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Heracleum dissectum Ledeb. ethanol extract attenuates metabolic syndrome symptoms in high-fat diet-induced obese mice by activating adiponectin/AMPK signaling

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ABSTRACT

The incidence of obesity has increased worldwide, leading to metabolic disorders such as hyperlipidemia and insulin resistance. Therefore, novel functional foods and therapeutic agents against obesity-related metabolic disorders are needed. We explored the effectiveness of the edible herb *Heracleum dissectum* Ledeb. (HD) against metabolic syndrome symptoms in a high-fat diet (60% of kcal) obesity mouse model. Metabolic syndrome was induced after 8 weeks of high-fat diet feeding, and HD extract (50 mg/kg) was administered by oral gavage during feeding. HD extract effectively decreased body weight gain and ameliorated the serum lipid status. HD extract also upregulated adiponectin/AMP-activated protein kinase (AMPK) signaling, and had anti-oxidation, anti-inflammation, and anti-insulin resistance effects. HD extract further elevated phosphorylation of AMPK and mitochondria biogenesis in the adipose tissue, resulting increased energy expenditure via the triglyceride/fatty acid cycle. Thus, HD extract could be considered a multi-targeting functional food candidate with novel molecular mechanisms.

1. Introduction

Recent environmental changes in lifestyle have drastically led to an increased prevalence of obesity, especially in developed countries, owing to affluence, excessive food consumption, and lack of exercise (Jeffery & Utter, 2003). In particular, the United States has been struggling with an explosive epidemic of obesity for decades, representing a serious public health issue, causing heavy social and economic burdens (Jia & Lubetkin, 2005). Moreover, obesity could progress to several complications, including metabolic syndrome, leading to a more complex pathophysiological state. Metabolic syndrome is diagnosed when patients meet three or more criteria of the following five criteria: abdominal obesity, hypertension, high triglyceride (TG), low high-density lipoprotein-cholesterol (HDL-C), and

Abbreviations: acyl-CoA, acyl coenzyme A; ALT, alanine aminotransferase; AdipoR1, adiponectin receptor 1; AdipoR2, adiponectin receptor 2; AMPK, AMP-activated protein kinase; Appl1, adaptor protein phosphotyrosine interacting with PH domain and leucine zipper 1; AST, aspartate aminotransferase; Atgl, adipose triglyceride lipase; ATP, adenosine triphosphate; AUC, area under the curve; BAT, brown adipose tissue; BSA, bovine serum albumin; C/EBPα, CCAAT/enhancer-binding protein α; CS, citrate synthase; CVD, cardiovascular disease; CPT-1, carnitine palmitoyltransferase-1; DIO, diet-induced obesity; DMEM, Dulbecco's modified Eagle medium; DsbA-L, disulfide-bond A oxidoreductase-like protein; ELISA, enzyme-linked immunosorbent assay; eWAT, epididymal white adipose tissue; FA, fatty acid; FBS, fetal bovine serum; GLUT4, glucose transporter type 4; GSK-3β, glycogen synthase kinase 3 beta Heracleum dissectum Ledeb; HDL-C, high-density lipoprotein cholesterol; HFD, high-fat diet; HO-1, heme oxygenase 1; Hsl, hormone-sensitive lipase; ICCT, Institute of Chemistry & Chemical Technology; IL-1β, interleukin-1β; IPITT, intraperitoneal insulin tolerance test; LDL-C, low-density lipoprotein cholesterol; MDA, malondialdehyde; MPO, myeloperoxidase; ND, normal diet; NHANES, National Health and Nutrition Examination Survey; NQO1, NAD(P)H quinone dehydrogenase 1; OGTT, oral glucose tolerance test; Pepck, phosphoenolpyruvate carboxykinase; PPARγ, peroxisome proliferator-activated receptor gamma; PRDM16, PR domain containing 16; qPCR, quantitative real-time polymerase chain reaction; Serca, Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; SOD, superoxide dismutase; SREBP-1, sterol regulatory element-binding transcription factor 1; TBST, Tris-buffered saline with Tween 20; TC, total cholesterol; TG, triglyceride; UCP1, uncoupling protein 1; UPLC-QTOF-MS/MS, ultra-high-performance liquid chromatography and quadrupole time-of-flight mass spectrometry; WAT, white adipose tissue.

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insulin resistance related to type 2 diabetes mellitus (Ascaso et al., 2003). Metabolic syndrome not only increases the occurrence of diabetes and cardiovascular disease (CVD) but is also associated with psychiatric illnesses such as depression (Reagan-Shaw, Nihal, & Ahmad, 2008; Simon et al., 2006). In the United States, almost one-fourth of the adult population suffers from metabolic syndrome, as per National Health and Nutrition Examination Survey (NHANES) data (Azizi, Salehi, Etemadi, & Zahedi-Asl, 2003). Given the pervasiveness of obesity worldwide, the development of anti-obesity drugs has progressed, with two main types of drugs now available patients: appetite suppressants that regulate noradrenergic or serotonergic signals, and lipase inhibitors that target the gastrointestinal tract (Yanovski & Yanovski, 2014). However, the side effects of these drugs with long-term use requires continued research to find safe functional food items for routine intake.

Although the therapeutic efficacy of appetite suppressors and lipase inhibitors have been proven in patients with obesity, energy metabolism-regulating agents have recently come into the spotlight as a novel target. For example, hydroxycitric acid derived from *Garcinia* plant is a typical energy metabolism regulator that disturbs *de novo* lipogenesis and enhances fatty acid oxidation, and its use resulted in significant weight loss in clinical studies (Tomar et al., 2019). Adiponectin, a major adipokine generated and secreted from the adipose tissues, is one of the key energy homeostasis regulators in the body, and patients with obesity have been reported to have reduced adiponectin levels and reduced adiponectin sensitivity (Kadowaki & Yamauchi, 2005). In a case study of a human population, the serum adiponectin level and body mass index showed a prominent negative correlation, and low adiponectin levels were associated with an increased incidence of type 2 diabetes mellitus (Arita et al., 1999). In addition, the plasma adiponectin level and TG content in the blood show a negative relationship associated with an increased CVD risk (Zhang et al., 2018). A critical role of adiponectin is activating AMP-activated protein kinase (AMPK) in energy homeostasis-related tissues such as the liver, muscle, and adipose tissue, and expedites glucose uptake and fatty acid (FA) oxidation in these tissues (Bonnard, Durand, Vidal, & Rieusset, 2008). In a series of energy metabolism mediation processes, AMPK promotes adenosine triphosphate (ATP) production by regulating glucose, protein, and lipid metabolism, and enhancing biogenesis of the mitochondria (Herzig & Shaw, 2018; Ke, Xu, Li, Luo, & Huang, 2018; Wang, Liu, Zhai, Zhang, & Tian, 2018). Moreover, AMPK activation improves antioxidative and anti-inflammatory responses in the cellular system (Wu et al., 2018), and relieves multiple symptoms of metabolic syndrome, especially insulin resistance (Teng, Chen, Fang, Yuan, & Lin, 2017).

Heracleum dissectum Ledeb. (HD) is a traditional vegetable of China, belonging to the Apiaceae family, and has long been used for treating rheumatoid diseases and headaches in folk medicine in China and Mongolia (Sun & Liu, 2007), and its anti-diabetic effect was revealed in a recent study (Zhang et al., 2017). We recently showed that HD extract had an anti-obesity effect based on *in vitro* screening; thus, we hypothesized that it would also be capable of mitigating metabolic syndrome symptoms. To test this hypothesis, we established a mouse model of diet-induced obesity (DIO); although this model takes longer to establish than other obesity models, it mimics the pathogenesis of obesity and metabolic syndrome in humans fairly well (Kuraji, Fujita, Ito, Hashimoto, & Numabe, 2018). Using a DIO model, we aimed to verify the effectiveness of HD in treating obesity and metabolic syndrome, along with the underlying molecular mechanisms. These findings can provide a scientific basis for developing HD as a multi-targeting functional food to alleviate obesity and metabolic syndrome.

