Emodin improves glucose and lipid metabolism disorders in obese mice via activating brown adipose tissue and inducing browning of white adipose tissue

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Abstract

Background and Purpose: Obesity has become a major health threat worldwide related to type 2 diabetes, hypertension, cardiovascular disease, etc. Activating brown adipocytes and inducing browning of white adipocytes has been proposed as a potential molecular target for obesity treatment. In the present study, we investigated the effects of emodin on browning in mice with high-fat diet (HFD) and explore its underlying pharmacological mechanisms. Experimental Approach: Male C57BL/6J mice were fed with HFD for 8 weeks to induce obesity. Then the obese mice were divided into four groups randomly, HFD or emodin (40, 80 mg/kg/day) or CL 316243 (1 mg/kg/day) for another 6 weeks. Body weight and food intake were recorded every week. After 6 weeks of treatment, fasting blood glucose, oral glucose tolerance, Lee's index, weight ratio (fat weight/body weight), blood lipids, and adipose tissues morphology were assayed. Then UCP1, CD36, FATP4, PPARα and PHB protein in subcutaneous white adipose tissue (scWAT) and brown adipose tissue (BAT) were analyzed. In addition, the lipid metabolites in adipose tissues were analyzed by Ultra Performance Liquid Chromatography with Electrospray Ionization Tandem Mass Spectrometry.

1. Introduction

Obesity is essentially caused by energy storage exceeding energy expenditure, which has been shown to be a major risk factor for various metabolic diseases (e.g. type 2 diabetes, non-alcoholic fatty liver disease, cardiovascular disease, and etc.) (Spiegelman and Flier,2001; Wang *et al* ., 2015; Georgianos and Zebekakis, 2018). One study showed that by 2025, the prevalence of obesity in men will reach to 18%, and the prevalence of obesity in women will exceed by 21% (NCD Risk Factor Collaboration, 2016). With rising worldwide, obesity has become a major health threat, which brings huge economic burden to individuals and the society. Therefore, it is necessary to develop more kinds of drugs to reduce the burden of diseases.

Adipose tissue is the main metabolic organ, which regulates lipid metabolism according to physiological energy requirements. Based on the cell morphology and function, mammalian adipose tissue can be divided into two types: white adipose tissue (WAT) and brown adipose tissue (BAT). As an endocrine organ, WAT can secrete hormones and cytokines which affect the body's glycolipid metabolism and inflammatory response etc. In addition, WAT stores a large amount of energy as triacylglycerols (TAGs) in unilocular white adipocytes, which can then be released as fatty acids when the body needs vast amounts of energy. In contrast to WAT, BAT is specialized for energy expenditure, which is characterized by small multi-atrial lipid droplets, abundant mitochondria and uniquely expresses uncoupling protein 1 (UCP1) (Nicholls and Locke, 1984; Cannon and Nedergaard, 2004). Located in the inner membrane of mitochondria, UCP1 acts to uncouple oxidative phosphorylation from ATP production, thereby releasing energy in the form of heat (Ricquier and Frédéric,2000; Lidell *et al* ., 2014). BAT as a thermogenesis tissue dissipates energy as heat

in a process called non-shivering thermogenesis, which plays a significant role in energy regulation and maintenance of the body's core body temperature (Andriy*et al* ., 2012; Carpentier *et al* ., 2018). In recent years, there has been a growing awareness for the potential benefits of acquiring brown adipocytes in non-classical BAT locations, such as WAT and skeletal muscles. It has been confirmed that when the body is stimulated by cold exposure (Matteis*et al* ., 2013), β-adrenoceptors activation (Ghorbani *et al* ., 1997) etc., brown-like phenotypic adipocytes called beige adipocytes can be detected in WAT, which is characterized by an increased number of mitochondria and increased expression of brown fat marker genes (e.g. UCP1, peroxisome proliferator activated receptor γ coactivator-1α, PGC-1α, positive regulatory domain-containing 16, PRDM16). The above process is called "Browning of WAT". Recent studies showed that increasing metabolic activity of brown and beige adipose tissue might be a novel way to ameliorate glucose and lipid metabolism in obese patients (Cypess*et al* ., 2009; Loyd and Obici, 2014; Schrauwen *et al* .,2015; Kaisanlahti and Glumoff, 2019). Therefore, the classic BAT activation and the recruitment of beige adipocytes may be used as a potential therapeutic target for metabolic diseases such as obesity and type 2 diabetes.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is one of the active anthraquinone, which mainly exists in *Rheum palmatum* and some other Chinese herbs with the ability to anti-inflammatory, anti-tumor and ameliorate metabolic disorders etc. (Izhaki, 2002; Heo *et al* ., 2008; Zhu *et al* ., 2013; Hwang *et al* ., 2015). Previous studies showed that emodin improved dyslipidemia by activating AMP activated protein kinase (AMPK) signaling pathway in rats with high-fat diet (HFD), and prevented diabetic cardiomyopathy by phosphorylation and regulating AKT/GSK-3 signaling pathway (Tzeng *et al* ., 2012; Wu *et al* .,2014). It was also reported that emodin ameliorated glucose tolerance and insulin resistance and reduced lipid levels in liver and adipose tissue in obese mice through regulating sterol regulatory element-binding protein (SREBP) pathway (Li *et al* ., 2016). However, the anti-obesity effect of emodin on obese mice induced by HFD and its underlying mechanisms have not been fully investigated. Therefore, in the present study, we evaluated the role of emodin in the treatment of obesity by using the HFD-induced obese mouse model in this study, and discussed its molecular mechanisms from two aspects of molecular biology and targeted metabolomics , Which provided a new way for emodin to treat obesity, T2DM and other metabolic diseases.

2. Materials and Methods

2.1 reagents

Emodin (purity 95%) was purchased from Shanghai Yuanye Biotechnology Co., Ltd. CL 316243 disodium salt was purchased from APExBIO Technology LLC (Houston, USA). Biochemical kits of serum total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-c), and low-density lipoprotein cholesterol (LDL-c) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Free fatty acid (FFA) ELISA assay kit was purchased from Jiangsu Kete Biotechnology Co., Ltd. (Jiangsu, China). Leptin ELISA assay kit and adiponectin ELISA assay kit were purchased from cloud-clone Corp. Wuhan (Wuhan, China).

