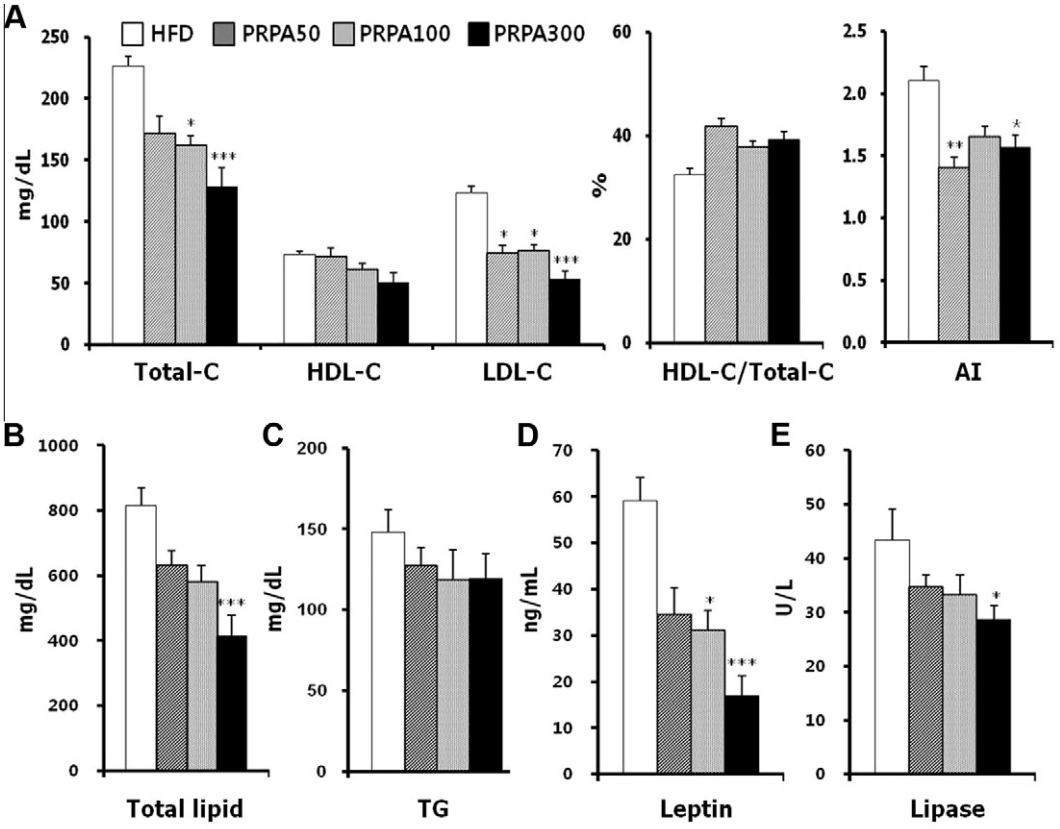

Fig. 3. PRPAs improve HFD-induced dysregulation of lipid metabolism. (A) The serum levels of Total-C, HDL-C, and LDL-C, the ratio of HDL-C/Total-C, and AI [(Total-C − HDL-C)/HDL-C]. (B) The serum levels of total lipids. (C) The serum levels of TGs. (D) The serum levels of leptin. (E) The serum levels of lipase were evaluated. Data are expressed as the mean ± SEM ($n \geqslant 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$ compared with the HFD control group.

TG levels (Fig. 3B and C) without altering the serum levels of hepatotoxic markers, such as glutamic oxaloacetic transaminase and glutamic pyruvic transaminase (data not shown). These results indicate that PRPAs suppress obesity-induced hyperlipidemia by decreasing the serum levels of lipids without altering those of liver toxicity markers.

The serum leptin levels reflect the amount of energy stored in adipose tissue, and a reduction in body weight induces a rapid decrease in serum leptin levels [22]. In this study, serum leptin levels were decreased in the PRPA 100 and 300 treatment groups (Fig. 3D), indicating that adipocyte size is decreased by body weight reductions. A gastrointestinal lipase inhibitor, such as Oristat, improves overall lipid profiles, including plasma LDL-C levels, by inhibiting dietary lipid absorption [23]. In the PRPA 300 treatment group, serum lipase levels were significantly decreased, and lipid profiles were improved (LDL-C and total lipid decrement) through the inhibition of dietary lipid absorption (Fig. 3E), consistent with the effects of Oristat. These data suggest that PRPAs improve the HFD-induced dysregulation of lipid metabolism, which results in protection against HFD-induced obesity.

