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Anti-insulin resistant effect of ferulic acid on high fat diet-induced obese mice

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ABSTRACT

Objective: To evaluate the insulin sensitivity action of ferulic acid (FA) in skeletal muscle and hypothalamus of high-fat diet (HFD)-induced obese mice. **Methods:** Obese mouse model was induced by HFD (45 kcal% lard fat) for 16 weeks. After 8 weeks of HFD feeding, these obese mice were orally treated with FA at doses of 25 and 50 mg/kg/day for 8 weeks. At the end of all treatments, the epididymal fat, pancreas, skeletal muscle and hypothalamus were removed for biochemical parameter and protein expression examinations. **Results:** FA treatment significantly decreased leptin level in fat tissue and insulin level in pancreas ($P < 0.05$). Interestingly, obese mice treated with FA increased the protein expressions of insulin receptor substrate-1, phosphatidylinositol 3-kinase, and phosphorylated-protein kinase B in both muscle and brain ($P < 0.05$). The phosphorylations of adenosine monophosphate-activated protein kinase and acetyl-CoA carboxylase in muscle, and leptin receptor protein in hypothalamus were also increased ($P < 0.05$). The pancreatic islets histology showed smaller size in obese mice treated with FA compared to untreated obese mice. **Conclusions:** These findings indicate the beneficial effect of FA in improving insulin resistance in HFD-induced obese mice. These effects are probably mediated *via* modulating the insulin receptor substrate/phosphatidylinositol 3-kinase/protein kinase B or adenosine monophosphate-activated protein kinase pathways.

1. Introduction

Insulin resistance is a main cause of type 2 diabetes mellitus (T2DM)[1]. Normally, glucose uptake is an important activity for glucose homeostasis regulation, this activity is associated with stimulation of glucose transporter 4 (GLUT4) translocation from the cytoplasm to the plasma membrane in skeletal muscle

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and adipose tissue[2]. In skeletal muscle, insulin plays roles in activating phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) which leads increased GLUT4 translocation to the plasma membrane[2]. Adenosine monophosphate-activated protein kinase (AMPK) also stimulates GLUT4 translocation by acting on exercise/muscle contraction[3] and antidiabetic agents such as metformin[4]. The action of insulin in brain can control energy homeostasis and peripheral insulin sensitivity[5]. Glucose uptake in hypothalamus is related to insulin activation *via* insulin receptor (IR) including IR substrate-1 (IRS-1) tyrosine phosphorylation[6]. Furthermore, insulin can suppress hepatic glucose production in hypothalamus[7].

There has been reported that leptin resistance suppressed hypothalamic response to insulin in obesity and thereby impaired peripheral glucose homeostasis, supporting the development of T2DM[8]. Leptin receptors (ObRs) are widely expressed in central nervous system, particularly in the hypothalamus[9]. The coordination between leptin and insulin may manage energy homeostasis *via* IRS/PI3K/Akt signaling pathway[9].

Ferulic acid (FA) is one of phenolic acid, which has several pharmacological activities. There has been many reports suggesting its effects as antioxidant[10], anti-inflammatory[11], and anti-diabetic activities[12-14]. Our previous study has shown that the FA treatment for 8 weeks reduced the weight gain, hyperglycemia, hyperlipidemia, hyperinsulinemia, and hyperleptinemia in high-fat diet (HFD)-induced obese mice[15]. Treatment of FA inhibited hepatic glucose production by suppressing phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, and reducing hepatic lipogenesis genes such as sterol regulatory element-binding protein 1c, fatty acid synthase, and acetyl-CoA carboxylase (ACC)[15]. Therefore, the administration of FA can improve glucose and lipid homeostasis in liver tissue of HFD-induced obese mice. Insulin sensitivity in skeletal muscle and hypothalamus is an important key for glucose homeostasis management in T2DM pathogenesis, it is thus essential to evaluate the insulin sensitivity action of FA in these tissues. The insulin sensitivity for regulating glucose homeostasis may be involved in insulin-dependent and -independent signal transduction pathways. Therefore, the effects of FA on activation of both PI3K and AMPK pathways were determined.