2.2 Animals and experimental protocol

Eight-week-old male C57BL/6J mice [Grade SPF, certificate No. SCXK (jing) 2016-0002], weighing 20 ± 2 g, were obtained from Sibeifu (Beijing) Biotechnology Co., Ltd. (Beijing, China). Male C57BL/6J mice were randomly assigned to two groups after 1 week of adaptation. The mice in the different groups were fed, respectively, a normal diet (3.65 Kcal/kg, n=8), high-fat diet (HFD; 60% of kilocalories from fat, 5.24 Kcal/kg n=8) for 8 weeks. After 8 weeks, the mice with HFD were randomly divided into four groups: HFD group (n=8), HFD with emodin treatment group (40 mg/kg/day, n=8, dissolved in 0.1% (w/v) carboxymethyl cellulose-Na (CMC-Na)), HFD with emodin treatment group (80 mg/kg/day, n=8) and HFD with CL 316243 disodium salt treatment group (1 mg/kg, n=8). Mice in both normal diet group and HFD model groups were given the same volume of 0.1% (w/v) CMC-Na. All mice except CL 316243 disodium salt treatment group were fed by oral gavage at the same time in each day for 6 weeks. Three days before the end of the experiment, mice in CL 316243 disodium salt treatment group were intraperitoneally injected with CL 316243.

All the mice were housed individually in a pathogen-free air-conditioned room (22°C) with a 12:12 h light/dark cycle with ad libitum access to food and water. All the animal studies were in accordance with ethics standards of the Animal Care and Welfare Committee of Beijing University of Chinese Medicine [Certificate No. BUCM-04-2018070603-3015].

2.3 Oral glucose tolerance test

After 6 weeks of intervention, the mice were fed by oral gavage with 50% D-glucose (2.0 g/kg) after overnight (12 h) fasting. Blood samples were taken from the tail 0, 30, 60, 90 and 120 min after oral gavage, and glucose levels were measured using the One touch Ultra blood glucose monitoring system (Johnson). The curve of blood glucose concentration and time was plotted and the area under circle (AUC) was calculated according to the formula:

$AUC_{0\text{-}2h}=[(G_{0h}+G_{0.5h})\times0.5h+(G_{0.5h}+G_{1h})\times0.5h+(G_{1h}+G_{1.5h})\times0.5h+(G_{1h}+G_{2h})\times0.5h]/2$. where 'G' is the blood glucose value.

2.4 Measurement of Lee's index

At the end of the treatment, the body mass of the mice was accurately weighed, and the body length (the distance from the tip of the nose to the anus) was accurately measured, and then the Lee's index was calculated according to the formula:

Lee's index=body weight (g)ˆ(1/3)/body length (cm) (Bunyan *et al* ., 1976).

2.5 Measurement of adipose tissues mass/ body weight

Subcutaneous WAT (scWAT) mass and scapular brown adipose tissues (BAT) mass were accurately weighed. The value of scWAT mass/body weight (BW) and BAT/body weight (BW) was calculated.

2.6 Serum Biochemicalanalysis

Serum TC, TG, HDL-c and LDL-c levels were measured with the use of biochemical kits (Nanjing Jiancheng, China). The levels of FFA were determined with a Mouse FFA ELISA kit (Kete, China). Serum Leptin and adiponectin levels were analyzed with Mouse Leptin and ELISA kit respectively (Cloud-clone, China).

2.7 Histological and immunohistochemical analysis

BAT and scWAT were fixed with 10% formalin, dehydrated, embedded in paraffin and sectioned. For histological analysis, sections were deparaffinized and stained with hematoxylin and eosin. Immunohistochemistry was performed using a polyclonal anti-UCP1 antibody (1:500, Abcam). Images were captured by a microscope (Leica, German). At least three pictures from different regions of each section.

2.8 Western blotting

The homogenates of scWAT and BAT were dissolved in RIPA lysate and protease inhibition for protein extraction. Sample protein concentrations were measured using the bicinchoninic acid (BCA) method (Beyotime). Total protein (10μg/Lane) was separated on a 12% acrylamide/acrylamide gel using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. PVDF membranes containing protein were incubated with specific anti-alpha tubulin antibody (1:5000, ab18251, Abcam), anti-UCP1 antibody (1:1000, ab10983, Abcam), anti-Prohibitin antibody (1:10000, ab75766, Abcam), anti-PPAR α antibody (1:2000, ab8934, Abcam), anti-CD36 antibody (1:5000, ab133625, Abcam), anti-slc27a4/FATP4 antibody (1:1000, ab200353, Abcam), respectively. Then membranes were incubated with HRP-conjugated Affinipure Goat Anti-Mouse IgG(H+L) (1:5000, 20000175, protentech) or HRP-conjugated Affinipure Goat Anti-Rabbit IgG(H+L) (1:5000, 20000174, protontech). Protein bands were visualized using the ECL kit (EMD millipore). Image analysis with Image J software.

2.9 Targeted Lipidomics analysis

BAT and scWAT samples were thawed at 4 for 30-60 min. Tissue samples were accurately weighed and then homogenized. 300μL of methanol (containing 4 internal standards SPLASH® II LIPIDOMIX® Mass Spec Standard (330709), Cer/Sph Mixture I (LM6002, Avanti), 12:0-13:0 PC (LM1000, Avanti), 12:0-13:0 PE (LM1100, Avanti)) was added to the sample, which extracted with 1mL of MBET for 1h. The sample was centrifuged for 5 min, then 100μL of the upper layer solution was taken and placed in a 1.5 mL centrifuge tube. The sample was concentrated by vacuum centrifugation for 4 h. The dried sample was reconstituted with 400 uL (isopropanol/acetonitrile (1:1)) and shaken for 40s. The sample was centrifuged for 5 min, then 100 μL of the upper layer solution was taken and placed in a 200 μL inner liner for testing. Separation of samples by Ultra Performance Liquid Chromatography (UPLC) (UPLC I-Class, Waters). Column: waters UPLC BEH C8 (1.7 μm 2.1 mm×100 mm). Mobile phase: A (acetonitrile/water 6:4, 0.1% formic acid, 5 mM ammonium formate) and B (acetonitrile/isopropanol 9:1, 0.1% formic acid, 5 mM ammonium formate). The elution procedure is shown in **Table 1** and **Table 2** . Electrospray ionization tandem mass spectrometry was used for mass spectrometry. Masslyxn4.1 was used for mass spectrometry data acquisition. The data of target metabolism group were processed through skyline 19.1. The allowable error of the retention time is 0.25 min.

2.10 Statistical analysis

All data were statistically analyzed using SAS 8.2 software. All data obtained by the experiment were expressed as mean ± SE. Statistics were performed using the one-way analysis of variance (ANOVA) test and SNK-$q$test. P -value$<$0.05 was considered statistically significant.