3.4. PRPAs prevent the development of nonalcoholic fatty liver and decreases ectopic fat accumulation through fat burning

The development of non-alcoholic fatty liver disease is one of the most important characteristics of obesity [24]. Several findings suggest that AMPK and SREBP are critical regulators of hepatic lipid metabolism, and they inhibit hepatic steatosis in a HFD-induced animal model [25]. The accumulation of fat droplets in the liver was observed in the HFD control group, indicating fatty liver (Fig. 4A); however, fat droplet size was decreased in the PRPA treatment groups. Consistent with the *in vitro* results, PRPA treatment increased the phosphorylation of AMPK and ACC in the liver; however, it inhibited SREBP-1 expression and its target proteins such as FAS and ACC (Fig. 4D). Moreover, PRPA treatment increased the expression of lipolytic proteins such as CPT-1L, UCP2, and UCP3 (Fig. 4E). This result indicated that PRPA treatment suppressed lipogenesis and activated fat burning through AMPK signaling activation. This leads to the decrement of fat accumulation in the liver and attenuation of fatty liver in HFD-induced obese mice.

Skeletal muscle is a major site of fatty acid catabolism, and abnormal fat accumulation in skeletal muscle causes metabolic disorders including obesity and type-2 diabetes [26]. Histological analysis and micro-CT analysis revealed that ectopic fat accumulation was reduced; however, muscle contents were increased in PRPA treatment groups (Fig. 4B and C). Consistent with the results in the liver, PRPA treatment increased the phosphorylation of AMPK and ACC in muscle tissue. Moreover, PRPA treatment activated AMPK signaling through the regulation of fat-burning proteins (CPT-1, UCP2, and UCP3) (Fig. 4F). This leads to reduced ectopic fat accumulation in the skeletal muscle of HFD-induced obese mice. In addition, these findings suggest that mice in the PRPA treatment groups more readily used intra-muscular TGs as a energy source compared to mice in the HFD control group by activating lipid transfer proteins (CPT-1) and fat-burning proteins (UCPs). These results suggest that PRPAs decrease fat accumulation in the liver and skeletal muscle by activating AMPK and fat-burning proteins. These actions may be associated with reductions in fat pad size and body weight in HFD-induced mice.

The present study demonstrates that PRPA administration attenuates HFD-induced weight gain, normalizes serum lipid parameters, and reduces excessive lipid accumulation in white adipose

