Nutrition Research and Practice (*Nutr Res Pract*) 2011;5(3):198-204
DOI: 10.4162/nrp.2011.5.3.198

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 PPARɣ, were measured. The results show that the expression of these two transcription factors was down regulated 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 ɣ (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 PPARɣ, C/EBPα, and KLF15, as well as the upregulation of anti-adipogenic regulators, including C/EBPɣ 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-PPARɣ antibody and secondary antibody were purchased from Cell Signaling (Beverly, MA, USA). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

This work was supported by the Biogreen 21 project (20070301034031) of Rural Development Administration and Sookmyung Women's University 2009.
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Received: December 24, 2010, Revised: April 6, 2011, Accepted: April 8, 2011

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℃ 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℃ 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'-CTCCGAGAGATG TGCAAACA-3', reverse, 5'-CAGTTCCACCTGCTCAGACA-3'; HSL forward, 5'-CTTCCTGCAAGAGTATGTCACG-3', reverse, 5'-TGGAGGTGAGATGGTGACTG-3; UCP2 forward, 5'-CTG GCAGGTAGCACCACAGGTG-3', reverse, 5'-GCATGGTAAG GGCACAGTGAC-3'; CPT1a forward, 5'-GTCTGGAATCAAC TCCTGGAAG-3', reverse, 5'-CAGTGACGTTGGAAGCTGTA G-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-methyl-xanthine, 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℃ 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℃, 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).

Weight of epididymal white adipose tissue was highly increased in the HFD group compared with the normal diet group,

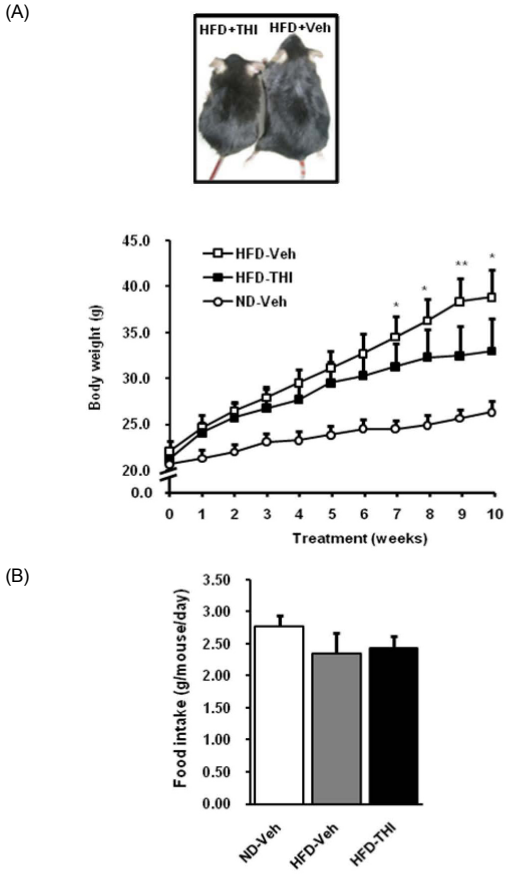

Fig. 1. THI administration reduces body weight in the diet-induced obesity (DIO) model. (A) Body weight changes were measured once a week, and mice were photographed after 10 weeks. HFD-Veh, high-fat diet with water administration; ND-Veh, normal diet with water administration; HFD-THI, high-fat diet 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.

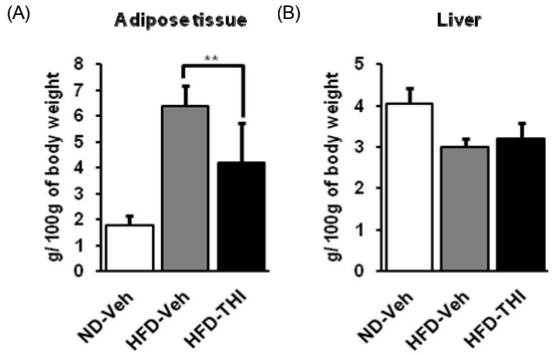

Fig. 2. THI administration decreases weights of epididymal adipose tissues. (A) Weights of two epididymal adipose tissues were measured after 10 weeks of high-fat diet and expressed as g/100 g of body weight. (B) Whole liver weights were measured and expressed as g/100 g of body weight. $^{**}P < 0.01$ indicates statistical significance between HFD-Veh, high-fat diet with water administration and HFD-THI, high-fat diet with THI administration. 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