2. Materials and methods

2.1. Plant materials

HD was collected from the vicinity of Ulaanbaatar, Mongolia. The collection of plant materials was conducted with the approval of Institute of Chemistry & Chemical Technology (ICCT) of Mongolia. The plant samples were classified by Dr. Batsuren of the ICCT, and a voucher specimen is stored at the Mongolian Academy of Sciences (2019/054). Approximately 200 g of dried powder of the entire epigeal part of the HD plant was mixed with 2 L of ethanol, and stored in the dark for 7 d at 20℃. After filtration of the liquid part, the extraction was repeated once more for the remaining powder using 2 L of ethanol. The final HD extract was obtained by completely evaporating the ethanol using a rotary evaporator (RE111; Büchi, Flawil, Switzerland).

2.2. Animal model and treatment

A total of 44 eight-week-old male C57BL/6N mice were provided by Orient Bio (Sungnam, Korea). The mice were placed in isolation cage and acclimated to the laboratory environment for 1 week under a 12-h light and 12-h dark cycle in a room maintained at 24 ± 0.5 ℃. The mice were then divided into four groups based on their body weight to standardize the initial body weight condition across experimental group. During the 8-week experimental period, the normal diet (ND) group (n = 12) was provided Teklad 10% kcal adjusted calories diet (TD.06416; Envigo, Indianapolis, IN, USA). The high-fat diet (HFD) group (n = 12), orlistat (positive control) group (n = 12), and HD group (n = 8) were provided Teklad 60% kcal adjusted calories diet (TD.06414; Envigo). Diet and water were provided *ad libitum* throughout the experiment.

To determine the dosage of HD extract, we first searched the relevant literature. The HD is mostly known for an edible plant and it has been used as a vegetable in China. We found only one animal study using HD extract, which reported that the 50% lethal dose of HD was over 5,000 mg/kg (Zhang et al., 2017). In addition, they fed 125–250 mg/kg HD to mice daily for a total of 3 weeks without toxic effects. Referring to this previous study, we set the dosage of HD extract for the mice to 50 mg kg^{-1} d^{-1}, which corresponds with a dose of 4.05 mg kg^{-1} body weight per day for an adult human (Reagan-Shaw et al., 2008). In turn, the vehicle solution (0.5% carboxymethyl cellulose) was administered to the ND and HFD groups, and a vehicle solution containing orlistat (30 mg/kg) or HD ethanol extract (50 mg/kg) was administered to the orlistat or HD group, respectively. Orlistat was used as a positive control drug owing to its broad use in the human population. The administration of each solution was performed once a day by oral gavage throughout the 8-week experimental period.

After the 8-week experimental period, the mice were anesthetized by intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (12 mg/kg). Blood was collected from the heart while under anesthesia, and the mice were euthanized thereafter. The animal study was planned and executed as per the National Institutes of Health guidelines. The animal experiments were approved by the Institutional Animal Care and Use Committee of Korea Institute of Science and Technology (Approval no.: KIST-2019–011).

2.3. Assessment of adipocyte cell size in the epididymal white adipose tissue (eWAT)

The eWAT was fixed in a paraffin block and a section of the eWAT was stained using hematoxylin and eosin. The images of tissues were reconstituted and assessed with ImageJ software (National Institutes of Health, Bethesda, MD, USA) as per a previous study (Parlee, Lentz, Mori, & MacDougald, 2014).

2.4. Colorimetric and enzyme immunosorbent assay (ELISA) kit analysis

The contents of alanine aminotransferase (ALT), aspartate aminotransferase (AST), TG, total cholesterol (TC), HDL-C, and ghrelin were determined using assay kits from Elabscience (Houston, TX, USA). The low-density lipoprotein cholesterol (LDL-C), malondialdehyde (MDA), and myeloperoxidase (MPO) were analyzed with assay kits from Bio-Vision (Milpitas, CA, USA). The citrate synthase (CS) activity was

detected using a kit from Sigma-Aldrich (St. Louis, MO, USA). The contents of leptin, insulin, interleukin-1β (IL-1β), and adiponectin were determined using assay kits from RayBiotech (Peachtree Corners, GA, USA).

2.5. Cell culture and adipogenesis induction

Murine pre-adipocytes (3T3-L1) were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were maintained using Dulbecco's modified Eagle medium (DMEM; Hyclone, Logan, UT, USA) containing 10% bovine calf serum (Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin–streptomycin (Hyclone). For the induction of adipogenesis, cells were seeded in a 6-well plate (1.5×10^5 cells/well). Two days after the cells reached 100% confluence, the culture medium was replaced by DMEM containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific), 1% penicillin–streptomycin solution, 0.1% 0.5 mM 3-isobutyl-1-methylxanthine, 0.05% 2.5 mM dexamethasone, and 0.05% 10 mg/mL insulin. After day 3, the culture medium was changed to DMEM containing 10% FBS, 1% penicillin–streptomycin, and 0.1% 10 mg/ml insulin, which was then maintained until day 9 with refreshing the same medium at day 6. The cells were treated with HD extract or bergenin (ChemFaces, Hubei, China) throughout the differentiation-inducing periods; that is, the cells were treated in differentiation-inducing media from day zero to day nine.

2.6. Determination of lipid accumulation in 3T3-L1 cells

The accumulation of lipids in 3T3-L1 cells was examined using Oil red O staining (Ramirez-Zacarias, Castro-Munozledo, & Kuri-Harcuch, 1992). To quantify lipid accumulation, the stained Oil red O solution was reconstituted with isopropanol and the absorbance was measured at 510 nm. The TG content was examined from the cell supernatants using a colorimetric assay kit (Biovision). Cell viability was determined using EZ-Cytox reagent (DoGEN, Seoul, Korea) following the manufacturer's protocol.

2.7. Western blot analysis

The mouse eWAT and liver tissues or 3T3-L1 cells were lysed in a protein inhibitors cocktail (Sigma-Aldrich) containing RIPA buffer, and the protein contents were quantified by the Bradford assay. Lysates were mixed with loading dye and electrophoresis was conducted using a sodium dodecyl sulfate–polyacrylamide gel electrophoresis system. After transferring the proteins to the polyvinylidene fluoride membrane, the membranes were soaked in blocking solution, 3% bovine albumin serum (BSA) in Tris-buffered saline with 0.1% Tween 20 (TBST), for 1 h. After washing the membranes with TBST solution for 5 min three times, they were incubated with primary antibodies in 1% BSA-TBST solution at 4℃ overnight. The membranes were rinsed with TBST solution three times, soaked in 1% BSA-TBST solution containing anti-mouse or anti-rabbit secondary antibodies for 1 h at 20℃, and then washed with TBST solution three times again. The chemiluminescence signals were detected using LAS-3000 Bio Imaging System (Fuji Film Co., Tokyo, Japan) by developing the membranes using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). The sterol regulatory element-binding transcription factor 1 (SREBP-1), carnitine palmitoyltransferase-1 (CPT1), peroxisome proliferator-activated receptor gamma (PPARγ), heme oxygenase 1 (HO-1), NAD(P)H quinone dehydrogenase 1 (NQO1), superoxide dismutase 1 and 2 (SOD1 and SOD2), glycogen synthase kinase 3 beta (GSK-3β), p-GSK-3β (Ser9), and glucose transporter type 4 (GLUT4) primary antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Primary antibodies against CCAAT/enhancer-binding protein α (C/EBPα), AMPK, p-AMPK, cytochrome *C*, Akt, and p-Akt (Ser473) were obtained from Cell Signaling Technology (Danvers, MA, USA). The antibodies for uncoupling protein 1 (UCP1) and PR domain containing 16 (PRDM16) were obtained from Abcam (Cambridge, UK).

2.8. Quantitative real-time polymerase chain reaction (qPCR)

RNA was extracted from the mouse eWAT tissue using an RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer instructions. The cDNA was synthesized using a PrimeScript 1st Strand cDNA Synthesis kit (Takara, Kusatsu, Japan). The gene expression of samples was analyzed by qPCR analysis with a LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland) using SYBR Green Master Mix solution (Hoffmann-La Roche Ltd., Schweiz, Switzerland). The primer sequences used in this study are shown in Table S1.