3. Results

3.1 Emodin inhibits obesity and appetite and reduces fat mass in obese mice induced by HFD

During the experiment, the body weight of each group showed an increasing trend. Mice with HFD developed moderate obesity. Mice in HFD group showed a significant increase in body weight compared with the normal diet group of mice. In contrast, after 3 weeks of treatment, emodin (80 mg/kg/day) treatment group induced marked body weight reduction compared with HFD group of mice. After 5 weeks of treatment, compared with HFD group of mice, the body weight was markedly reduced in the emodin (40 mg/kg/day) treatment group. CL 316243 (1mg/kg/day) treatment group also significantly decreased the body weight compared with HFD group of mice (Fig 1A). We aimed to investigate the effect of emodin on energy intake in mice. Emodin (40 mg/kg/day) and emodin (80 mg/kg/day) treatment group induced marked anorectic effects, resulting in a 11.5% or 11.4% reduction in food intake compared with the HFD mice (Fig 1B). Lee's index can be used as an indicator to evaluate the degree of obesity in adult obese model mice (Bunyan *et al* ., 1976). We found that emodin (40 mg/kg/day) and emodin (80 mg/kg/day) significantly reduced the Lee's index (Fig 1C). Mice in HFD group showed a significant increase in the ratio of scWAT/BW compared with the normal diet group of mice. In particular, the ratio of scWAT/BW was drastically reduced by emodin (40 mg/kg/day), emodin (80 mg/kg/day) and CL 316243 (1 mg/kg/day) treatment group (Fig 1D). The function of BAT is consuming glucose and lipids, mediating the thermogenic effects of non-shivering, thereby increasing energy expenditure (Okla*et al* ., 2017). Interestingly, compared with HFD mice, emodin (80 mg/kg/day) and CL 316243 (1 mg/kg/day) treatment group significantly decreased the ratio of BAT/WB, with a reduction ratio of 37.5% and 47.5%, respectively (Fig 1E). Above results suggest that emodin can prevent the rapid growth of body weight by affecting the appetite of mice with HFD.

3.2 Emodin amelioratesabnormal blood glucose and

blood lipid in mice fed with HFD

In this study, we investigated whether emodin improved glucose tolerance in mice with HFD. The results showed that fasting blood glucose levels were significantly increased in HFD mice compared with the normal diet group (Fig 2A). It was confirmed that fasting blood glucose levels were significantly reduced by emodin (40 mg/kg/day, 80 mg/kg/day) and CL316243 (1 mg/kg/day) treatment group compared with the HFD group. After 6 weeks of intervention, we performed a glucose tolerance test and found that the AUC of

HFD mice was significantly increased compared with the normal diet group of mice. Compared with HFD group, emodin (40 mg/kg/day), emodin (80 mg/kg/day) and CL316243 (1 mg/kg/day) treatment group respectively reduced the AUC by 31%, 38%, 45%, (Fig 2B). These results suggest that emodin can ameliorate glucose metabolism in mice with HFD. To investigate whether emodin improved lipid metabolism disorders in mice with HFD, we further measured indicators related to blood lipids. Lipid metabolism in mice with HFD was disordered, and serum levels of TC, TG, LDL-c and FFA were significantly higher than those in the normal diet group (Fig 2C-2E). Emodin (40 mg/kg/day) treatment group significantly decreased TG, LDL-c levels in serum of mice compared with HFD mice. Furthermore, compared with the HFD group, the levels of TC, TG, LDL-c and FFA were markedly decreased in emodin (80 mg/kg/day) and CL316243 (1 mg/kg/day) treatment group. However, HDL-c levels of emodin (40 mg/kg/day, 80 mg/kg/day) and CL316243 (1 mg/kg/day) treatment group were not changed significantly compared with HFD group (Fig 2F). Leptin has the function of maintaining energy metabolism and regulating adipose ratio, and plays a critical role in regulating lipid metabolism (Dodd *et al* ., 2015). We also analyzed leptin in serum (Fig 2G). It was demonstrated that the serum leptin content of HFD mice was significantly increased compared with the normal diet group of mice, which may be related to leptin resistance. Particularly, emodin (40 mg/kg/day, 80 mg/kg/day) and CL316243 (1 mg/kg/day) treatment group significantly decreased leptin levels in serum of mice compared with HFD group. As an endogenous insulin sensitizer secreted by adipose tissue, adiponectin decreased is an independent risk factor for hyperlipidemia and diabetes (Ziemke and Mantzoros, 2010). It was showed that the HFD mice had lower the serum adiponectin content than that of the normal diet group. However, compared with HFD group, emodin (40 mg/kg/day, 80 mg/kg/day) and CL316243 (1 mg/kg/day) treatment group drastically increased adiponectin levels in serum of mice (Fig 2H). Therefore, we believe that emodin can ameliorate abnormal blood glucose and blood lipid in mice fed with HFD.

3.3 Emodin induces a multilocular phenotype and increases the expression of thermogenic protein UCP 1 in adipose tissues

We also analyzed the morphology of adipose tissues (Fig 3A). The H&E staining revealed that the cells in scWAT of HFD mice were single chamber and irregular compared with normal diet group. However, emodin (40 mg/kg/day, 80 mg/kg/day) and CL316243 (1 mg/kg/day) treatment group induced the transition of unilocular adipocytes to multilocular ones and increased cell density compared with HFD group. The H&E staining of BAT demonstrated that compared with normal diet group, the cells in BAT of HFD mice were more large single vacuolar adipose chambers, which had a tendency from brown to white. In particular, emodin (40 mg/kg/day, 80 mg/kg/day) and CL316243 (1 mg/kg/day) markedly increased brown adipocytes density and enriched blood vessels between cells and cells compared with HFD mice. We further performed an immunohistochemical analysis of adipose tissues (Fig 3B). As an important thermogenic protein, UCP1 is specifically expressed in BAT (Lowell and Spiegelman, 2000; Carpentier *et al* ., 2018). Therefore, we analyzed the expression of UCP1 protein in adipose tissues (Fig 3C and 3D). It was showed that the expression of UCP1 protein was significant decreased in both scWAT and BAT in HFD mice compared with the normal diet group mice. However, emodin (80 mg/kg/day) and CL316243 (1 mg/kg/day) treatment group significantly enhanced the expression of UCP1 protein in scWAT and BAT compared with HFD group. The above results suggest that emodin can induce the browning of scWAT and increase the thermogenesis of BAT by increasing the expression of UCP1 protein.

3.4 Emodin induces browning of scWAT and activates BAT of mice fed with HFD by up regulating the expression of thermogenic protein and lipoproteins

In order to further confirm that emodin can improve the lipid metabolism of mice fed with HFD by activating BAT and inducing browning of WAT, we analyzed the thermogenic proteins and lipoproteins in scWAT and BAT (Fig 4A and 4C). The western blotting results showed that emodin (40 mg/kg/day) treatment group significantly up-regulated the expression of UCP1 and Prohibitin protein in scWAT compared with HFD group. Moreover, emodin (80 mg/kg/day) and CL 316243 (1 mg/kg/day) treatment group significantly increased the expression of UCP1, Prohibitin, PPAR alpha, CD36 and FATP4 protein in scWAT and BAT of mice compared with HFD group (Fig 4B and 4D).

