SUPPLEMENTARY MATERIAL

Flavonoid derivative exerts an antidiabetic effect via AMPK activation in diet induced obesity mice

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Abstract

In our previous study, a derivative of tiliroside, 3-O-[(E)-4-(4-ethoxyphenyl)-2-oxobut-3-en-1-yl]kaempferol (Fla-OEt) significantly enhanced glucose consumption in insulin resistant HepG2 cells. This paper deals with the anti-hyperglycemic and anti-hyperlipidemic effects of Fla-OEt in diet induced obesity (DIO) mice. Daily administration of Fla-OEt significantly decreased oral glucose tolerance test (OGTT), intraperitoneal insulin tolerance test (IPITT), and serum lipids. Hyperinsulinemic–euglycemic clamp and the ratio of high-density-lipoprotein (HDL)/low-density-lipoprotein (LDL) with Fla-OEt treatment were increased comparing with high fat diet (HFD) group, so lipid metabolism was improved. Histopathology examination showed that the Fla-OEt restored the damage of adipose tissues and liver in DIO mice. Moreover, compared with HFD group, Fla-OEt treatment significantly increased the phosphorylation of AMPK and ACC in adiposity tissues, liver, and muscles. The mechanism of its action might be the activation of AMPK pathway. It appears that Fla-OEt is worth further study for development as a lead compound for a potential antidiabetic agent.

Keywords: Fla-OEt, insulin resistant, lipid metabolism, anti-obesity

1. Experimental section

1.1. Flavonoid derivative

Fla-OEt was synthesized as described before (Qin et al. 2011). The structure of the compound was determined by ^1H , ^{13}C NMR, and 2D NMR spectral data analysis, including COSY, HSQC, HMBC, and ROESY spectra. The purity of Fla-OEt was $\geq 95\%$.

1.2. Animals and treatments

Male C57BL/6 mice (4 weeks old) were obtained from the Academy of Military Medical Science. All mice were adapted for one week to specific temperature ($23 \pm 2^\circ\text{C}$), humidity ($50 \pm 5\%$), and a 12h light/dark cycle (lights on at 8:00am and off at 8:00pm) conditions. The mice were housed in plastic cages (5/cage) and given free access to drinking water and food. After this adaptation, they were randomly divided into two groups and fed different diets during a 12 week period: one group (low fat diet, LFD; $n = 10$) was fed with a D12450B 10% kcal fat diet (Research Diets, New Brunswick, NJ, USA; protein: 19.2%, carbohydrate: 67.3%, fat: 4.3% and others: 3.85kcal/g). The others were fed with a D12492 60% kcal fat diet (Research Diets; protein: 26.2%, carbohydrate: 26.3%, fat: 34.9%, and others: 5.24kcal/g).

After 12 weeks, high-fat diet mice exhibiting obvious obesity and insulin resistance were selected and randomly divided into 3 groups of 10 animals per group. One group served as high-fat diet control (HFD); one group received 5 mg/kg Fla-OEt (HFD+Fla-OEt) daily, and the positive model group was administrated 200 mg/kg metformin (Met, Tianjin Pacific Chemical & Pharmaceutical Co. Ltd) for 4 weeks. The purity of metformin was $\geq 95\%$. The mice in the LFD and HFD groups were given the same volume PBS. The vehicle and test drugs were administered orally.

Food intakes of the mice were recorded daily, and the body weights were monitored every week. At the end of the experiment, mice were anesthetized after 12h fasting period. The visceral fat pads of epididymal, perirenal, and mesenteric were weighed after being rapidly removed from the sacrificed mice. Serum samples were collected and centrifuged at 5000 rpm/min for 15min at 4°C . Serum, liver, muscle and epididymal adipose tissues were frozen in liquid nitrogen immediately, and stored at -80°C until analysis.

1.3. Biochemical analysis

Serum concentration of triglyceride (TG), total cholesterol (TC), high-density-lipoprotein (HDL), low-density-lipoprotein (LDL), and nonesterified fatty acid (NEFA) were assayed enzymatically using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

1.4. Oral glucose tolerance test (OGTT)

Glucose tolerance test was performed at the end of fourth week in overnight-fasted animals by injecting 2g of glucose/kg. Blood samples were taken by tail bleeding at 0, 30, 60, 90, and 120min after glucose loading to determine the glucose concentrations. Area under the glucose–time curve (AUC) was calculated.

1.5. Intraperitoneal insulin tolerance test (IPITT)

The mice were fasted for 2h and then injected with 0.75IU/kg human short insulin subcutaneously. Blood samples were collected from the tails at 0, 30, 60, 90, and 120min after the insulin loading to determine glucose concentrations. AUC was then calculated.

1.6. Histology

The liver and epididymis adipose tissue were fixed for 48h in a 10% neutral formalin solution. The tissue was subsequently dehydrated with a graded ethanol series (75 to 100%) and embedded in paraffin wax. The embedded tissue was sectioned 8μm thick sections, stained with hematoxylin and eosin, and examined using a BX51 light microscope (Olympus Optical, Tokyo, Japan) before being photographed at a final magnification of 100.