2.9. Glucose and insulin tolerance test

The oral glucose tolerance test (OGTT) and intraperitoneal insulin tolerance test (IPITT) were both performed on the 7th week of the experiment. Before testing, the mice were fasted for 6 h and 2 g/kg glucose solution was orally administered for the OGTT and 0.8 U/kg insulin was injected intraperitoneally for the IPITT. Glucose levels were tested in the tail vein blood using Accu-Chek meter (Roche) at 0, 30, 60, 120, and 180 min after treatment.

2.10. Ultra-high-performance liquid chromatography and quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS/MS)

Chemical analysis of the HD extract was performed using UPLC-QTOF-MS/MS. The chromatogram of the HD extract was examined by UltiMate 3000 (Thermo Fisher Scientific) with a Phenomenex Kinetex C18 column (2.1 × 150 mm, 1.7 μm). Solvent A was water with 0.1% formic acid and solvent B was acetonitrile with 0.1% formic acid. The gradient of solvents A:B was as follows: 0–10 min, 99:1; 15 min, 90:10; 30 min, 70:30; 40 min, 30:70; 41–44 min, 0:100; 45–50 min, 99:1. The flow rate was 0.2 mL/min and detection was conducted at 254 nm. The mass signal was detected using a Triple TOF 5600 system (AB SCIEX, Foster City, CA, USA). Both positive and negative modes were analyzed and an electrospray ionization source was used. The mass scan range was set to 50–2,000 *m/z*, and was operated in full scanning mode. Nitrogen gas was used as the collision gas and the desolvation temperature was set to 500 ℃. The ion-spray voltage was 5.5 kV and 4.5 kV for the positive and negative mode, respectively. The collision-induced dissociation energy was set as 35 ± 15 eV for positive mode and at –35 ± 15 eV for negative mode.

2.11. Statistical analysis

All data are expressed as mean ± standard error of the mean. Statistical comparison across experimental groups was conducted using one-way analysis of variance and Duncan's multiple range test. Statistical analysis was conducted using SPSS Statistics v25 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. HD intake decreased weights of the body and WATs in the DIO mouse model

After eight weeks of inducing obesity in mice, the difference in final body weight between the ND and HFD groups was 8.5 g (Table 1). Both the HD extract and the positive control orlistat caused significant loss of body weight compared to the HFD ($p < 0.05$), and the weights of three kinds of WATs were significantly reduced in both the orlistat and HD groups ($p < 0.05$). Daily calorie intake was the highest in the orlistat group, and there was no significant difference between that in HFD and HD groups. The serum levels of leptin and ghrelin were significantly decreased in the HD group compared with those of the HFD group ($p <$

Table 1
Whole-body and fat-tissue weights, daily diet intake, and hormone levels of HD-administered diet-induced obesity (DIO) mice.

	ND	HFD	ORL	HD
Body weight				
Initial body weight (g)	22.23 ± 0.28	22.38 ± 0.37	22.27 ± 0.23	22.36 ± 0.37
Final body weight (g)	26.95 ± 0.44^{c}	35.47 ± 0.73^{a}	32.34 ± 0.56^{b}	33.75 ± 1.00^{b}
Weight gain (g)	4.72 ± 0.35^{c}	13.08 ± 0.77^{a}	10.07 ± 0.44^{b}	11.39 ± 0.79^{b}
White adipose tissue weight				
Epididymal (g)	0.75 ± 0.04^{c}	2.20 ± 0.12^{a}	1.56 ± 0.13^{b}	1.43 ± 0.18^{b}
Mesenteric (g)	0.34 ± 0.02^{c}	0.74 ± 0.05^{a}	0.51 ± 0.04^{b}	0.47 ± 0.04^{b}
Perirenal (g)	0.33 ± 0.02^{c}	0.94 ± 0.04^{a}	0.74 ± 0.05^{b}	0.63 ± 0.08^{b}
Daily diet intake				
Daily diet intake (g body weight^{-1})	0.10 ± 0.00^{a}	0.08 ± 0.00^{b}	0.09 ± 0.00^{a}	0.08 ± 0.00^{b}
Daily calorie intake (Kcal body weight^{-1})	0.35 ± 0.01^{c}	0.41 ± 0.02^{b}	0.46 ± 0.02^{a}	0.41 ± 0.02^{b}
Hormone level in serum				
Leptin (pg/mL)	28.19 ± 5.42^{c}	142.50 ± 13.65^{a}	133.96 ± 13.24^{a}	96.07 ± 38.41^{b}
Ghrelin (pg/mL)	348.19 ± 24.16^{b}	442.47 ± 49.47^{a}	317.39 ± 21.15^{b}	293.68 ± 3.67^{b}

Different lowercase superscripts within rows indicate significant differences between groups at $p < 0.05$.

0.05), although the ratio of leptin to ghrelin remained unchanged. The weights of the major organs (liver, kidney, spleen, and heart) and levels of serum ALT and AST were determined to check the potential toxicity; there was no evident toxic effect observed in all mice groups (Table S2).

3.2. Adipocyte size in the eWAT and serum lipid parameters were ameliorated by HD intake in DIO mice

The morphology and adipocyte size of the eWAT were verified using images of tissue sections (Fig. 1A–C). Administration of orlistat and HD reduced the proportion of larger adipocytes, and the average size of adipocyte was significantly decreased in these groups compared with that of the HFD group ($p < 0.05$). There was no change regarding adipogenesis (PPARγ and C/EBPα), lipid metabolism (SREBP-1), and lipid oxidation (CPT-1)-related protein expression in the orlistat and HD groups (Fig. 1D). Administration of HD extract significantly reduced TG, TC, and LDL-C levels in the serum ($p < 0.05$), but there was no decrease of serum HDL-C levels in the HD group compared with that in the HFD group (Fig. 1E–H). Therefore, HD extract notably improved hyperlipidemia in the DIO mouse model.

3.3. HD extract elevated adiponectin/AMPK signaling and stimulated biogenesis of the mitochondria in the eWAT

Although there was loss of weight in the HD group, there was no significant difference in calorie intake between the HFD and HD groups (Table 1). These results suggest that HD extract could stimulate energy expenditure mechanisms. To test this possibility, we determined the serum level of adiponectin, an adipokine that is an upstream hormone for AMPK activation (Fig. 2A). The HD extract highly increased the serum adiponectin level, and also upregulated the mRNA expression of adiponectin-related genes (Fig. 2B). The mRNA levels of *adiponectin*, the adiponectin synthesis-related gene, and disulfide-bond A oxidoreductase-like protein (*DsbA-L*), related to the maturation and secretion of adiponectin, were significantly elevated in the HD group compared with those of the HFD group ($p < 0.05$). In addition, the mRNA expression levels of adiponectin receptor-related genes such as adiponectin receptor 1 (*AdipoR1*) and adiponectin receptor 2 (*AdipoR2*), and adaptor protein phosphotyrosine interacting with PH domain and leucine zipper 1 (*Appl1*) in the HD group were significantly higher than those of the HFD group ($p < 0.05$). To verify the stimulation of HD extract on adiponectin/AMPK signaling and energy expenditure, the mitochondria content was first examined by a CS activity assay (Fig. 2C). The HD group showed significantly increased CS activity in the eWAT compared with that of the other groups ($p < 0.05$). At the molecular level, the phosphorylation of AMPK was significantly increased by HD extract treatment ($p < 0.05$), and protein levels of PRDM 16 and cytochrome C were also significantly increased (Fig. 2D). However, the level of UCP1, a major thermogenesis channel, was not elevated by HD treatment. Therefore, we predicted that the HD extract upregulates thermogenesis mechanisms via AMPK stimulation rather than via UCP1-dependent signaling. The stimulation of ATP synthesis in the eWAT and UCP1-independent thermogenesis mechanisms generally require enhanced mitochondrial functions; therefore, we further evaluated the mRNA expression of genes related to mitochondrial biogenesis. The HD group showed significantly elevated mRNA expression levels of mitochondrial biogenesis-related markers (Fig. 2E; $p < 0.05$), similar to the result of CS activity. Lastly, we also determined the effects of HD extract on lipid catabolism, FA synthesis, and glyceroneogenesis-related genes. As shown in Fig. 2F, genes related to the TG/FA cycle, such as adipose triglyceride lipase (*Atgl*), hormone-sensitive lipase (*Hsl*), and phosphoenolpyruvate carboxykinase (*Pepck*), were significantly increased in the HD group compared with those in the HFD group ($p < 0.05$). The circulation of this cycle requires futile energy input; therefore, the activation of this cycle may have caused the usage of synthesized ATPs.