3.5 Phospholipid metabolism is altered in scWAT with emodin

Based on the above experimental results, we further analyzed scWAT samples by targeted metabolomics. We selected the biomarkers that have changed and made a heat map (Fig 5). Specifically, the lipid levels of SM (36:2) was significantly up-regulated in mice with HFD, when compared with normal mice. Meanwhile, lipid levels of 2 PCs, 11 PCs-O,1 LPC, 4 PEs, 13 PEs-O, 1 LPE, 2 Cer and 3SMs, including PC (38:6), PC (39:2), PC-O (30:1), PC-O (32:1), PC-O (32:2), PC-O (34:2), PC-O (34:3), PC-O (36:3), PC-O (36:5), PC-O (38:0), PC-O (38:6), PC-O (40:6), PC-O (40:7), LPC-O (32:2), PE(38:3), PE(38:4), PE(40:7), PE-O (36:2), PE-O (36:3), PE-O (36:5), PE-O (38:3), PE-O (38:6), PE-O (38:7), PE-O (40:4), PE-O (40:5), PE-O (40:6), PE-O (40:7), PE-O (40:8), PE-O (42:7), LPE-O (18:2), Cer (34:1), Cer (38:1), SM (39:2), SM (41:0) and SM (42:1), were remarkably reduced in mice with HFD, when compared with normal mice.

After emodin treatment, lipid levels of 2PCs-O, 5 PEs-O, 1 Cer and 1 SM, including PC-O (38:0), PC-O (40:7), PE-O (36:3), PE-O (38:6), PE-O (40:4), PE-O (40:5), PE-O (40:6), Cer (34:1) and SM (41:0), were significantly altered and exhibited a normal-level tendency.

3.6 Phospholipid metabolism is altered in BAT with emodin

We also analyzed BAT samples by targeted metabolomics. We selected the biomarkers that have changed and made a heat map (Fig 6). Specifically, the lipid levels of 6 PCs, 1 PC-O and SM, including PC (32:4), PC (38:4), PC (38:5), PC (38:8), PC (39:5), PC (40:5), PC-O (38:3) and SM (32:0), were significantly up-regulated in mice with HFD, when compared with normal mice. Meanwhile, lipid levels of 9 PCs, 1 PC-O,7 LPCs and1 PE, including PC (30:0), PC (32:1), PC (32:2), PC (33:4), PC (34:3), PC (38:0), PC (41:2), PC (41:6), PC (41:7), PC-O (38:7), LPC (19:0), LPC (20:0), LPC (20:1), LPC (22:0), LPC (22:1), LPC (24:0), LPC (24:1) and PE(38:3), were remarkably reduced in mice with HFD, when compared with normal mice. Results showed that after emodin treatment, the lipid levels of PC-O (38:0), PC-O (40:7), PE-O (36:3), PE-O (38:6), PE-O (40:4), PE-O (40:5), PE-O (40:6), Cer (34:1) and SM (41:0) in scWAT changed significantly and showed a positive trend. At the same time, the lipid levels of PC (30:0), PC (32:1), PC (32:2), PC (32:4), PC (33:4), PC (38:0), PC (38:8), PC (39:5), LPC (20:0), LPC (22:0) and LPC (22:1) in BAT also changed significantly , which was similar to those in normal group mice

After emodin treatment, lipid levels of 8PCs and 3 LPCs, including PC (30:0), PC (32:1), PC (32:2), PC (32:4), PC (33:4), PC (38:0), PC (38:8), PC (39:5), LPC (20:0), LPC (22:0) and LPC (22:1), were significantly altered and exhibited a normal-level tendency.

4. Discussion

With the deepening understanding of WAT and BAT function, the strategy that activating BAT and inducing browning of WAT to treat obesity, hypertension, type 2 diabetes and other metabolic diseases have aroused great interest of scientists. Browning of WAT can inhibit obesity by increasing energy consumption, and reduce the adverse effects caused by excessive accumulation of WAT, such as insulin resistance (IR), inflammation etc. (Huiying *et al* ., 2018; Babaei *et al* ., 2018). In this study, for the first time, we studied emodin's new opinion on the treatment of obesity by activating BAT and promoting the formation of beige fat of obese mice induced by HFD. At the same time, we analyzed the metabolites of BAT and scWAT through targeted metabolomics, and predicted the signal pathway that emodin may play a therapeutic role in the treatment of obesity.

In the present study, emodin and decreased body weight in obese mice compared with the HFD group, which was consistent with the results of previous studies in *vivo*(Feng *et al* ., 2010; Wang e*t al* ., 2012).In addition, the food intake was significantly increased in emodin treatment groups compared with the HFD group, suggesting that the reduction in body weight gain in emodin treatment groups was due to reduces in food consumption. Lee's index can be used as an index to evaluate the degree of obesity in adult obesity model mice (Bernardis and Patterson, 1986). Compared with HFD mice, emodin decreased of Lee's index of HFD mice and lowered the obesity degree of HFD mice. The imbalance ratio of WAT and BAT can lead to obesity. Our results demonstrated that emodin reduced the ratio of scWAT/BW and BAT/BW,

which might be associated with emodin promoting lipolysis. Obese mice induced by HFD have disorders of glycolipid metabolism. However, glycolipid metabolism is regulated by multiple genes and proteins, which is a complex process. Previous studies revealed that emodin reduced the levels of ALT, AST, TC and TG in serum of rat with nonalcoholic fatty liver (Dong e*t al* ., 2005), and significantly ameliorated glucose tolerance and IR in diabetic mice (Xue e*t al* ., 2010). High free fatty acidemia caused by high FFA in the body is related to the occurrence and development of obesity, non-alcoholic fatty liver, type 2 diabetes and other diseases. The results of oral glucose tolerance and blood lipid test showed that emodin decreased the AUC of fasting blood glucose value and the contents of TC, TG, LDL-C and FFA in the serum of HFD mice, which might be related to emodin enhancing energy consumption via activating BAT and induce browning of WAT. It has been reported that leptin maintains energy metabolism and regulates fat ratio. It acted on hypothalamic neuropeptide Y (NPY)/agouti-related peptide (AgRP) to suppress appetite and promote energy consumption, and cooperated with insulin to act on proopiomelanocortin (POMC) in hypothalamus neurons, so that POMC neurons participated in fat browning and increased energy consumption (Dodd*et al* ., 2015). Our results showed that emodin significantly reduced the content of leptin in the serum of mice fed with HFD, which might be related to the two-way regulation of leptin. Adiponectin is an endogenous insulin sensitizer secreted by adipose tissue, which can increase insulin sensitivity and fatty acid oxidation in peripheral tissues. The reduction of adiponectin level is an independent risk factor for hyperlipidemia and diabetes (Ziemke and Mantzoros, 2010; Konishi *et al* ., 2011). The results suggested that emodin significantly increased the content of adiponectin in the serum of obese mice, thereby reducing the risk of disease.