1.7. Hyperinsulinemic–euglycemic clamp

The experimental process was performed as previously described (Tao et al. 2009) with some modification. After fasting for 4h, the mice were anesthetized with 27 mg/kg sodium pentobarbital (i.p.) and heparinized with 1U/kg heparin (i.v.). An indwelling catheter was inserted into the left internal jugular vein for infusing insulin and glucose. Insulin and glucose were infused by programmable syringe pumps (Cole Parmer, Vernon Hills, ILL, USA)

The hyperinsulinemic-euglycemic clamp technique was carried out with a prime-continuous infusion of human short insulin at the rate of 60pmol/kg/min and 20% glucose. When the blood glucose had maintained a steady state (6 ± 0.5 mmol/L) for at least 20min, the glucose infusion rate (GIR) was measured three times and was averaged.

1.8. Immunoblotting

Tissues were homogenized in ice-cold RIPA buffer containing protease inhibitor and phosphatase inhibitors. Tissue homogenates were centrifuged (13,000 rpm, 20 min, 4°C), and the supernatants were used for Western blot analyses. Protein concentrations were measured using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). Protein samples (60µg/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were blocked with 10% nonfat milk in TBS containing 0.1% Tween 20 (TBS-T) for 1h and incubated at 4°C overnight with primary antibodies for p-AMPK, p-ACC, and beta-actin. It followed by secondary antibodies conjugated with horseradish peroxidase. The bands were visualized by using enhanced chemiluminescence Western blotting detection system (GE Healthcare Life Sciences, Buckinghamshire, UK). All antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA).

1.9 Statistical analyses

The one-way ANOVA analysis (SPSS version 11.0, USA) was performed to assess data differences among various groups. $P < 0.05$ was considered statistically significant.

Reference

Tao R, Ye F, He Y, Tian J, Liu G, Ji T, Su Y. 2009. Improvement of high-fat-diet-induced metabolic syndrome by a compound from *Balanophora polyandra* Griff in mice. *Eur J Pharmacol.* 616:328–333.

Figure S1. The chemical structure of Fla-OEt.

Figure S2. Effects of Fla-OEt on body weight, food intake and visceral fat weight in high fat diet-induced insulin resistance mice. (A) body weight gain. (B) food intake. (C) visceral fat weight (sum of epididymis, mesenteric, and perirenal adipose). Values are expressed as mean $\pm$ S.D (n = 10). *** $P < 0.001$ vs. LFD. # $P < 0.05$ and ## $P < 0.01$ vs. HFD.

Figure S3. Effects of Fla-OEt on glucose tolerance, insulin tolerance and hyperinsulinemic-euglycemia clamp test in mice. Test were taken according to the “Materials and Methods” described. (A) The changes of blood glucose after intraperitoneal glucose loading. (B) The values of area under the curves in fig (A). (C) The changes of blood glucose after intraperitoneal insulin. (D) The values of area under the curves in fig (C). (E) The glucose infusion rates (GIR) in mice. Values are expressed as mean $\pm$ S.D (n = 10). *** P < 0.001 and ** P < 0.01 vs. LFD. # P < 0.05, ## P < 0.01 and ### P < 0.001 vs. HFD.

Figure S4. Histological analyses of epididymal adipose and liver tissue in mice. (A) epididymis adipose tissue and liver of mice fed with LFD. (B) epididymis adipose tissue and liver of mice fed with HFD. (C) epididymis adipose tissue and liver of mice in HFD+Fla-OEt. (D) epididymis adipose tissue and liver of mice in HFD+Met. Samples were stained with hematoxylin and eosin and photographed at 100× magnification.

Figure S5. Effects of Fla-OEt on the phosphorylations of AMPK and ACC in adipose tissue (A), liver (B), and muscle (C). The p-AMPK and p-ACC protein expressions in adipose tissue, liver, and muscle were determined by Western blot analysis. The relative band density was determined by densitometry with Image J software. The p-AMPK and p-ACC protein expression was normalized to the AMPK expression level and β -actin, respectively. Relative protein expression levels were from 3 independent experiments of 6 mice. Values are expressed as mean $\pm$ S.D, * $P < 0.05$ and ** $P < 0.01$ vs. LFD. # $P < 0.05$ and ## $P < 0.01$ vs. HFD.

Table S1. Effects of Fla-OEt on serum general characteristics

Group	LFD	HFD	HFD+Fla-OEt	HFD+Met
Insulin (μ IU/ml)	1.73 $\pm$ 0.44	10.07 $\pm$ 0.99 ^{***}	2.71 $\pm$ 0.66 ^{###}	3.58 $\pm$ 0.71 ^{###}
TG (mg/dl)	0.44 $\pm$ 0.048	2.17 $\pm$ 0.17 ^{***}	0.39 $\pm$ 0.19 ^{###}	0.35 $\pm$ 0.02 ^{###}
TC (mg/dl)	2.71 $\pm$ 0.51	8.43 $\pm$ 1.60 ^{**}	4.26 $\pm$ 0.55 ^{##}	5.99 $\pm$ 0.69 ^{##}
HDL/LDL	1.48 $\pm$ 0.24	0.84 $\pm$ 0.10 ^{**}	1.33 $\pm$ 0.25 [#]	0.94 $\pm$ 0.27
NEFA (μ mol/dl)	56.91 $\pm$ 11.49	406.51 $\pm$ 22.93 ^{***}	83.33 $\pm$ 18.87 ^{###}	111.78 $\pm$ 12.87 ^{###}

Values are expressed as mean $\pm$ S.D (n = 10). * P < 0.05, ** P < 0.01 and *** P < 0.001 vs. LFD. # P < 0.05, ## P < 0.01 and ### P < 0.001 vs. HFD.