3.4. HD extract upregulated anti-oxidation-related proteins and alleviated inflammation in DIO mice

Excessive accumulation of lipids in animal tissues causes oxidative stress and inflammatory responses. Administration of HD extract resulted in a decreased MDA content (a lipid peroxidation marker), and enhanced expression of anti-oxidative-related proteins (Fig. 3A, B). In addition, the HD group showed significantly reduced serum IL-1β and MPO levels in eWAT, and reduced mRNA expression levels of inflammatory response genes in the eWAT ($p < 0.05$) (Fig. 3C–E). The total macrophage and M1 macrophage markers (*F4/80* and *CD11c*) were also significantly mitigated in the HD group ($p < 0.05$).

3.5. HD treatment improved insulin sensitivity in the eWAT and liver of DIO mice

In the OGTT, the fasting glucose level was higher in the HFD group than in the HD group, and the HD group showed faster recovery of the serum glucose level after oral glucose intake; the area under the curve (AUC) of the HD group was significantly decreased compared with that of the HFD group ($p < 0.05$) (Fig. 4A, B). Similarly, HD administration enhanced insulin sensitivity in the IPITT, with a significant decrease in the AUC compared with that of the HFD group ($p < 0.05$; Fig. 4C, D). Although there was no significant difference in the fasting serum insulin level, the HOMA-IR, an important marker of insulin resistance, was significantly reduced in the HD group ($p < 0.05$) (Fig. 4E, F). At the molecular level, phosphorylation of Akt was upregulated in the eWAT by HD treatment (Fig. 4G). We also verified the glycometabolism of the liver, and found that the HD extract upregulated the phosphorylation of Akt and GSK-3β along with GLUT4 protein expression in the liver (Fig. 4H). Hence, HD extract alleviated insulin resistance in DIO mice, mediating related signaling in the eWAT and liver.

3.6. HD extract and its major compound, bergenin, alleviated lipid accumulation in vitro

Using UPLC-MS/MS, we identified five major compounds in the HD ethanol extract (Figure S1). The largest peak in the HPLC chromatogram

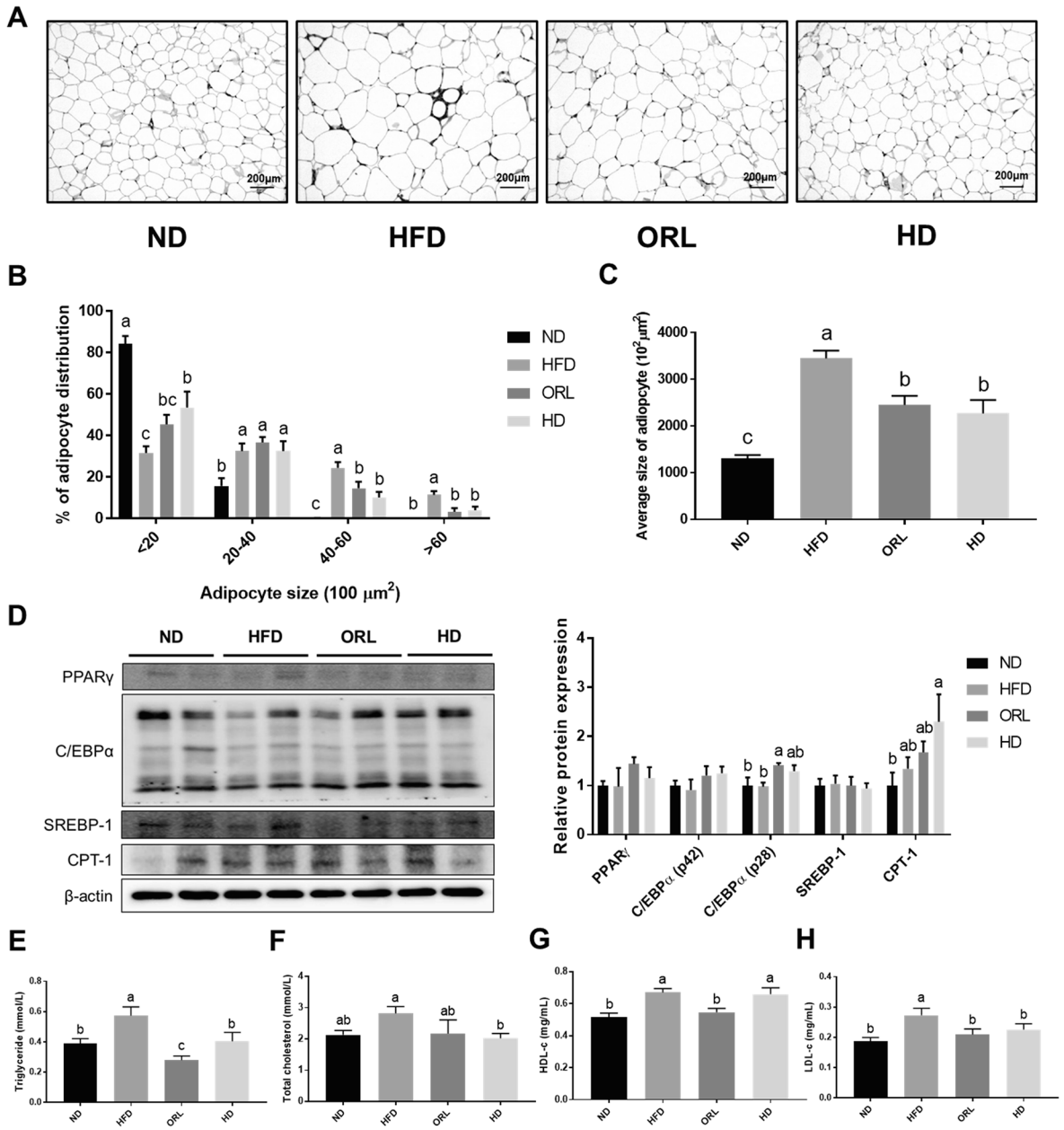

Fig. 1. HD treatment decreased adipocyte size of epididymal white adipose tissue (eWAT) and alleviated hyperlipidemia in DIO mice. (A) eWAT tissue section was visualized with H&E staining (×100 magnification). (B) Distribution of adipocyte sizes and (C) average size of adipocytes of eWAT were calculated by ImageJ software. (D) Expression of proteins related to adipogenesis, lipid synthesis, and lipid oxidation in eWAT. (E) Serum triglyceride levels. (F) Serum total cholesterol levels. (G) Serum high-density lipoprotein cholesterol (HDL-C) levels. (H) Serum low-density lipoprotein cholesterol (LDL-C) levels. Statistical differences within groups were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple range test. Different lowercase superscripts indicate significant differences between groups at $p < 0.05$.

was revealed to bergenin, and its effectiveness on obesity and type 2 diabetes were reported in previous studies (Ambika & Saravanan, 2016; Shikov et al., 2012). Since the most abundant chemical compound in the HD extract was bergenin, we additionally verified whether bergenin could alleviate lipid accumulation in the 3T3-L1 cell line. First, we checked the cell toxicity of the HD extract and bergenin in 3T3-L1 cells, and found no significant differences in cell viability compared with negative control cells up to 100 μg/mL or 100 μM for HD extract and bergenin treatment, respectively (Figure S2). The HD extract decreased lipid accumulation and TG synthesis of 3T3-L1 cells (Fig. 5A, B), and similar effects were found with bergenin treatment (Fig. 5C, D). We also found the bergenin successfully regulates adipogenesis, lipid metabolism, and lipid oxidation related proteins (Fig. 5E), and the result was opposite to our animal study data. Therefore, we conjecture that the therapeutic efficacies of HD extract stemmed from an integrated effect of multiple constituents in HD extract, and not solely the contribution of the major component, bergenin. However, further study is needed to verify it.