The morphological results showed that the fat cells of emodin treated mice in scWAT were smaller than those of obese mice, which indicated that emodin could decrease the lipid accumulation in scWAT of obese mice induced by HFD. It is recognized that obesity is characterized by abnormal fat deposition, which indicates that it is necessary to study the changes of genes or proteins in adipose tissue. In order to explore the possible mechanisms of emodin in preventing obesity, we analyzed the thermogenic proteins and lipoproteins in adipose tissues. The results of immunohistochemistry and Western blotting analysis indicated that emodin promoted the up-regulation of UCP1 protein in scWAT and BAT to increase Thermogenesis. PPAR α is the key factor of BAT thermogenesis, which can regulate lipid catabolism and thermogenic gene expression in coordination with PGC-1 α and PRDM16 (Hondares *et al* ., 2011). In addition, PPAR α can enhance the expression of PGC-1α and UCP-1 by increasing the activity of erythropoietin (EPO). PPAR α also plays a coordinating role with SIRT1 activated by EPO and jointly regulates the level of NAD^+ to heighten the metabolic activity (Wang *et al* ., 2013). The results of Western blotting demonstrated that emodin increased metabolic activity of obese mice by increasing the expression of PPAR α protein in scWAT and BAT. Mitochondria is the core functional units of metabolic control in many cells, such as White adipocytes and brown adipocytes. Mitochondrial function is related to the endocrine function of adipocytes. In addition, brown adipocytes rely on mitochondria to maintain intracellular metabolism. Located in mitochondrial inner membrane, Prohibitin (PHB) plays a critical role in maintaining the shape and function of mitochondria and regulating energy metabolism (Vessal *et al* ., 2006; Artal and Tavernarakis, 2009). Our findings indicated that emodin enhanced the activity of mitochondria in both scWAT and BAT through increasing the expression of PHB in adipose tissues. As a fatty acid translocase, CD36 acts a pivotal part in the uptake and transport of long-chain fatty acids (LCFA) in heart and adipose tissues (Habets*et al* ., 2007; Wan *et al* ., 2013). It was found that cold exposure drastically accelerated plasma clearance of triglycerides as a result of increased uptake into BAT, a process crucially dependent on local LPL activity and transmembrane receptor CD36 (Bartelt *et al* ., 2011). Fatty acid transporter 4 (FATP 4) is a member of the fatty acid transport proteins (FATPs), which plays a significant role in the transport of long-chain fatty acids with more effectively compared with FATP1. It was found that FAT/CD36 and FATP4 were the most effective fatty acid transporters (Stahl *et al* ., 2001; Nickerson *et al* ., 2009). In this study, emodin accelerated the transport and consumption of fatty acids and improved the disorder of lipid metabolism by increasing the expression of CD36 and FATP4 protein in both scWAT and BAT.

In order to further explore how emodin affected the changes of lipid content and types, thus activating BAT and inducing WAT browning, we applied UPLC-MS targeted metabolomics to comprehensively characterize

the effects of emodin on structural lipid content and composition in scWAT and BAT. Targeted metabolomics analysis revealed some species-specific changes in phospholipids (PCs, PEs) in scWAT and BAT in obese mice after emodin treatment. PCs accounts for nearly 50% of membrane phospholipids and is necessary for cell membrane structure, cell division and interaction with intact membrane proteins. It was confirmed that regulation and metabolism of PC, PS and PE prevented inflammation of adipose tissue, hyperlipidemia and obesity (Body, 1988). One class of enzymes that regulate phospholipid metabolism is the phospholipase, which is an enzyme that catalyzes the cleavage of phospholipids and, in some cases, catalyzes the cleavage of TAG. Our results showed that after emodin treatment, the lipid levels of PC-O (38:0), PC-O (40:7), PE-O (36:3), PE-O (38:6), PE-O (40:4), PE-O (40:5), PE-O (40:6), Cer (34:1) and SM (41:0) in subcutaneous adipose tissue changed significantly and showed a positive trend. At the same time, the lipid levels of PC (30:0), PC (32:1), PC (32:2), PC (32:4), PC (33:4), PC (38:0), PC (38:8), PC (39:5), LPC (20:0), LPC (22:0) and LPC (22:1) in BAT also changed significantly, which was similar to those in normal group mice. Although we did not see large changes in the total amount of fat subtypes, the specific changes in individual molecular species reflected the selective remodeling of emodin after treatment.

We evaluated the role of emodin in the treatment of obesity and explored its molecular mechanism via using HFD-induced obese mouse models in this study. We found that emodin could increase energy consumption and ameliorate glucose and lipid metabolism in obese mice by activating BAT and inducing browning of scWAT. Moreover, we analyzed the differential metabolites of BAT and scWAT by targeting metabolomics, and improved metabolic activity by changing the type and content of some lipids in adipose tissues, which suggested that emodin might play a therapeutic role in the treatment of obesity. With the further study on the mechanisms of emodin activating BAT and inducing browning of WAT, it is believed that emodin can be used as a new drug in the treatment of obesity, T2DM and other metabolic diseases.

Authors' contributions

Jianning Sun and Shifen designed experiments. Long Cheng, Shuofeng Zhang, Fei Shang, and Shifen Dong performed experiments. Fei Shang and Long Cheng performed UPLCQ-MS/MS analysis. All authors read and approved the final manuscript. Long Cheng and Shifen Dong performed statistical analysis. Long Cheng wrote the paper.

Competing interests

The authors declare that they have no competing interest.

Availability of data and supporting materials

The datasets used and analyzed during the current study available from the corresponding author or the first author on reasonable request.

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References

Andriy F, Lishko PV, Yuriy K. (2012). Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria. Cell 151: 400-413.

Artal S M, Tavernarakis N. (2009). Prohibitin and mitochondrial biology. Trends Endocrinol Metab 20: 394-401.

Babaei R, Schuster M, Meln I, Lerch S, Ghandour RA, Pisani DF, et al. (2018). Jak-TGFβ cross-talk links transient adipose tissue inflammation to beige adipogenesis. Sci Signal 11: eaai7838.

Bartelt A, Bruns O T, Reimer R, Hohenberg H, Ittrich H, Peldschus K, et al. (2011). Brown adipose tissue activity controls triglyceride clearance. Nat Med 17: 200-205.

Bernardis LL, Patterson BD. (1968). Correlation between 'Lee index' and carcass fat content in weanling and adult female rats with hypothalamic lesions. J Endocrinol 40: 527-528.

Body DR. (1988). The lipid composition of adipose tissue. Prog Lipid Res 27: 39-60.