2. Materials and methods

2.1. Chemical and reagents

Low-fat diet (LFD) and HFD were from Research Diets (New Brunswick, NJ, USA). The diet composition was shown in Table 1. All chemicals were from Sigma Aldrich (St. Louis, MO, USA). TPER® and Halt® protease inhibitor cocktails were from Thermo Scientific (Rockford, IL, USA). Anti-ObR and anti-actin primary antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA). Polyvinylidene difluoride membrane, anti-pAMPK α , anti-total AMPK (anti-tAMPK), anti-pACC, anti-IRS-1, anti-pAkt, anti- tAkt, and anti-PI3K were from EMD Millipore (Billerica, MA, USA). Anti- tACC was from Cell Signaling (Danvers, MA, USA).

Table 1

Composition of experimental diets.

Composition	LFD	HFD
Ingredients (g/kg diet)		
Casein	200	200
L-cystine	3	3
Corn starch	452.2	72.8
Sucrose	172.8	172.8
Cellulose	50	50
Soybean oil	25	25
Lard	20	177.5
Mineral mix S10026	10	10
Vitamin mix V10001	10	10
Choline bitartrate	2	2
Energy (kcal%)		
Protein	20	20
Carbohydrate	70	35
Fat	10	45

2.2. Experimental design

All animal experimental protocols were approved by the Animal Ethics Committee of Thammasat University (Rec. No. AE 002/2015). Thirty-two male ICR mice (weighing 20-25 g) were obtained from the National Laboratory Animal Center of Mahidol University (Nakhon Pathom, Thailand). The animals were housed at Thammasat University Laboratory Animal Center (AAALAC accredited facility), in a standard room with the temperature (25 ± 2) ℃ and a 12-h dark/light cycle. Mice were allowed to have freely access to food and water. Mice were divided into 4 experimental groups. Group 1 was a normal control group; mice were fed with LFD for 16 weeks and designated as LFD group. Group 2 was an obese control group; mice were fed with HFD for 16 weeks and designated as HFD group. In the experimental Group 3 and Group 4, mice were fed with HFD for 16 weeks with the oral administration of FA at 25 and 50 mg/kg/day, respectively, everyday over the last 8 weeks. FA was suspended in 5% gum arabic. Once the treatment was completed, the mice were fasted for 6 h prior to being anesthetized with isoflurane. Soleus muscle, pancreas, epididymal fat and hypothalamus were removed for further studies.

2.3. Concentration of insulin, leptin, and triglyceride (TG) in tissues

Pancreas and epididymal fat were homogenized followed by an extraction using TPER® mixed with Halt® protease inhibitor cocktails. Pancreatic tissue was measured for an insulin level while a leptin level was measured from the epididymal fat extraction using the leptin kit assay (EMD Millipore, Billerica, MA, USA).

Soleus muscle was homogenized and extracted with isopropanol (50 mg/mL, w/v). The supernatant was collected and measured for muscle TG using an enzymatic colorimetric method (Wako, Osaka, Japan) as previously described[16].

2.4. Protein expression in hypothalamus and muscle

After being homogenized, hypothalamus and soleus muscle were extracted with TPER® mixed with Halt® protease inhibitor

cocktail. The extracted proteins were separated by 8% or 12% SDS-polyacrylamide gel electrophoresis and blotted on polyvinylidene difluoride membranes, followed by blocking with 5% non-fat powdered milk in Tris-buffered saline/Tween-20. The membranes were incubated overnight at 4 ℃ with anti-ObR, anti-pAMPK, anti-tAMPK, anti-pACC, anti-tACC, anti-IRS-1, anti-PI3K, anti-pAkt, anti-tAkt, and anti-actin primary antibodies (diluted 1:500). The blots were detected after incubation with horseradish peroxidase-conjugated secondary antibody (diluted 1:5 000) using Clarity™ Western ECL Blot Substrate kit (Bio-rad, Brea, CA, USA). Images were obtained using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Band intensity was performed using densitometry and a Gel-Pro™ Analyzer version 3.1 software (MediaCybernetics, Rockville, MD, USA). The blots were normalized with actin to ensure equal protein loading.