4. Discussion

Metabolic syndrome represents a range of several disorders, and is mostly caused by obesity. Although the pathogenesis of metabolic syndrome from obesity is not yet completely elucidated, some obesity-related complications such as increased serum lipid levels and inflammatory cytokines are explicit risk factors of metabolic syndrome

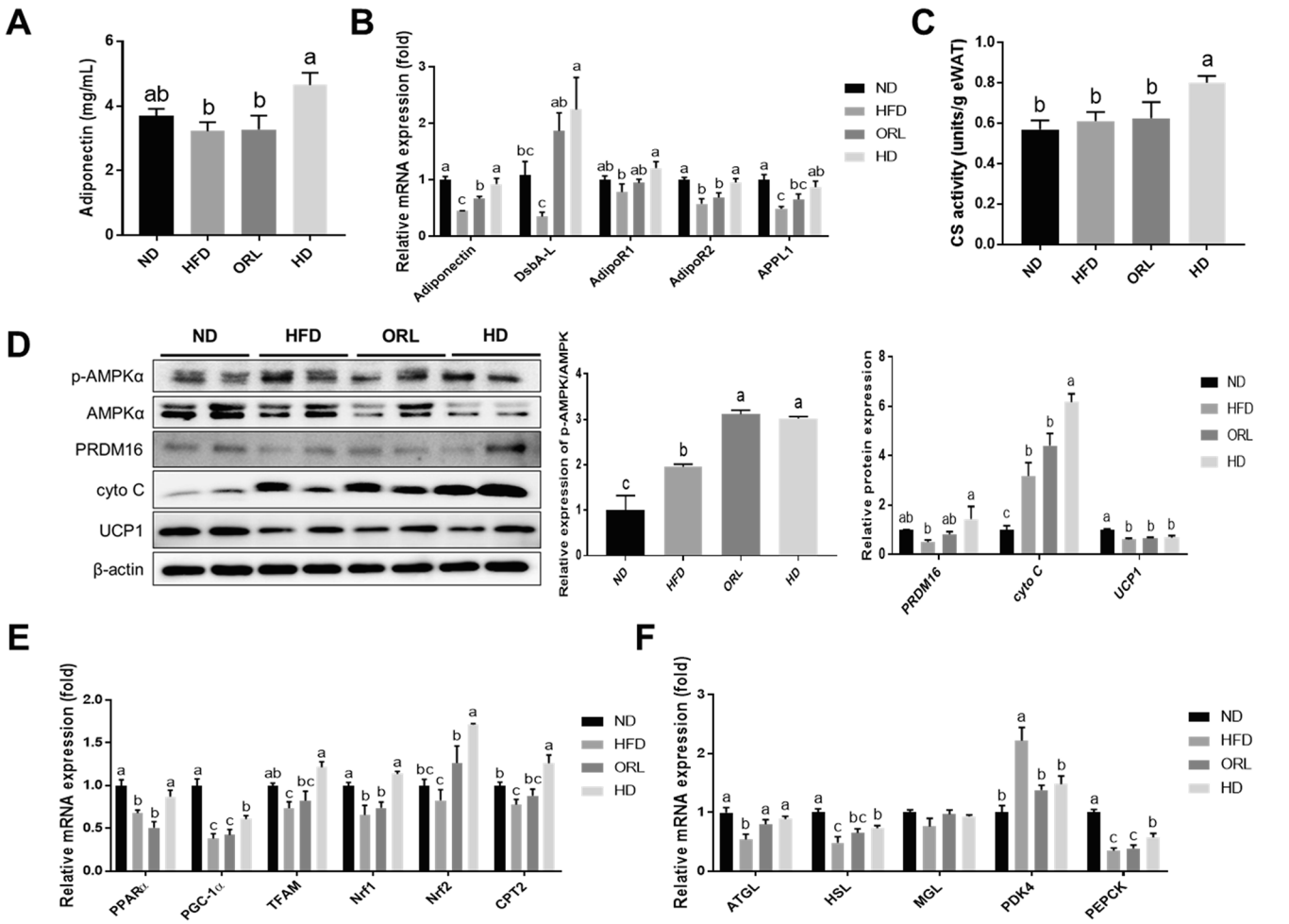

Fig. 2. HD intake stimulated adiponectin/AMP-activated protein kinase (AMPK) signaling and it evoked uncoupling protein 1 (UCP1)-independent energy consumption in eWAT of DIO mice. (A) Adiponectin content in serum. (B) The mRNA level of genes related with adiponectin synthesis, secretion, and its receptors in eWAT. (C) Citrate synthase activity of eWAT. (D) Protein expression of AMPK and p-AMPK, and other energy expenditure related markers in eWAT. (E) The relative mRNA expression of mitochondrial biogenesis and lipid oxidation related genes in eWAT. (F) The relative mRNA expression of tryglyceride (TG)/fatty acid (FA) cycle related genes in eWAT. Statistical differences within groups were examined by ANOVA and Duncan's multiple range test. Different lowercase superscripts indicate significant differences between groups at $p < 0.05$.

(Grundy, 2004). This indicates that the first goal for treating metabolic syndrome is to lose weight. Here, we showed that HD extract effectively reduced body weight in DIO mice and also decreased the weights of WATs from different locations. HD extract also mitigated the elevated TG and TC content in the serum, and decreased the serum LDL-C level (compared with those of the HFD group) but without a decrease in the HDL-C level. A high TG content and low HDL-C level are the main criteria for a diagnosis of metabolic syndrome, and are closely related to CVD risk (de Freitas et al., 2011). The ratios of TC/HDL-C and LDL-C/HDL-C have been used as CVD risk factors (Millán et al., 2009), and HD administration ameliorated both of these factors. Interestingly, both obesity and serum lipid status were improved in the HD group, whereas adipogenesis and lipid synthesis-related protein expression remained unchanged. For this reason, we suspect that the loss of weight in the HD group was mainly due to energy expenditure mechanisms rather than by disturbing adipogenesis and lipid synthesis in the WAT. The increased burn in calories in HD group is in line with the diet intake results since there was no difference in total energy intake between the HFD and HD groups.

There are two major types of adipose tissues, namely the WAT and the brown adipose tissue (BAT), with opposite roles of fat storage and energy expenditure with thermogenesis, respectively (Marlatt & Ravussin, 2017). However, some previous studies that investigated energy expenditure in animal models noted the expression of UCP1-dependent thermogenesis in the WAT (Shabalina et al., 2013). There is a new type of adipocyte tissue proposed, named "beige" or "brown-like", whose function resembles that of the BAT but is converted from the WAT (Desjardins & Steinberg, 2018). PRDM16 is a key mediator of brown fat differentiation that is selectively expressed in the BAT (Seale et al., 2007); therefore, PRDM16 is commonly used as a marker of switching energy expenditure mechanisms in the WAT to the brown-like adipose tissue (Hilton, Karpe, & Pinnick, 2015). In this study, the HD extract strongly stimulated the expression of PRDM16 in the eWAT, and cytochrome C protein expression and AMPK phosphorylation were also significantly elevated. The increase of cytochrome C represents excitation of the electron transport system, further supporting that HD extract stimulates energy consumption in the WAT. However, the expression level of UCP1 remained unchanged despite the increase of PRDM16 in the HD group, which generally accompanies activation of thermogenesis reactions via the UCP1 channel. Hence, we concluded that HD extract likely stimulates the electron transport system, with the translocated H^+ ions mainly passing through ATP synthase rather than the UCP1 channel (Letts & Sazanov, 2017). This pathway is known as UCP1-independent energy expenditure and its mechanism is unusual and unclear; however, a previous study indicated that *Ucp1*$^{-/-}$ mice can also exploit thermogenic responses in a cold-acclimated mice model (Ukropec,

