Herbal extract THI improves metabolic abnormality in mice fed a high-fat diet

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Abstract
Target herbal ingredient (THI) is an extract made from two herbs, Scutellariae Radix and Platycodi Radix. It has been developed as a treatment for metabolic diseases such as hyperlipidemia, atherosclerosis, and hypertension. One component of these two herbs has been reported to have anti-inflammatory, anti-hyperlipidemic, and anti-obesity activities. However, there have been no reports about the effects of the mixed extract of these two herbs on metabolic diseases. In this study, we investigated the metabolic effects of THI using a diet-induced obesity (DIO) mouse model. High-fat diet (HFD) mice were orally administered daily with 250 mg/kg of THI. After 10 weeks of treatment, the THI-administered HFD mice showed reduction of body weights and epididymal white adipose tissue weights as well as improved glucose tolerance. In addition, the level of total cholesterol in the serum was markedly reduced. To elucidate the molecular mechanism of the metabolic effects of THI in vitro, 3T3-L1 cells were treated with THI, after which the mRNA levels of adipogenic transcription factors, including C/EBPα and PPARγ, were measured. The results show that the expression of these two transcription factors was downregulated by THI in a dose-dependent manner. We also examined the combinatorial effects of THI and swimming exercise on metabolic status. THI administration simultaneously accompanied by swimming exercise had a synergistic effect on serum cholesterol levels. These findings suggest that THI could be developed as a supplement for improving metabolic status.

Key Words: Triglyceride, baicalin, glucose tolerance, exercise

Introduction

 Platycodi Radix has long been used as an expectorant in traditional Oriental medicine. Recently, it was reported that Platycodi Radix has anti-inflammatory, anti-allergy, anti-tumor, apoptosis-inducing, and immune-stimulating activities [1-4]. Several reports have also shown that platycodin saponins, a major component of Platycodi Radix, have beneficial effects on the treatment of metabolic disorders, including obesity and hyperlipidemia [5]. Platycodi Radix has been found to reduce hepatic and serum triglyceride levels in Sprague-Dawley rats fed a high-fat diet (HFD) [6] as well as inhibit adipogenesis by modulating kruppel-like factor 2 (KLF2) and peroxisome proliferator-activated receptor γ (PPAR-gamma) [7].

 Scutellariae Radix has also been widely used for the clinical treatment of hyperlipidemia, atherosclerosis, and hypertension in East Asian countries, including China, Korea, and Japan. The active component of Scutellariae Radix is baicalin, which is well known as an anti-inflammatory and antioxidant agent [8-10]. Baicalin inhibits adipogenesis through the downregulation of pro-adipogenic genes, including PPARγ, C/EBPα, and KLF15, as well as the upregulation of anti-adipogenic regulators, including C/EBPγ and KLF2 [11]. It was also reported that baicalin exerts an anti-adipogenic effect through the maintenance of β-catenin expression, which is reduced during normal adipogenesis [12].

 Although Platycodi Radix and Scutellariae Radix show anti-adipogenic effects, studies have not been performed on the combinatorial effects of Platycodi Radix and Scutellariae Radix on metabolic disease. Thus, we examined the metabolic effects of a mixture of Platycodi Radix and Scutellariae Radix using a diet-induced obesity model.

Materials and Methods

 Chemicals and reagents

 Cell culture reagents were obtained from Life Technologies (Grand Island, NY, USA). Anti-C/EBPα and anti-C/EBPβ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PPARγ antibody and secondary antibody were purchased from Cell Signaling (Beverly, MA, USA). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

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Animals and experimental groups

All animals were obtained from the Korea Research Institute of Bioscience and Biotechnology, Daejoen, Korea. Seven-week-old C57BL/6 male mice were subdivided, acclimatized for 1 week, and divided randomly into five groups: (1) sedentary, normal diet, and vehicle treatment (ND-Veh), (2) sedentary, high-fat diet, and vehicle treatment (HFD-Veh), (3) sedentary, high-fat diet, and THI treatment (HFD-THI), (4) exercise, high-fat diet, and vehicle treatment (E/HFD-Veh), and (5) exercise, high-fat diet, and THI treatment (E/HFD-THI). Bedding was changed once a week, and the temperature and humidity were controlled. Mice were housed under 12 hr light/12 hr dark conditions. The plans and protocols for animal experiments were approved by the Institutional Animal Care and Use Committee of Sookmyung Women’s University, Seoul, Korea.