2.5. Pancreas histology

Pancreas was fixed with 10% formalin and embedded in paraffin. Sections of 3-micron thickness were cut and stained with hematoxylin and eosin (H&E). Histological changes were performed using a light microscope (CX31; Olympus, Tokyo, Japan).

2.6. Statistical analyses

The results were expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Tukey's *post hoc* test were used for multiple comparisons. All statistical analyses were performed using SigmaStat (Systat Software, CA, USA). The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Effect of FA on biochemical parameters and pancreas histology

The concentrations of TG in muscle, leptin in epididymal fat, and insulin in pancreas were decreased in FA-treated groups compared to HFD group ($P < 0.05$) (Table 2). Moreover, the obese mice treated with FA showed the smaller size of pancreatic islets compared to the enlarged pancreatic islets of mice in the HFD group (Figure 1).

Table 2

Effect of FA on metabolic parameters in HFD-induced obese mice.

Groups	Muscle TG (mg/g tissue)	Fat leptin (ng/mg protein)	Pancreas insulin (ng/mg protein)
LFD	16.7 ± 1.6	7.6 ± 0.6	0.40 ± 0.02
HFD	36.4 ± 2.3$^{\#}$	14.4 ± 1.2$^{\#}$	1.50 ± 0.20$^{\#}$
HFD + FA 25 (mg/kg)	28.4 ± 1.0$^{\#*}$	9.4 ± 1.1*	0.50 ± 0.02*
HFD + FA 50 (mg/kg)	24.8 ± 1.9$^{\#*}$	8.3 ± 0.6*	0.50 ± 0.03*

Values are represented as mean ± SEM (n=8). $^{\#}P < 0.05$ compared to LFD group. $^{*}P < 0.05$ compared to HFD group. LFD group: normal control mice fed with low-fat diet, HFD group: obese mice fed with high-fat diet.

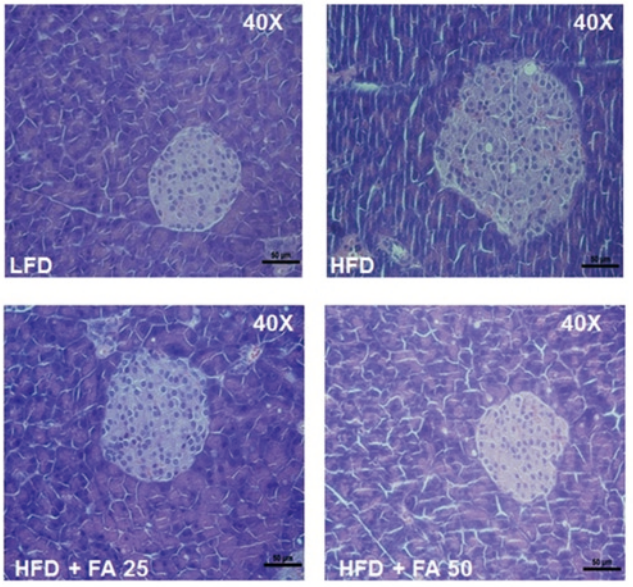

Figure 1. Effect of FA on pancreas histology in HFD-induced obese mice. Pancreas tissue was stained with H&E and performed under microscope (40 ×). The islet size of FA-treated groups was smaller than that of HFD group. LFD group: normal control mice fed with low-fat diet, HFD group: obese mice fed with high-fat diet.

3.2. Effect of FA on protein expressions of muscle IRS-1, PI3K, pAkt, pAMPK, and pACC

The protein expressions of muscle IRS-1, PI3K, pAkt (ratio of pAkt/tAkt), pAMPK (ratio of pAMPK/tAMPK), and pACC (ratio of pACC/tACC) were significantly decreased in the HFD group (0.29-, 0.27-, 0.30-, 0.33-, and 0.60-fold of control, respectively) as compared to the LFD group ($P < 0.05$) (Figure 2a-2e). Interestingly, obese mice treated with FA (25 and 50 mg/kg) significantly increased IRS-1 (0.92- and 1.08-fold of control), PI3K (0.77- and 0.95-fold of control), pAkt (0.95- and 1.04-fold of control), pAMPK (1.08- and 1.10-fold of control), and pACC (1.06- and 1.40-fold of control) ($P < 0.05$) (Figure 2a-2e).