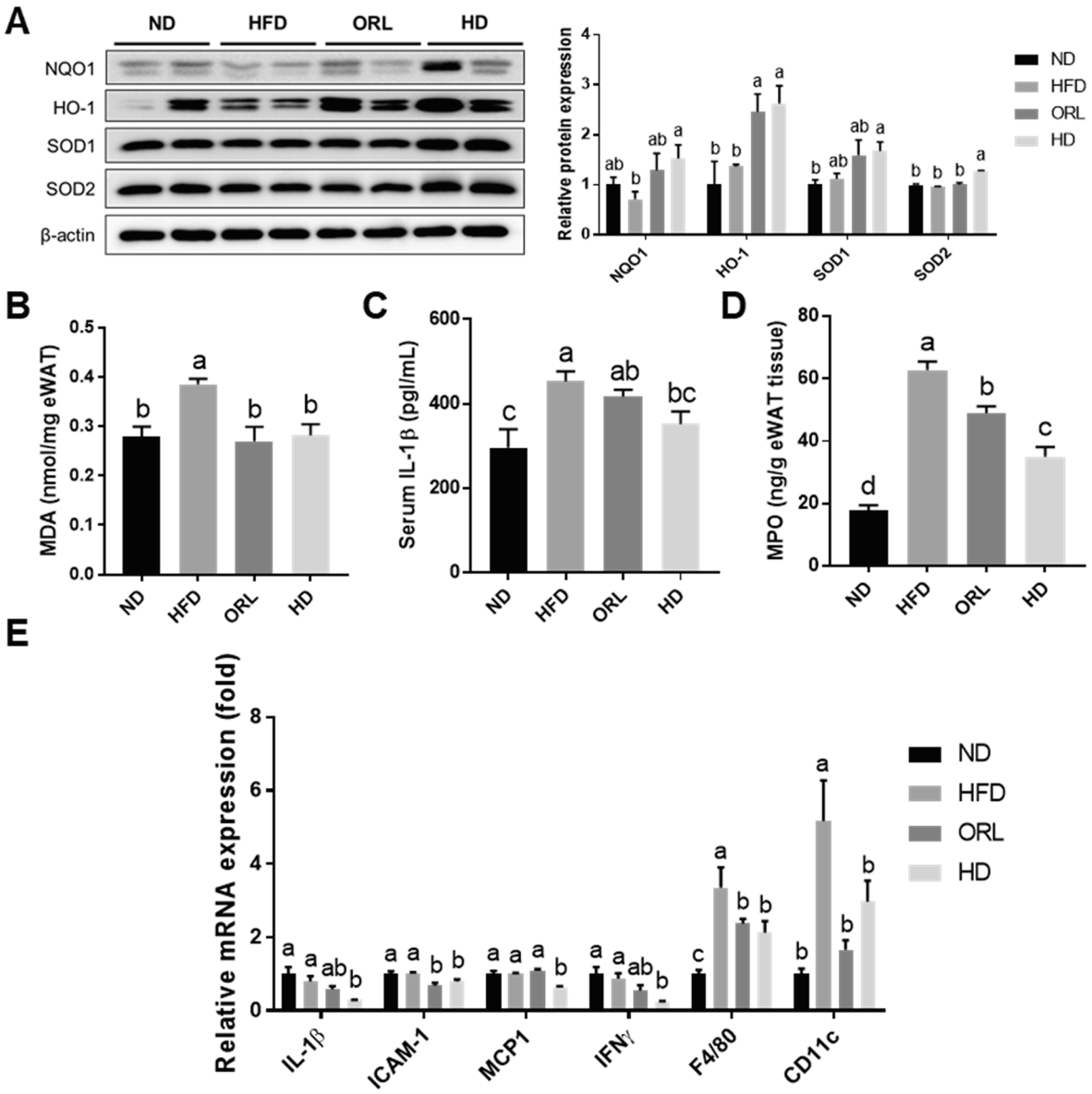

Fig. 3. HD extract upregulated anti-oxidation related proteins and mitigated inflammatory responses in DIO mice. (A) Expression of anti-oxidative proteins in eWAT (B) The malondialdehyde content of eWAT. (C) Serum IL-1β content. (D) Myeloperoxidase amounts in eWAT. (E) The relative mRNA expression of cytokine, chemokine, adhesive molecules, and macrophage markers in eWAT. Statistical differences within groups were determined by ANOVA and Duncan's multiple range test. Different lowercase superscripts indicate significant differences between groups at $p < 0.05$.

Anunciado, Ravussin, Hulver, & Kozak, 2006). Another study suggested that UCP1-independent energy expenditure may involve ATP-related mechanisms through chronic AMPK activation, which increases mitochondrial biogenesis and the oxidative capacity of the WAT (Ceddia, 2013). In line with this proposed mechanism, HD extract treatment upregulated the mRNA expression of *Pgc-1α/Nrf1/Tfam,* which is a key signaling pathway of mitochondrial biogenesis (Chen, Tao, Li, & Yao, 2018; Kang, Chu, & Kaufman, 2018), and the increased CS activity further indicated an increase in mitochondria abundance in the eWAT. The swelling of mitochondria enables the WAT to generate energy by increasing the oxidative capacity; however, it is still unclear how ATP could be consumed in the WAT in this study.

Recent studies have proposed some of possible UCP1-independent thermogenesis mechanisms related to the consumption of energy through specific futile cycles (Chang, Song, Choi, Yun, & Park, 2019; Ikeda & Yamada, 2020). For example, Ca^{2+} cycling-mediated thermogenesis via Ca^{2+}-ATPase transport in the WAT is a well-established mechanism (Ikeda et al., 2017). Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (Serca) 1 or 2 is a central mediator of this signaling, and stimulates respiratory reactions in the mitochondria, accompanied by enhanced tricarboxylic acid metabolism and glycolysis (Pollard et al., 2019). However, we identified another possible energy consumption mechanism in this study related to AMPK signaling given the strong AMPK activation in the HD group.

The futile TG/FA cycle is a UCP1-independnt energy expenditure mechanism, which mainly involves AMPK as a key modulator (Flachs, Rossmeisl, Kuda, & Kopecky, 2013; Mottillo et al., 2014). Previous studies using omega-3 FAs or palmitoleic acid also proposed the model that upregulation of the TG/FA cycle is associated with abundant ATPs induced by elevated AMPK phosphorylation in the WAT (Cruz et al., 2018; Flachs et al., 2013). There are two opposite metabolic functions for FA homeostasis in the WAT: lipolysis and re-esterification. Their co-activation initiates the TG/FA cycle, and their net rates control the amount of FAs released from the WAT (Cadoudal et al., 2005). Approximately 20–30% of FAs are recycled by co-activation of these antagonistic reactions in the WAT in a normal state, but the increment of