Preparation of herbal mixture

The roots of *Platycodon grandiflorum* and *Scutellariae baicalensis* were obtained from Namil Farm & Ginseng Co. (Geumsam, Korea); voucher specimens were preserved at Chug-Ang University College of Medicine, Seoul, Korea (No. 2009.11-12). The two herbs were mixed in a 1:1 ratio and extracted with 70% ethanol. The extract was concentrated by evaporating solvent under low pressure conditions. The final yield of extract compared with raw herbal material was 25% (w/w).

Diet and THI treatment

Purina Diet (Koatech, Seoul, Korea) was provided to mice in the normal diet group, whereas a pellet rodent diet with 60% K cal fat (Central Lab. Animal Inc., Seoul, Korea) was provided for 10 weeks to the HFD group. Each mouse was administered 25 mg of THI orally, as determined in previous studies. For the vehicle treatment group, the same volume of distilled water was administered orally every day for 10 weeks by the same method used for THI treatment. Each group comprised six mice, and all mice were allowed free access to the described diet and water during experimental periods. Body weights and food intake were measured weekly at regular times.

Tissue preparation and blood chemistry

Mice were dissected to collect tissues for analysis. Blood was collected from the retro-orbital sinus by using sodium-heparinized microhaematocrit capillary tubes (Marienfeld-superior, Lauda-Königshofen, Germany) and then transferred to Eppendorf tubes and incubated at room temperature. After 3 hr, all samples were centrifuged at 13,500 rpm for 5 min, after which serum was taken to measure triglyceride, glucose, and total cholesterol levels using an automatic blood chemistry analyzer Dry-Chem 4000i (Fujifilm, Saitama, Japan) at the Yonsei Laboratory Animal Research Center.

Swimming exercise

For exercise experiments, exercise groups of HFD mice were divided into two groups. One group was subjected to exercise without administration of THI while the other group was subjected to swimming exercise with oral administration of 25 mg of THI to each mouse. Mice underwent compulsory swimming exercise at 9:00 am and 5:00 pm twice a day, 5 days per week for 10 weeks. In the first week, mice swam voluntarily for 40 min, and this time was increased gradually up to 60 min for intensification of exercise. Mice swam at 31°C in a temperature-controlled water bath, and the water bath was cleaned once a week.

RT-PCR analysis

Total RNA samples were prepared by homogenizing epididymal tissues and liver tissues with 500 μL of RNA iso-plus (Takara, Shiga, Japan). Prepared total RNAs were reverse-transcribed using M-MuLV reverse transcriptase (Promega, Madison, WI, USA) at 37°C for 1 hr. PCR for amplification of mRNAs encoding hormone-sensitive lipase (HSL), adipose triglyceride lipase (ATGL), mitochondrial uncoupling protein 2 (UCP2), and carnitine palmitoyltransferase 1a (CPT1a) was performed using appropriate primer pairs: ATGL forward, 5'-CTCCGAGAGATGTGCAAACA-3', reverse, 5'-CAGTTCCACCTGCTCAGACA-3'; HSL forward, 5'-CTTCCTGCAAGAGTATGTCACG-3', reverse, 5'-TGGAGGTGAGATGGTGACTG-3; UCP2 forward, 5'-CGTAGTAGTACATGCTCAGACA-3'; HSL forward, 5'-CTTCCTGCAAGAGTATGTCACG-3', reverse, 5'- TGGAGGTGAGATGGTGACTG-3'; CPT1a forward, 5'-GCACTTGGAACCTGAC-3', reverse, 5'-CATGGTAGTACATGCTCAGACA-3'; RT-PCR products were visualized by 1% agarose gel electrophoresis, and the intensity of the bands was measured using a DNR Bio-Imaging system (Kiryat Anavim, Jerusalem, Israel).