3.3. Effect of FA on protein expressions of hypothalamus IRS-1, PI3K, pAkt, and ObR

As shown in Figure 3a-3d, the protein expressions of IRS-1, PI3K, pAkt, and ObR in hypothalamus were decreased in HFD group (0.34-, 0.58-, 0.52 and 0.32-fold of control, respectively) as compared to LFD group ($P < 0.05$). Remarkably, obese mice treated with FA (50 mg/kg) could reverse these protein expressions. The IRS-1, PI3K, pAkt, and ObR (1.03-, 1.05-, 1.1-, and 1.32-fold of control, respectively) were significantly increased as compared to HFD group ($P < 0.05$). However, FA at dose of 25 mg/kg significantly increased only the protein expressions of PI3K and pAkt (1.12- and 0.91-fold of control) ($P < 0.05$) but not IRS-1 and ObR (Figure 3a-3d).

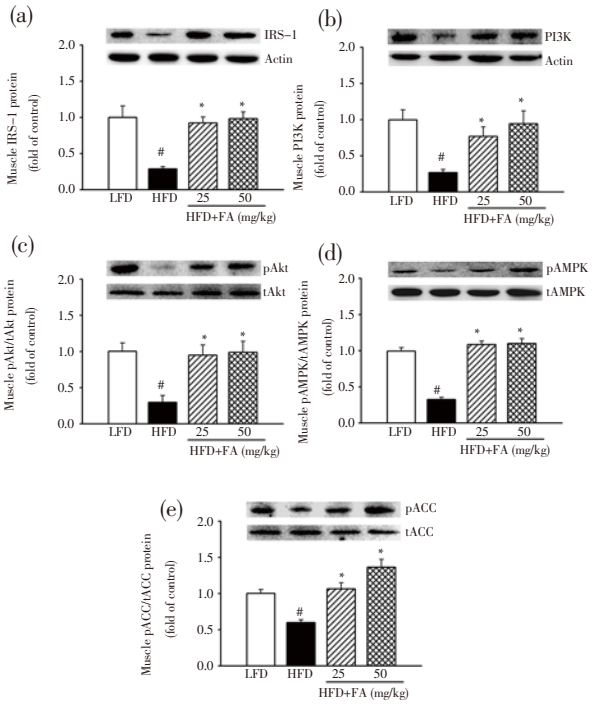

Figure 2. Effect of FA on protein expressions of muscle IRS-1 (a), PI3K (b), pAkt (c), pAMPK (d) and pACC (e) in HFD-induced obese mice.
All values represent mean ± SEM (n=8). $^{\#}P < 0.05$ compared to LFD group. $^{*}P < 0.05$ compared to HFD group. LFD group: normal control mice fed with low-fat diet, HFD group: obese mice fed with high-fat diet.

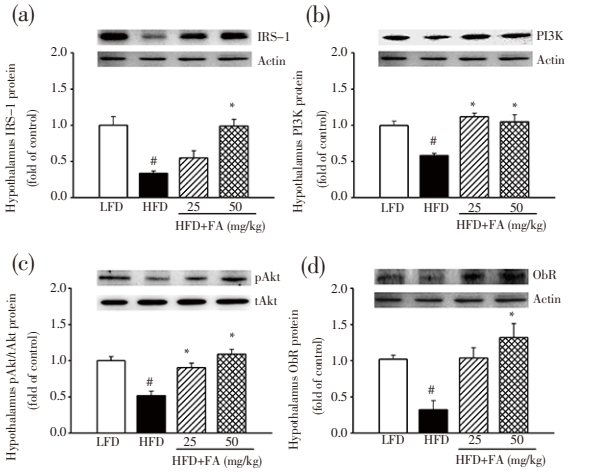

Figure 3. Effect of FA on protein expressions of hypothalamus IRS-1 (a), PI3K (b), pAkt (c) and ObR (d) in HFD-induced obese mice.
All values represent mean ± SEM (n=8). $^{\#}P < 0.05$ compared to LFD group. $^{*}P < 0.05$ compared to HFD group. LFD group: normal control mice fed with low-fat diet, HFD group: obese mice fed with high-fat diet.