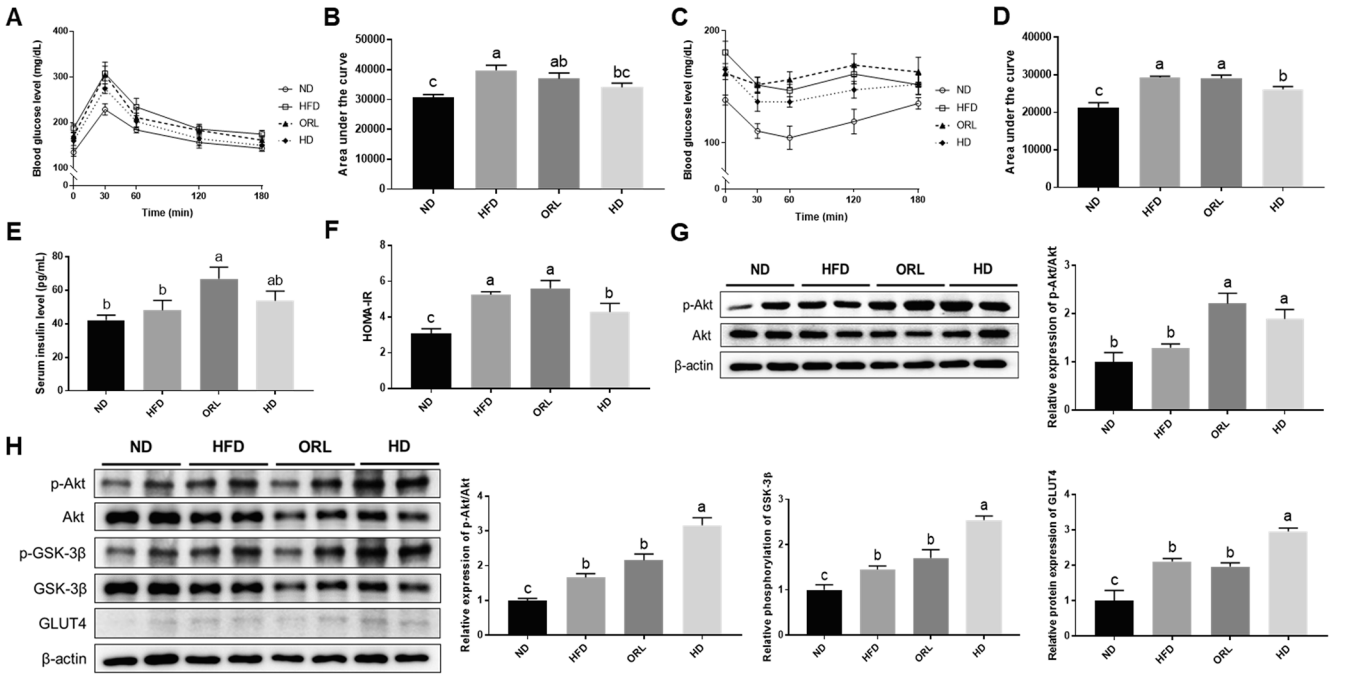

Fig. 4. HD treatment improved insulin sensitivity and ameliorated glycometabolism of eWAT and liver in DIO mice. (A) The results of oral glucose tolerance test (OGTT). (B) The area under the curve (AUC) of OGTT. (C) The results of intraperitoneal insulin tolerance test (IPITT). (D) The AUC of IPITT. (E) The serum insulin level. (F) The graph of HOMA-IR. (G) Protein expression of p-Akt (ser473) and Akt in eWAT. (H) Protein expression of glycometabolism related markers in liver tissue. Statistical differences within groups were determined by ANOVA and Duncan's multiple range test. Different lowercase superscripts indicate significant differences between groups at $p < 0.05$.

metabolic flux (e.g., exercise) enhances the recycling rate to facilitate elimination of excessive ATPs (Gauthier et al., 2008; Reshef et al., 2003). Although there is no alteration of energy level during lipolysis, the requirement of ATP in this cycle is approximately 2.7 ATP/FA, which is consumed when FAs are acetylated to acyl coenzyme A (acyl-CoA) by acyl-CoA synthetase, resulting in a waste of ATP through the TG/FA cycle spends (Mottillo et al., 2014; Townsend, Knuth, & Wright, 2017). We found that HD extract activated genes related to lipolysis (*Atgl, Hsl*) and re-esterification (*Pepck*) in the WAT of mice; therefore, we assume that the elevated AMPK activation by HD extract eliminated redundant ATPs via the TG/FA cycle, and this futile dissipation of energy alleviated obesity in DIO mice.

Adiponectin is a hormone that originates from the adipose tissue, and has been identified as a novel metabolic messenger in numerous organs (e.g., the liver, heart, kidney, brain, beta cell, adipose tissue, and muscle). Adiponectin regulates broad biological responses, including anti-hyperglycemia, anti-inflammatory, anti-apoptotic, and anti-insulin resistance, and has therefore been considered a possible therapeutic target for several diseases (Ghadge, Khaire, & Kuvalekar, 2018; Ng et al., 2016; Straub & Scherer, 2019). The full length adiponectin comprises three main domains: an N-terminal domain, collagen-like fibrous domain, and a globular domain, and several molecular chaperones are associated with its biosynthesis and secretion (Achari & Jain, 2017). Adiponectin is mainly secreted from the adipose tissue, although other cell types, including skeletal and cardiac cells, have also recently been shown to secrete adiponectin for metabolic regulation (Diniz et al., 2019). Although the precise mechanism is not yet clear, the main factor for augmentation of adiponectin secretion appears to be acute aerobic exercise leading to an energy-deficient state in the body (Simpson & Singh, 2008). The metabolic regulation of adiponectin is mainly mediated via AMPK phosphorylation, and because of the broad regulatory roles of AMPK, adiponectin can evoke downstream signaling of AMPK in target organs (Wang, Li, Qiao, Li, & Qiao, 2019). Interestingly, we found that HD extract markedly stimulated the expression of genes related to the maturation and secretion of adiponectin, and the adiponectin content in serum was significantly elevated in the HD group. In addition, the mRNA levels of adiponectin receptors (*AdipoR1* and *AdipoR2*) and *Appl1*, the link between adiponectin and AMPK activation, were also increased by HD extract. Thus, HD extract clearly upregulated adiponectin/AMPK signaling cascades in the DIO mouse model.

Adiponectin/AMPK signaling mediates versatile downstream mechanisms. First, the vigorous stimulation of AMPK could promote UCP-1-independent energy expenditure through the TG/FA cycle as stated above, which can cause loss of calories without exercise. Moreover, AMPK activation is highly associated with relief of insulin resistance, a representative symptom of metabolic syndrome. Insulin resistance in patients with obesity is mainly caused by loss of insulin sensitivity of the receptor owing to continuous oxidative stress and inflammatory responses (Shoelson, Lee, & Goldfine, 2006). There is ample evidence suggesting an association of anti-oxidation and anti-inflammation-related responses with AMPK activation (Huang et al., 2015; Zimmermann et al., 2015); the HD extract also showed anti-oxidative and anti-inflammatory responses in the eWAT. Adiponectin/AMPK signaling regulates glucose utilization and FA oxidation throughout the body (Berg, Combs, & Scherer, 2002; Ceddia, 2013), and APPL1 (an intermediate protein of adiponectin/AMPK signaling) can facilitate binding of insulin receptor and insulin receptor substrate proteins (Ryu et al., 2014). The interaction between APPL1 and insulin sensitivity was identified in *Appl1* knockout mice and its expression regulating model, and the deficiency of APPL1 reduced glucose uptake in adiponectin-sensitive organs; conversely, overexpression of APPL1 resulted in enhanced activation of Akt in target organs, in turn mitigating insulin resistance (Cheng et al., 2009; Hosch, Olefsky, & Kim, 2006). Akt is an instrumental mediator for glucose metabolism that is activated by the insulin signaling cascade; therefore, its irregular lowered activation is a principal indicator of insulin resistance (Carvalho-Filho et al., 2005). The crucial role of Akt is to enhance glucose uptake by promoting GLUT4 translocation to the cell membrane in the major organs, and Akt also stimulates glycogen synthesis by converting GSK-3β to its inactive form (phosphorylated form at Ser9) (Hou et al., 2019; Kim, Nikoulina,