Bunyan J, Murrell EA, Shah PP. (1976). The induction of obesity in rodents by means of monosodium glutamate. Br J Nutr 35: 25-39.

Cannon B, Nedergaard J. (2004). Brown adipose tissue: function and physiological significance. Physiol Rev 84: 277-359.

Carpentier AC, Blondin DP, Virtanen KA, Richard D, Haman F, Turcotte ÉE, et al. (2018). Brown adipose tissue energy metabolism in humans. Front Endocrinol 9: 447.

Cong HY, Zhong WX, Wang YY, Ikuyama S, Fan B, Gu JQ. (2018). Pycnogenol induces browning of white adipose tissue through the PKA signaling pathway in apolipoprotein E-deficient mice. J Diabetes Res 2018: 9713259.

Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. (2009). Identification and importance of brown adipose tissue in adult humans. N Engl J Med 360: 1509-1517.

Dodd GT, Decherf S, Loh K, Simonds SE, Wiede F, Balland E, et al. (2015). Leptin and insulin act on POMC neurons to promote the browning of white fat. Cell 160: 88-104.

Dong H, Lu FE, Gao ZQ, Xu LJ, Wang KF, Zou X. (2005). Effects of emodin on treating murine nonalcoholic fatty liver induced by high caloric laboratory chaw. World J Gastroenterol 11: 1339-1344.

Feng Y, Huang SL, Dou W, Zhang S, Chen JH, Shen Y, et al. (2010). Emodin, a natural product, selectively inhibits 11b-hydroxysteroid dehydrogenase type 1 and ameliorates metabolic disorder in diet-induced obese mice. Br J Pharmacol 161: 113-126.

Georgianos PI, Zebekakis PE. (2018). General obesity, abdominal adiposity, and the risk of incident hypertension-From anthropometry to modern imaging techniques. J Clin Hypertens 20: 1427-1429.

Ghorbani M, Claus TH, Himmshagen J. (1997). Hypertrophy of brown adipocytes in brown and white adipose tissues and reversal of diet-induced obesity in rats treated with a beta3-adrenoceptor agonist. Biochem Pharmacol 54: 121-131.

Habets DD, Coumans WA, Voshol PJ, Boer MA, Febbraio M, Bonen A, et al. (2007). AMPK-mediated increase in myocardial long-chain fatty acid uptake critically depends on sarcolemmal CD36. Biochem Biophys Res Commun 355: 204-210.

Heo SK, Yun HJ, Park WH, Park SD. (2008). Emodin inhibits TNF-A-induced human aortic smooth-muscle cell proliferation via caspase and mitochondrial-dependent apoptosis. J Cell Biochem 105: 70-80.

Hondares E, Rosell M, Díaz-Delfín J, Olmos Y, Monsalve M, Iglesias R, et al. (2011). Peroxisome proliferator-activated receptor α (PPARα) induces PPARγ coactivator 1α (PGC-1α) gene expression and contributes to thermogenic activation of brown fat: involvement of PRDM16. J Biol Chem 286: 43112-43122.

Hwang SY, Heo K, Kim JS, Im JW, Lee SM, Cho M, et al. (2015). Emodin attenuates radio-resistance induced by hypoxia in HepG2 cells via the enhancement of PARP1 cleavage and inhibition of JMJD2B. Oncol Rep 33: 1691-1698.

Izhaki I. (2002). Emodin-a secondary metabolite with multiple ecological functions in higher plants. New Phytol 155: 205-217.

Kaisanlahti A, Glumoff T. (2019). Browning of white fat: agents and implications for beige adipose tissue to type 2 diabetes. J Physiol Biochem 75: 1-10.

Konishi M, Haraguchi G, Ohigashi H, Ishihara T, Saito K, Nakano Y, et al. (2011). Adiponectin protects against doxorubicin-induced cardiomyopathy by anti-apoptotic effects through AMPK up-regulation. Cardiovasc Res 89: 309-319.

Li JM, Ding LL, Song BL, Xiao X, Qi M, Yang QL, et al. (2016). Emodin improves lipid and glucose metabolism in high fat diet-induced obese mice through regulating SREBP pathway. Eur J Pharmacol 770: 99-109.

Lidell ME, Betz MJ, Enerbäck S. (2014). Brown adipose tissue and its therapeutic potential. J Intern Med 276: 364-377.

Lowell BB, Spiegelman BM. (2000). Towards a molecular understanding of adaptive thermogenesis. Nature 404: 652-660.

Loyd C, Obici S. (2014). Brown fat fuel use and regulation of energy homeostasis. Curr Opin Clin Nutr Metab Care 17: 368-372.

Matteis RD, Lucertini F, Guescini M, Polidori E, Zeppa S, Stocchi V, et al. (2013). Exercise as a new physiological stimulus for brown adipose tissue activity. Nutr Metab Cardiovasc Dis 23: 582-590.

NCD Risk Factor Collaboration (NCD-RisC). (2016). Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 192 million participants. Lancet 387: 1377-1396.

Nicholls DG, Locke RM. (1984). Thermogenic mechanisms in brown fat. Physioll Rev 64: 1-64.

Nickerson JG, Alkhateeb H, Benton CR, Lally J, Nickerson J, Han XX, et al. (2009). Greater transport efficiencies of the membrane fatty acid transporters FAT/CD36 and FATP4 compared with FABPpm and FATP1 and differential effects on fatty acid esterification and oxidation in rat skeletal muscle. J Biol Chem 284: 16522-16530.

Okla M, Kim J, Koehler K, Chung S. (2017). Dietary factors promoting brown and beige fat development and thermogenesis. Adv Nutr 8: 473-483.

Ricquier D, Frédéric B. (2000). The uncoupling protein homologues: UCP1, UCP2, UCP3, StUCP and AtUCP. Biochem J 345: 161-179.

Schrauwen P, van Marken Lichtenbelt WD, Spiegelman BM. (2015). The future of brown adipose tissues in the treatment of type 2 diabetes. Diabetologia 58: 1704-1707.

Spiegelman BM, Flier JS. (2001). Obesity and the regulation of energy balance. Cell 104: 531-543.

Stahl A, Gimeno RE, Tartaglia LA, Lodish HF. (2001). Fatty acid transport proteins: a current view of a growing family. Trends Endocrinol Metab 12: 266-273.

Tzeng TF, Lu HJ, Liou SS, Chang CJ, Liu IM. (2012). Emodin protects against high-fat diet-induced obesity via regulation of AMP-activated protein kinase pathways in white adipose tissue. Planta Med 78: 943-950.

Vessal M, Mishra S, Moulik S, Murphy LJ. (2006). Prohibitin attenuates insulin-stimulated glucose and fatty acid oxidation in adipose tissue by inhibition of pyruvate carboxylase. FEBS J 273: 568-576.