Oral glucose tolerance test (OGTT)

Mice were fasted for 14 hr before experiments, and D-glucose (Duchefa Biochemie, Haarlem, Netherlands) was administered to mice at a dose of 1 g per kg of body weight. Glucose level of the blood taken from the tail vein was measured using Accu-Chek (Roche Diagnostics, Basel, Switzerland) at 30 min intervals for 120 min.

Cell culture

The 3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Two days after reaching confluence (day 0), 3T3-L1 cells were cultured in Dulbecco's
Modified Eagle’s Medium (DMEM) containing 1 μg/mL of insulin, 0.25 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 10% fetal bovine serum (differentiation induction medium) for 2 days. Cells were then maintained in DMEM containing 1 μg/mL of insulin and 10% fetal bovine serum (differentiation maintenance medium). The differentiation maintenance medium was changed every 2 days until the cells were harvested on day 7. To test the effects of THI on adipogenesis, various concentrations of THI were added to the differentiation induction and maintenance media until cells were harvested. Differentiated cells in each well of 6-well plates were harvested in 500 μL of phosphate-buffered saline (PBS), frozen, and then sonicated. The triglyceride contents of the cell lysates were measured using a TG-S reaction kit (Asan Pharm., Seoul, Korea). Lipid droplets in cells were stained with Oil Red O, as previously described [13].

**Protein extraction and Western blotting**

Cultured and differentiated cells were harvested using a cell scraper and lysed with ice-cold RIPA buffer containing 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail (Sigma-Aldrich). The total cell lysates were centrifuged at 14,000 rpm for 20 min at 4°C to remove insoluble materials. The protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Fifty micrograms of protein extract was resolved by 10% SDS-polyacrylamide gel electrophoresis at 150 mA for 1 hr and then transferred to nitrocellulose membranes. The membranes were next blocked for 2 hr at room temperature with PBS containing 5% skim milk and 0.1% Tween 20, incubated with 1:1,000-dilutions of primary antibodies overnight at 4°C, and then with horseradish peroxidase-conjugated anti-rabbit secondary antibody for 1 hr at room temperature. Peroxidase activity was visualized using an ECL kit (Pierce, Rockford, IL, USA).

**Statistical analysis**

Statistical analysis was performed using SPSS statistical software. When differences among the groups were detected by one-way factorial ANOVA, Turkey’s test was used. A level of significance of $P < 0.05$ was chosen for all statistical comparisons. Data are presented as means ± SEM.

**Results**

**Effects of THI on body weight and food intake**

Many herbal extracts were screened for the inhibition of fat accumulation in 3T3-L1 cells (data not shown). Two herbs, Scutellariae Radix and Platycodi Radix, were finally selected due to their strong activities. In this study, we examined the effects of the mixed extract of these two herbs, named THI, on metabolic abnormality in mice fed a high-fat diet. There are two types of obesity animal models available. One includes genetic models such as the leptin-deficient ob/ob mouse model and the leptin receptor deficient db/db mouse model. The other type includes the DIO model. In this study, we used the DIO model to determine the effects of THI on obesity since DIO is an experimental model with much higher applicability to human obesity. Mice were subdivided into three groups: the normal diet group with vehicle treatment, the 60% HFD group with vehicle treatment, and the 60% HFD group with THI treatment. There were significant differences in body weight between the HFD and normal diet groups from the first week of the experiment. The THI-administered mice showed a more modest increase in size and body weight than did the HFD group, although food intake was the same for both groups (Fig. 1A and 1B).