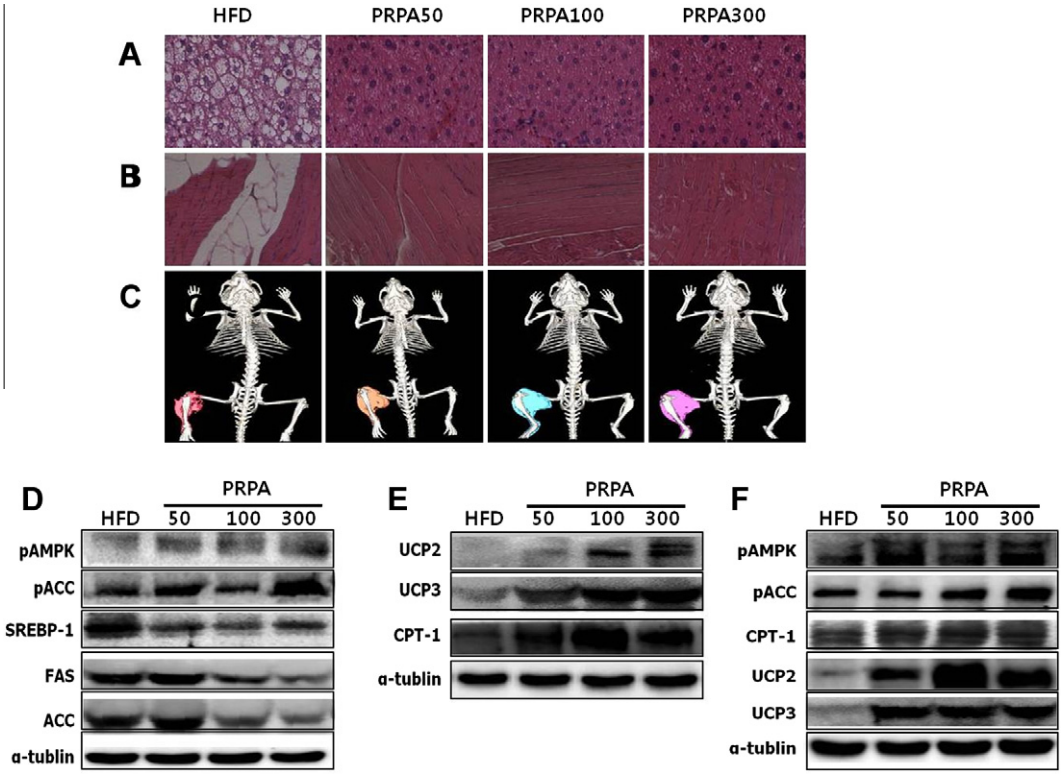

Fig. 4. PRPAs prevent the development of nonalcoholic fatty liver and decrease ectopic fat accumulation in skeletal muscle through fatty acid oxidation. (A) Histological analysis of liver of mice fed a HFD, PRPA 50, PRPA 100, or PRPA 300 (magnification, ×200) (each treatment group, $n \geqslant 6$). (B) Histological analysis of muscle tissue in mice fed a HFD, PRPA 50, PRPA 100, or PRPA 300 (magnification, ×200). (C) Micro-CT images of thigh muscle, the mice fed a HFD, PRPA 50, PRPA 100, or PRPA 300. (D) Western blot analysis of pAMPK, pACC, SREBP-1, FAS, and ACC expression in the liver. α-Tubulin was used as a protein loading control. (E) Western blot analysis of UCP2, UCP3, and CPT-1 expression in the liver. α-Tubulin was used as a protein loading control. (F) Western blot analysis of pAMPK, pACC, CPT-1, UCP2, and UCP3 expression in the skeletal muscle. α-Tubulin was used as a protein loading control. Western blots are representative of the findings for the six mice in each group.

and liver tissue by activating AMPK and regulating the expression of proteins involved in lipid metabolism. Piperidine alkaloids including PP, DPN, and PN were determined to be the anti-obesity constituents of *P. retrofractum*. Although the accurate molecular mechanism by which PRPAs exert anti-obesity effects remains to be elucidated, PRPAs exhibit potential as novel natural agents for the prevention and treatment of HFD-induced obesity and fatty liver disease.

Acknowledgments

This work was supported by a Grant from the Translational Research Center for Protein Function Control, NSF (2010-0001928) and in part by the Yonsei Biomolecule Research Initiative of the two-step Brain Korea 21 Project.

References

[1] J.P. Després, I. Lemieux, Abdominal obesity and metabolic syndrome, Nature 444 (2006) 881–887.

[2] C.F. Semenkovich, Insulin resistance and atherosclerosis, J. Clin. Invest. 116 (2006) 1813–1822.

[3] J.M. Olefsky, Fat talks, liver and muscle listen, Cell 134 (2008) 914–916.

[4] H. Choi, H. Eo, K. Park, M. Jin, E.J. Park, S.H. Kim, J.E. Park, S. Kim, A water-soluble extract from *Cucurbita moschata* shows anti-obesity effects by controlling lipid metabolism in a high-fat diet-induced obesity mouse model, Biochem. Biophys. Res. Commun. 359 (2007) 419–425.

[5] Y. Shi, P. Burn, Lipid metabolic enzymes: emerging drug targets for the treatment of obesity, Nat. Rev. Drug Discovery 3 (2004) 695–710.

[6] S.A. Kliewer, H.E. Wu, M.H. Lambert, T.M. Willson, Peroxisome proliferator-activated receptors: from genes to physiology, Recent Prog. Horm. Res. 56 (2001) 239–263.

[7] Y.S. Wang, C.L. Zhang, R.T. Yu, H.K. Cho, M.C. Nelson, C.R. Bayuga-Ocampo, J. Ham, J.H. Kang, R.M. Evans, Regulation of muscle fiber type and running endurance by PPAR delta, PLoS. Biol. 2 (2004) 1532–1539.

[8] D.G. Hardie, AMPK: a key regulator of energy balance in the single cell and the whole organism, Int. J. Obes. (Lond) 32 (2008) 7S–12S.

[9] M.E. Osler, J.R. Zierath, Adenosine 5′-monophosphate-activated protein kinase regulation of fatty acid oxidation in skeletal muscle, Endocrinology 149 (2008) 935–941.

[10] A.B. Ouadda, E. Levy, E. Ziv, G. Lalond, A.T. Sané, E. Delvin, M. Elchebly, Increased hepatic lipogenesis in insulin resistance and type 2 diabetes is associated with AMPK signaling pathway up-regulation in *Psammomys obesus*, Biosci. Rep. 29 (2009) 283–292.