Wan Z, Matravadia S, Holloway GP, Wright DC. (2013). FAT/CD36 regulates PEPCK expression in adipose tissue. Am J Physiol Cell Physiol 304: C478-C484.

Wang C, Li JX, Xue HF, Li Y, Huang JF, Mai JZ, et al. (2015). Type 2 diabetes mellitus incidence in Chinese: contributions of overweight and obesity. Diabetes Res Clin Pract 107: 424-432.

Wang L, Teng RF, Di LJ, Rogers H, Wu H, Kopp JB, et al. (2013). PPARα and Sirt1 mediate erythropoietin action in increasing metabolic activity and browning of white adipocytes to protect against obesity and metabolic disorders. Diabetes 62: 4122-4131.

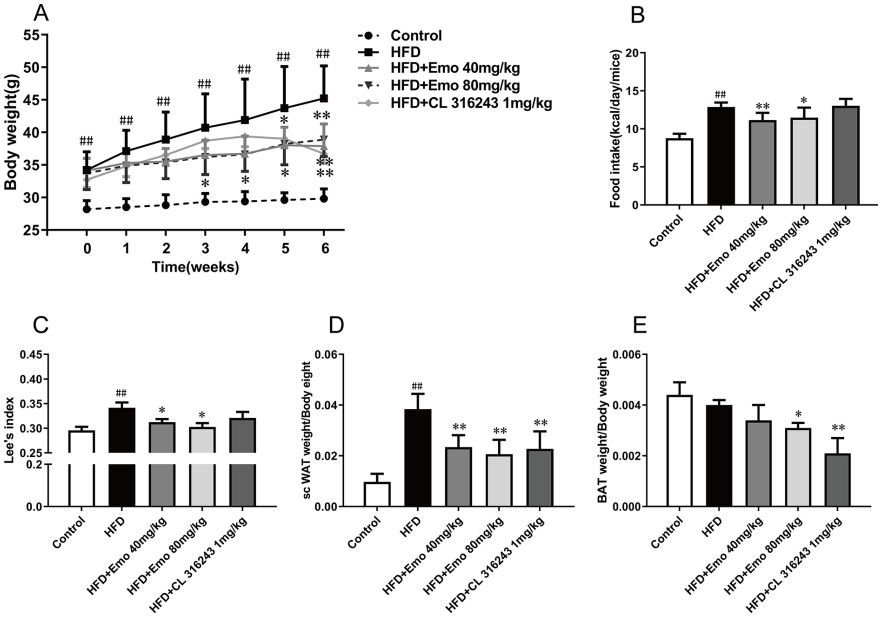
Wang YJ, Huang SL, Feng Y, Ning MM, Leng Y. (2012). Emodin, an 11β-hydroxysteroid dehydrogenase type 1 inhibitor, regulates adipocyte function in vitro and exerts anti-diabetic effect in Ob/Ob mice. Acta Pharmacol Sin 33: 1195-1203.

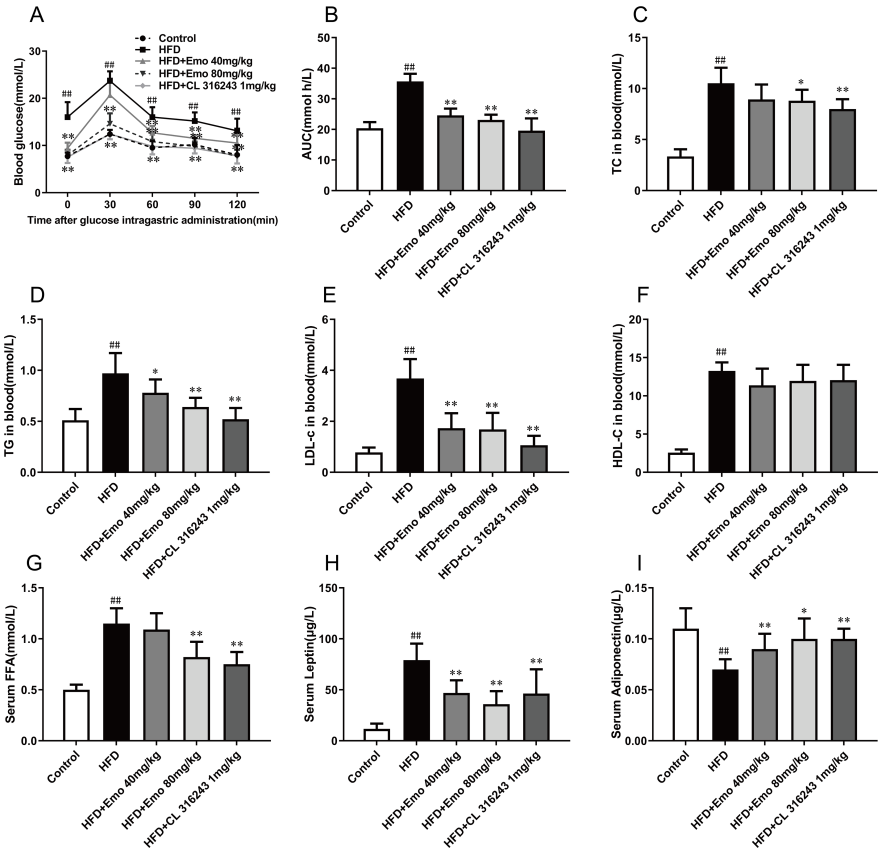
Wu ZQ, Chen QW, Ke DZ, Li GQ, Deng W. (2014). Emodin protects against diabetic cardiomyopathy by regulating the AKT/GSK-3β signaling pathway in the rat model. Molecules 19: 14782-14793.