**Fig. 1.** THI administration reduces body weight in the diet-induced obesity (DIO) model. (A) Body weight changes were measured once a week, and mice were photographed after 10 weeks. HFD-Veh, high-fat diet with water administration; ND-Veh, normal diet with water administration; HFD-THI, high-fat diet with THI administration. *$P<0.05$ and **$P<0.01$ indicate statistical significance between the HFD-Veh and HFD-THI groups. (B) Food intake per mouse per day was measured. Data show average food intake over 10 weeks. All values are given as mean ± SEM (n = 7) for all groups.
and the THI-administered HFD group showed a significant decrease in adipose tissue weight by as much as 40% compared with the HFD group (Fig. 2A). However, liver tissue weights were not significantly different among the three groups (Fig. 2B).

Effects of THI on serum levels of triglycerides, total cholesterol, and glucose

Since THI administration reduced adipose tissue weight, we wondered whether or not THI could induce changes in the serum levels of triglycerides and total cholesterol. The level of total cholesterol was lower compared with the HFD group (Fig. 3A and 3B), whereas the triglyceride level was not reduced to a statistically significant level. Next, to determine whether or not administration of THI enhances energy expenditure, total RNA was isolated from epididymal white adipose tissues. The mRNA levels of hormone-sensitive lipase (HSL), adipose triglyceride lipase (ATGL), mitochondrial uncoupling protein 2 (UCP2), and carnitine palmitoyltransferase 1a (CPT1a) in epididymal white adipose tissues were determined using RT-PCR (n = 3). Values are given as mean ± SEM **P<0.01.
THI improves metabolic abnormality

levels of hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), which are involved in the mobilization of free fatty acids from adipose triacylglycerol stores, were analyzed by RT-PCR. There was no significant increase in HSL and ATGL mRNA expression (Fig. 3C). The mRNA levels of mitochondrial uncoupling protein 2 (UCP2) and carnitine palmitoyltransferase 1a (CPT1a), which are responsible for energy expenditure, were also analyzed by RT-PCR. The levels of UCP2 and CPT1a mRNA in adipose and muscle tissues were not changed statistically (Fig. 3E and 3F).

THI was also found to inhibit Oil red O-stained fat droplet formation, which is a marker of adipogenesis, during adipogenic differentiation of 3T3-L1 cells in vitro (Fig. 4A). THI also significantly reduced the intracellular fat content in a dose-dependent manner (Fig. 4B). To elucidate the molecular mechanism of THI action in vitro, the expression levels of major transcription factors of adipogenesis were analyzed. It was found that THI downregulated the expression of C/EBPα and PPARγ, whereas C/EBPβ levels were not changed (Fig. 4C).

Next, we performed an oral glucose tolerance test. All groups of mice were fasted during the night cycle, and glucose was orally injected 14 hr after starvation. Blood glucose levels were measured at the indicated time points. The THI-administered group showed improved oral glucose tolerance compared with the HFD group, although the glucose tolerance of the THI-administered group was not improved to the levels of the normal diet group (Fig. 5).

**Effect of exercise on the THI-administered HFD group**

Since it is well-known that exercise effectively improves the metabolic index, we examined whether or not THI administration...
could act synergistically with exercise. The HFD group was orally administered with THI and subjected to swimming exercise for 10 weeks or swimming exercise without THI administration. Exercise induced dramatic weight loss in the THI-administered high-fat group (Fig. 6A) but did not alter the amount of food intake (Fig. 6B). Weight loss in the THI-administered exercised group was due to the dramatic reduction of adipose tissue (Fig. 6C) and triglyceride levels (Fig. 6D). The simultaneous combination of exercise and THI administration induced lower levels of total cholesterol compared with the group that only exercised (Fig. 6E). These results indicate that the simultaneous combination of exercise and THI administration has a synergistic effect on the reduction of total cholesterol levels and that THI does not affect the beneficial effect of exercise. In addition, the results also confirm an improvement in fasting blood glucose levels and oral glucose tolerance (Fig. 6F).