4. Discussion

Our previous study reported that FA administration decreased the hepatic lipogenesis and gluconeogenesis in HFD-fed mice[15]. In the present study, we further investigated the possible mechanisms of FA in improving insulin sensitivity in the skeletal muscle and hypothalamus of HFD-induced obese mouse model.

There has been reported that hyperleptinemia is related to development of leptin resistance in diet-induced obese mice[17]. The leptin resistance, impaired leptin function, and reduced leptin receptor expression are found in obesity condition[18]. The leptin effect is associated with insulin sensitivity and insulin-dependent glucose uptake[9,19]. Leptin and insulin signaling systems work together at the level of PI3K and Akt in the hypothalamus[9,19]. The present study showed that FA administration significantly reduced leptin concentration in epididymal fat of HFD-induced obese mice. We further found that obese mice treated with FA could increase the ObR expression in hypothalamus. These results may contribute to decreasing the overstimulation of the leptin receptors as well as the downstream signaling, thus improving central leptin sensitivity. Activation of ObR can sensitize insulin action in the hypothalamus *via* IRS/PI3K/Akt pathway[9]. Our findings are consistent with the hypothesis that leptin may directly regulate insulin sensitivity in the hypothalamus, at least in part, through specific modulation of IRS-1/PI3K/Akt pathway. The present study also showed that the decreased leptin content in adipose tissue was related to the decreased content of insulin in pancreas. We propose that FA may improve leptin sensitivity which is essential for insulin action in the hypothalamus, and simultaneously enhance the central insulin signal to maintain blood glucose homeostasis. Our data support the concept that central insulin signaling is an important factor for peripheral glucose homeostasis regulation. The IRS/PI3K/Akt pathway is associated with insulin-dependent action[20]. Akt is a key downstream of PI3K pathway that inhibits the hepatic glucose production and stimulates the muscle glucose uptake[20]. The present study showed that FA could increase the expression of IRS-1, PI3K and phosphorylation of Akt in skeletal muscle of obese mice. These results indicate that FA improves insulin sensitivity in muscle of obese mice by stimulating IRS/PI3K/Akt pathway.

Furthermore, the stimulation of AMPK is another pathway that has also been reported to control hepatic glucose homeostasis *via* suppressing gluconeogenic enzyme and glucose production[21], and enhancing glucose uptake in skeletal muscle[22]. Previously, we showed that FA treatment significantly decreased hepatic gluconeogenic enzymes, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase[15]. In the present study, FA was found to increase the expression of AMPK phosphorylation in skeletal muscle of obese mice. These findings indicate that FA also has the ability to improve the impaired glucose homeostasis through enhancing AMPK phosphorylation in skeletal muscle. It has been reported that leptin activates AMPK which leads to phosphorylation of ACC and subsequent stimulation of fatty acid oxidation[23,24]. Since fatty acid plays a central role in the state of insulin resistance, the potentiality of leptin to stimulate fatty acid oxidation in skeletal muscle may result in prevention of lipotoxicity and consequent insulin resistance[23,24]. The present study showed that FA could increase phosphorylation of ACC in muscle. This may increase fatty acid oxidation and finally improve insulin resistance. The elevated intramyocellular lipid accumulation is associated with insulin resistance[25]. Evidently, the present study demonstrated that FA administration reduced TG accumulation in the skeletal muscle, possibly by decreasing the insulin resistance.

When the insulin resistance happens, β-cells are activated to

release more insulin in order to maintain the normal glycemic level. This eventually increases β-cell mass[26]. Consistent with the aforementioned study, the hypertrophic pancreatic islets were found in insulin resistant mice, and this hypertrophy was minimized in FA-treated mice. Moreover, the decreased insulin content in pancreatic tissue was also found in FA-treated groups.

In conclusion, this study provides evidence supporting that FA improves the impaired glucose homeostasis and insulin sensitivity in both skeletal muscle and hypothalamus *via* activating IRS/PI3K/Akt and AMPK signaling pathways in HFD-induced obese mice. FA is thus a potential alternative agent for an alleviation of obesity-related insulin resistant condition.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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