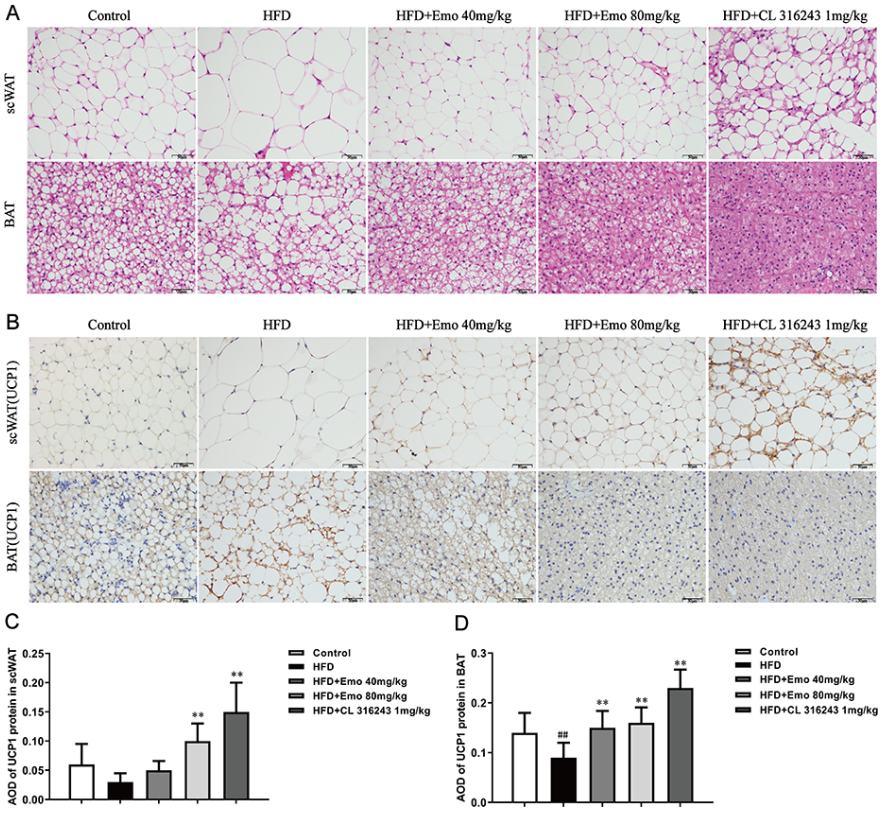
Xue J, Ding W, Liu Y. (2010). Anti-diabetic effects of emodin involved in the activation of PPARγ on high-fat diet-fed and low dose of streptozotocin-induced diabetic mice. Fitoterapia 81: 173-177.

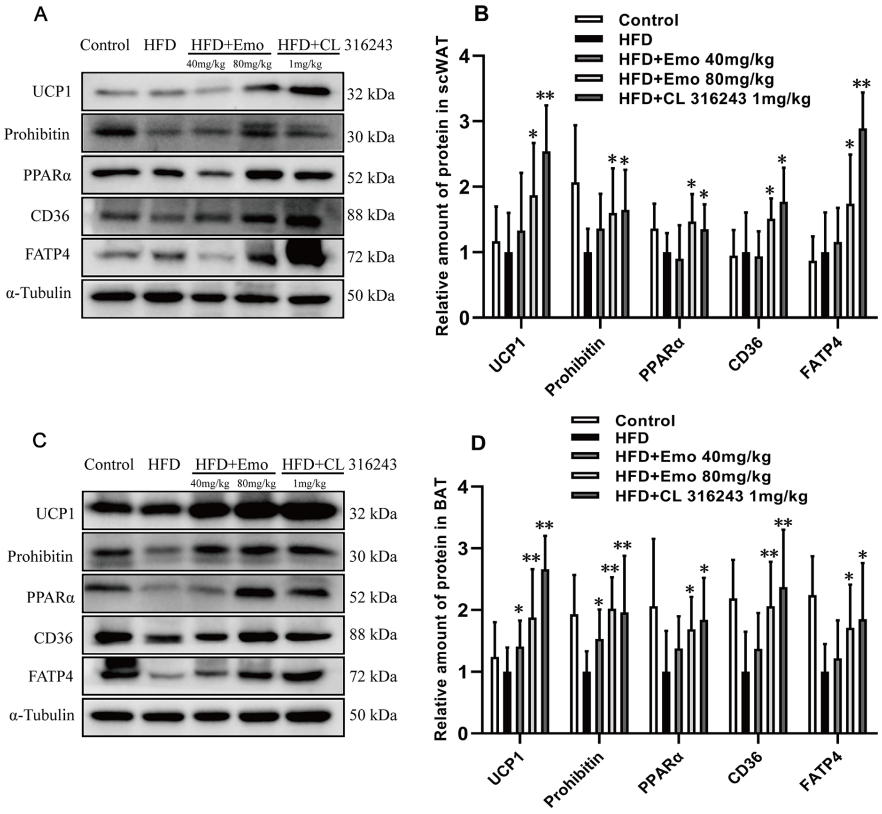
Zhu XF, Zeng K, Qiu Y, Yan FH, Lin CZ. (2013). Therapeutic effect of emodin on collagen-induced arthritis in mice. Inflammation 36: 1253-1259.

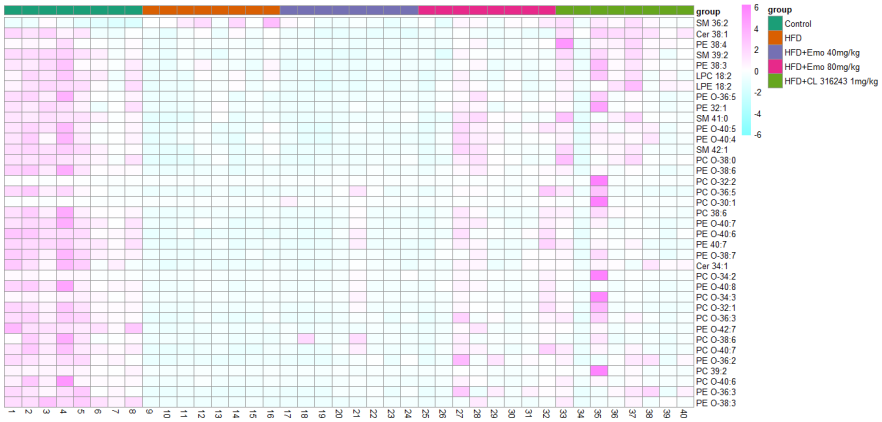
Ziemke F, Mantzoros CS. (2010). Adiponectin in insulin resistance: lessons from translational research. Am J Clin Nutr 91: 258S-261S.

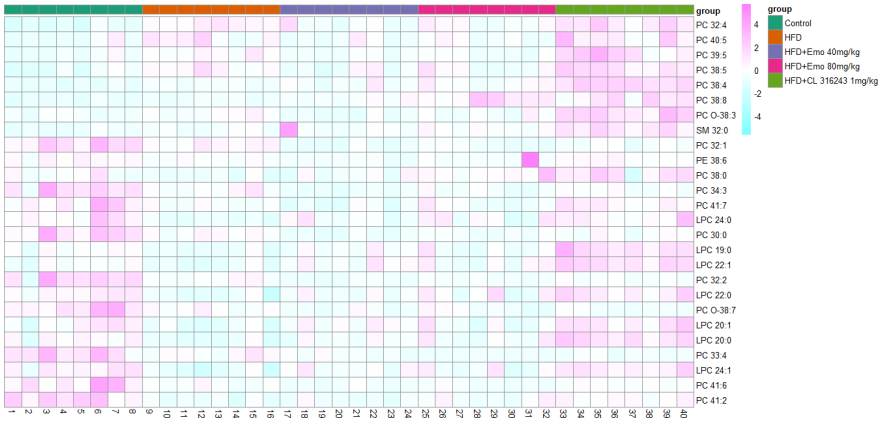












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