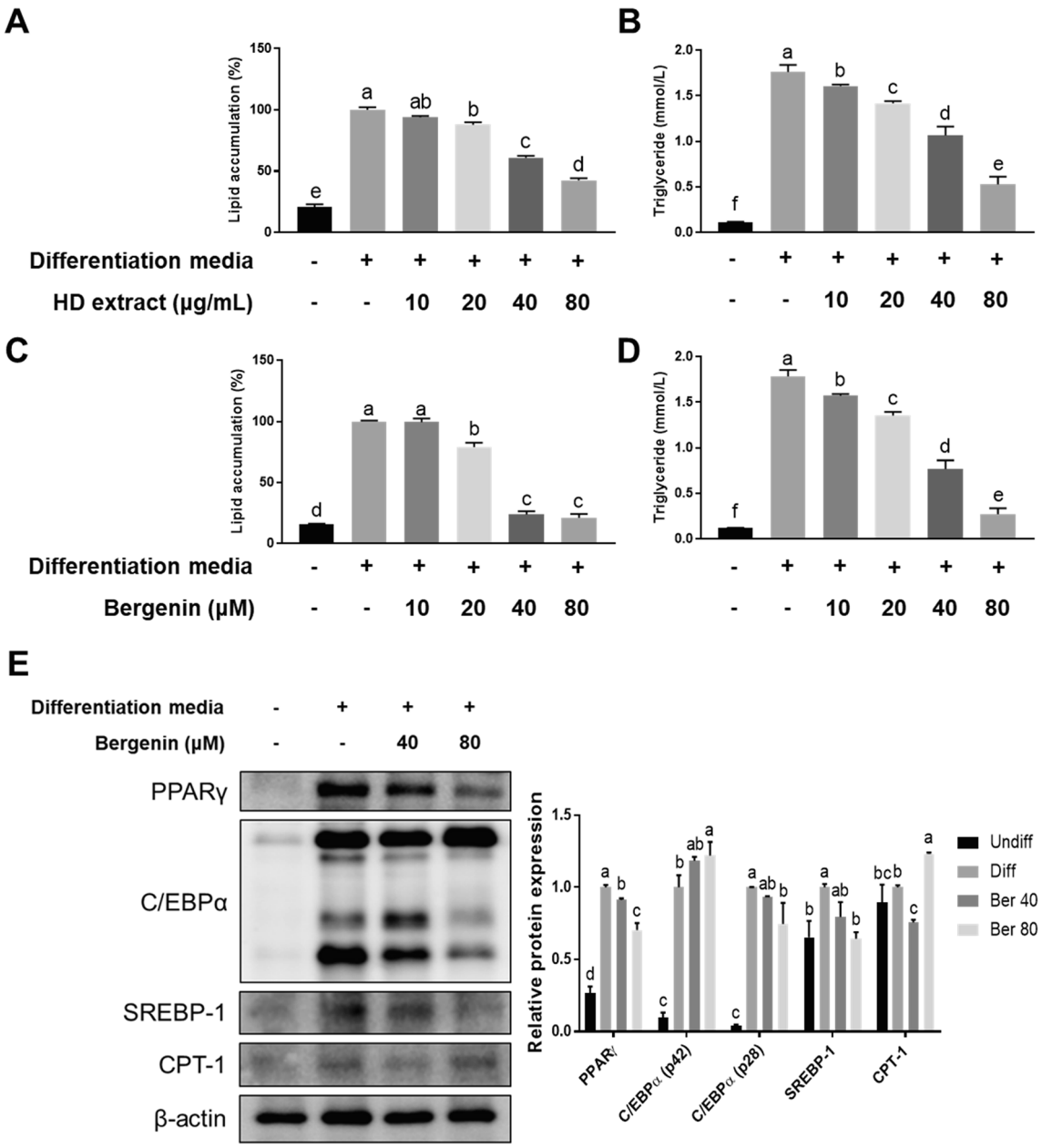

Fig. 5. HD ethanol extract and the bergenin, a major compound of HD ethanol extract, impeded lipid accumulation in 3T3-L1 cell line. (A) The lipid accumulation result and (B) triglyceride contents after HD extract treatment. (C) The lipid accumulation result and (D) triglyceride contents after bergenin treatment. (E) Lipid metabolism related proteins were analyzed with Western blot. Statistical differences within groups were analyzed by ANOVA and Duncan's multiple range test. Different lowercase superscripts indicate significant differences between groups at $p < 0.05$.

Ciaraldi, Henry, & Kahn, 1999). HD extract treatment resulted in the phosphorylation of Akt in the eWAT of DIO mice, and also found changes in protein expression in the liver due to its central role in glucose metabolism in the body; Akt and GLUT4 were significantly stimulated by HD treatment, which led to inactivation of GSK-3β. In turn, HD treatment improved insulin tolerance, and lowered fasting glucose and HOMA-IR levels in DIO mice.

In conclusion, we verified the versatile effectiveness of HD extract, suggesting its use as a potent functional food resource that can relieve metabolic syndrome, and propose the underlying molecular mechanism (Fig. 6). HD extract administration in DIO mice resulted in the loss of body weight and fat tissue weight, and ameliorated lipid status in the plasma. HD treatment highly activated AMPK, as well as the related mechanisms, including anti-oxidation and anti-inflammation processes. Although HD did not evoke UCP1-dependent thermogenic signaling in the eWAT, the increased number of mitochondria and their capacity in the WAT may cause UCP1-independent, but AMPK-dependent, energy expenditure via the TG/FA cycle. As a stimulator of AMPK phosphorylation, we revealed upregulation of adiponectin related signaling from HD extract treatment. Furthermore, the promotion of adiponectin/AMPK signaling by HD treatment also alleviated insulin resistance and reinforced glycometabolism in the eWAT and liver. Therefore, daily intake of HD as part of a regular diet could mitigate the symptoms of metabolic syndrome with multi-targeting effects.

5. Ethics statements

The animal study was planned and executed as per the National Institutes of Health guidelines. The animal experiments were approved by the Institutional Animal Care and Use Committee of Korea Institute of

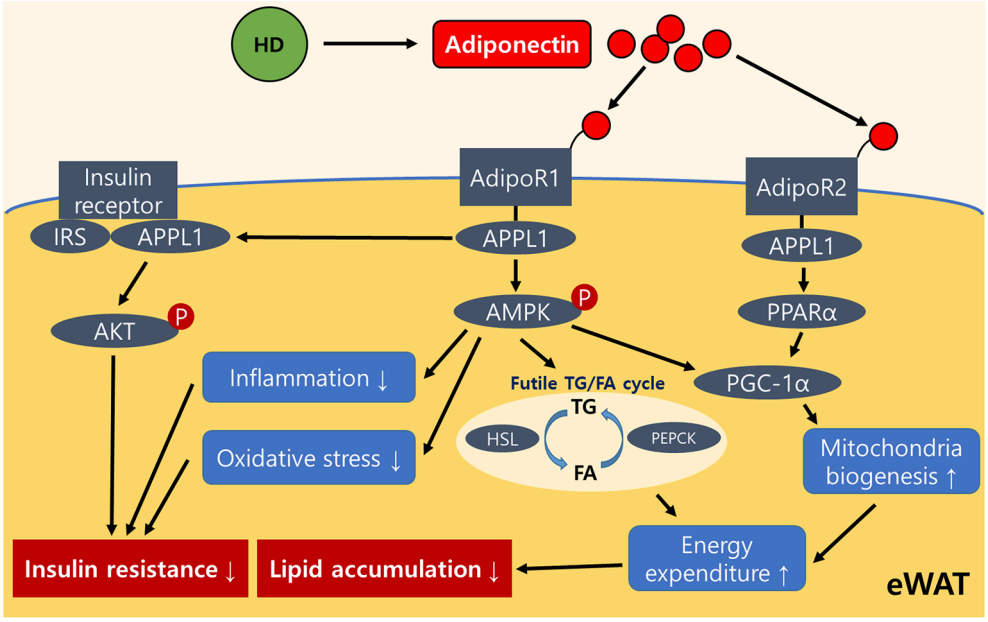

Fig. 6. The proposed mechanism mediated by HD ethanol extract administration in DIO mice.

Science and Technology (Approval no.: KIST-2019-011).

CRediT authorship contribution statement

Yang-Ju Son: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Da Seul Jung:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation. **Ji Min Shin:** Conceptualization, Validation, Formal analysis, Investigation, Data curation. **Saruul Erdenebileg:** Validation, Formal analysis, Investigation, Data curation. **Chu Won Nho:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2021.104581.

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