**Discussion**

Herbal extracts have been widely used for the treatment of metabolic diseases, including obesity, type II diabetes, and hyperlipidemia. The effects of THI on metabolic diseases were examined using a DIO mouse model. In our system, although the concentration of THI required for inhibition of adipogenesis was quite high in vitro, potent THI metabolite was generated in vivo and thus may be involved in the reduction of adipose tissue. It is also conceivable that the reduction of body weight was due to reduced adipose tissue weight, as THI effectively inhibited adipogenesis in 3T3-L1 preadipocytes through the downregulation of C/EBPα and PPARγ, which are the main transcription factors involved in adipogenesis. We also examined the possibility that THI reduced adipose tissue weight through increased fatty acid oxidation. However, the levels of HSL, ATGL, UCP-2, and CPT1a, which are involved in lipid mobilization and fatty acid oxidation, were not significantly changed. However, we cannot rule out the possibility that THI increases the consumption of fatty acids through another mechanism involving PPARδ or AMPK activation, as reduced adipogenesis could increase the level of fatty acids.

The anti-obesity effects of many natural compounds are reported to be mediated by inhibition of adipogenesis. For instance, epigallocatechin gallate (EGCG), genistein, esculetin, berberine, resveratrol, guggulsterone, conjugated linoleic acid, capsainc, baicalin, and procyanidins are all reported to inhibit adipogenesis [14]. Among these, genistein, EGCG, and capsainc have been shown to inhibit adipogenesis by activating AMP-activated protein kinase [15], and resveratrol was reported to increase the expression of sirtuin 1, a gene that represses expression of PPARγ [16]. It may be very difficult to identify every molecular target of a multi-component herbal composition like THI, but the combined effects of its diverse actions are clearly the downregulation of PPARγ and C/EBPα, major transcription factors, as shown in Fig. 4C. PPARγ, a transcription factor of the nuclear-receptor superfamily, is known to be the master regulator of adipogenesis since it is both necessary and sufficient for adipogenesis [17]. The expression of PPARγ alone has been shown to induce adipogenesis in fibroblasts [18]. PPARγ is also known to induce the expression of C/EBPα by binding to its promotor region [19], and the THI-induced reduction of C/EBPα expression may be the result of decreased PPARγ expression. The results of this study suggest that the combined effects of THI are mediated through the downregulation of the major transcription factors of adipogenesis, PPARγ and C/EBPα.

Orally administered THI markedly reduced serum cholesterol levels and fasting glucose levels while improving glucose tolerance. Reportedly, Scutellariae Radix contains wogonin, a flavone that is known to have anti-inflammatory properties in mice [20] and potentiate the anti-tumor action of etoposide by ameliorating adverse effects [21]. However, there has been report that Scutellariae Radix has any anti-adipogenic effects. On the other hand, Scutellariae Radix contains baicalin [22], which is known to exhibit anti-inflammatory activity by binding to chemokines [23] or by inhibiting NF-kB [24]. Baicalin suppresses lung carcinoma and lung metastasis by SOD mimic and HIF-1alpha inhibition [25] and exerts anti-adipogenic functions through the maintenance of β-catenin expression [12]. Therefore, the observed effects of the THI extracts, such as loss of body weight and improved glucose tolerance, may be attributed to baicalin.

Although platycodin saponins are known to ameliorate obesity and hyperlipidemia [5] as well as reduce serum triglyceride levels in HFD rats [6], the administration of THI-containing platycodin saponins did not result in decreased serum triglyceride levels. In the case of cholesterol, the exercised THI-administered high-fat group showed a decrease in cholesterol levels compared with the exercised high-fat group. This implies that when exercise and THI administration are performed simultaneously, the metabolic index can be improved compared to that obtained with only THI administration. Although it was reported that swimming exercise does not significantly increase glucose uptake in isolated skeletal muscles [26], swimming exercise alone or in combination with THI administration markedly improved HFD-induced glucose intolerance in vivo.

In conclusion, THI administration effectively reduced body weight, improved cholesterol level typically increased by a HFD, decreased fasting glucose levels, and enhanced glucose tolerance. Exercise training combined with THI administration also synergistically enhanced the effect of THI on cholesterol levels. These results show that THI has potential as a powerful health improvement substance.

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