[11] J.L. Goldstein, R.B. Rawson, M.S. Brown, Mutant mammalian cells as tools to delineate the sterol regulatory element-binding protein pathway for feedback regulation of lipid synthesis, Arch. Biochem. Biophys. 397 (2002) 139–148.

[12] H. Lee, Pesticidal constituents derived from Piperaceae fruits, Agric. Chem. Biotechnol. 48 (2005) 65–74.

[13] S. Tewtrakul, K. Hase, S. Kadota, T. Namba, K. Komatsu, Fruit oil composition of *Piper chaba* Hunt. *P. longum* L. and *P. nigrum* L., J. Essent. Oil Res. 2 (2000) 603–608.

[14] T. Morikawa, H. Matsuda, I. Yamahuchi, Y. Pongpiriyadacha, M. Yoshikawa, New amides and gastroprotective constituents from the fruit of *Piper chaba*, Planta Med. 70 (2004) 152–159.

[15] H. Matsuda, K. Ninomiya, T. Morikawa, D. Yasuda, I. Yamaguchi, M. Yoshikawa, Protective effects of amide constituents from the fruit of *Piper chaba* on D-galactosamine/TNF-α-induced cell death in mouse hepatocytes, Bioorg. Med. Chem. Lett. 18 (2008) 2038–2042.

[16] S.W. Lee, M.C. Rho, H.R. Park, J.H. Choi, J.Y. Kang, J.W. Lee, K. Kim, H.W. Lee, Y.K. Kim, Inhibition of diacylglycerol acyltransferase by alkamides isolated from the fruits of *Piper longum* and *Piper nigrum*, J. Agric. Food Chem. 54 (2006) 9759–9763.

[17] S. Wu, C. Sun, S. Pei, Y. Lu, Y. Pan, Preparative isolation purification of amides from the fruits of *Piper longum* L. by upright counter-current chromatography and reversed-phase liquid chromatography, J. Chromatogr. 1040 (2004) 193–204.

[18] K.L. Han, J.S. Choi, J.Y. Lee, J. Song, M.K. Joe, M.H. Jung, J.K. Hwang, Therapeutic potential of peroxisome proliferators-activated receptor-alpha/gamma dual agonist with alleviation of endoplasmic reticulum stress for the treatment of diabetes, Diabetes 57 (2008) 737–745.

[19] Y.S. Lee, W.S. Kim, K.H. Kim, M.J. Yoon, H.J. Cho, Y. Shen, J.M. Ye, C.H. Lee, W.K. Oh, C.T. Kim, C. Hohnen-Behrens, A. Gosby, E.W. Kraegen, D.E. James, J.B. Kim, Berberine, a natural plant product, activates AMP-activated protein kinase with beneficial metabolic effects in diabetic and insulin-resistant states, Diabetes 55 (2006) 2256–2264.
[20] V.A. Narkar, M. Downes, R.T. Yu, E. Embler, Y. Wnag, E. Banayo, M.M. Mihaylova, M.C. Nelson, Y. Zou, H. Juguilon, H. Kang, R.J. Shaw, R.M. Evans, AMPK and PPARδ agonists are exercise mimetics, Cell 134 (2008) 405–415.
[21] J.R. Downs, M. Clearfield, S. Weis, E. Whitney, D.R. Shapiro, P.A. Beere, A. Langendorfer, E.A. Stein, W. Kruyer, A.M. Gotto, Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol level, J. Am. Med. Assoc. 279 (1998) 1615–1622.
[22] R. Geldszus, B. Mayr, R. Horn, F. Geisthövel, A. Von zur Mühled, G. Brabant, Serum leptin and weight reduction in female obesity, Eur. J. Endocrinol. (1996) 659–662.
[23] T.D. Filippatos, D.P. Mikhailidis, Lipid-lowing drugs acting at the level of the gastrointestinal tract, Curr. Pharm. Des. 15 (2009) 490–516.
[24] H. Tilg, A.R. Moschen, Adipocytokines: mediators linking adipose tissue, inflammation and immunity, Nat. Rev. Immunol. 6 (2006) 772–783.
[25] M.H. Ahmed, C.D. Byrne, Modulation of sterol regulatory element binding proteins (SREBPs) as potential treatments for non-alcoholic fatty liver disease (NAFLD), Drug Discovery Today 12 (2007) 740–747.
[26] H. Yki-Järvinen, Ectopic fat accumulation: an important cause of insulin resistance in humans, J. R. Soc. Med. 95 (2002) 39–45.