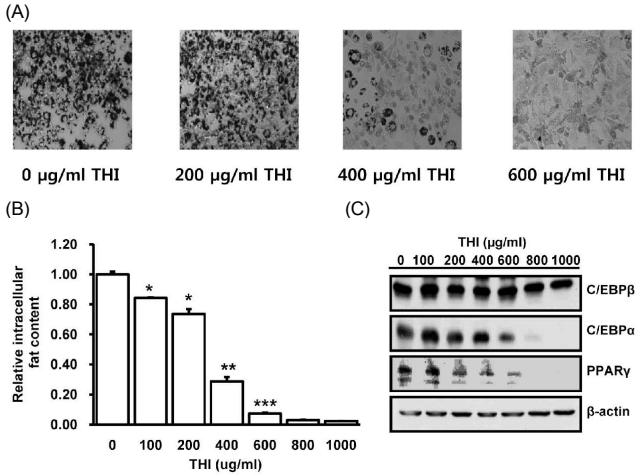

Fig. 4. THI reduces adipogenic differentiation of 3T3-L1 cells *in vitro*. (A) Intracellular fat droplets in 3T3-L1 cells were stained with Oil red O dye after adipogenic differentiation for 7 days. (B) Intracellular fat content was compared in 3T3-L1 cells after treatment with various concentrations of THI. (C) Protein expression levels of adipogenic transcription factors, C/EBPα, C/EBPβ, and PPARγ, were measured in 3T3-L1 cells. β-actin was used as a loading control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with untreated sample.

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

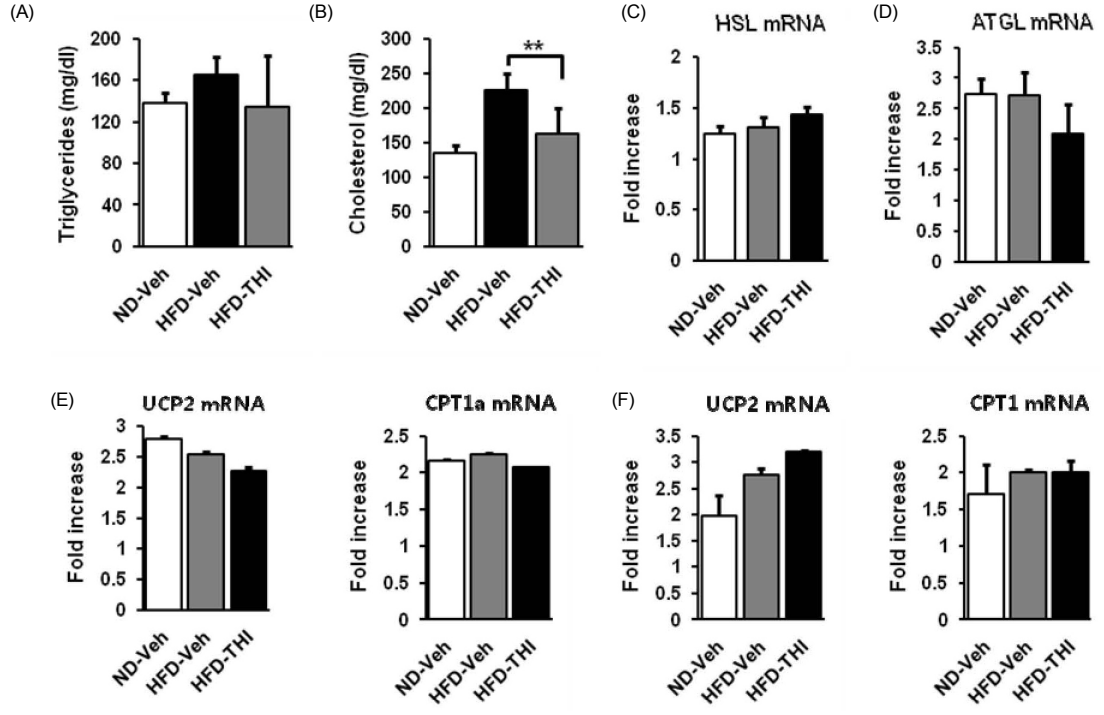

Fig. 3. THI reduces total cholesterol levels. (A) Serum triglyceride levels were measured (n = 7). (B) Serum total cholesterol levels were measured (n = 7). (C-F) Relative expression levels of hormone-sensitive lipase (HSL), adipose triglyceride lipase (ATGL), mitochondrial uncoupling protein 2 (UCP2), and carnitine palmitoyltransferase 1a (CPT1a) mRNA in epididymal white adipose tissues and skeletal muscle tissues were determined using RT-PCR (n = 3). Values are given as mean ± SEM $^{**}P < 0.01$.

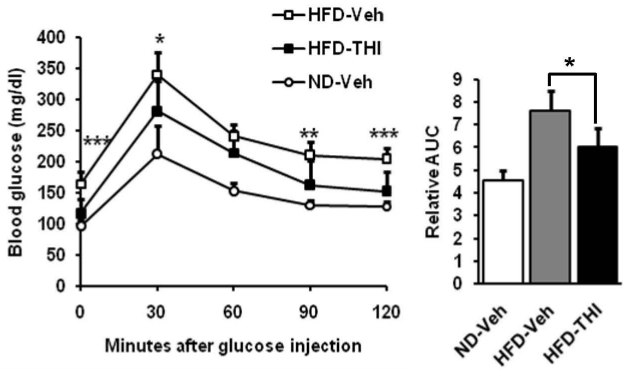

Fig. 5. THI administration improves glucose tolerance. Mice were fasted for 14 hr before OGTT. Blood glucose levels were measured every 30 min for 120 min, and relative areas under curves (AUC) were calculated. Data are expressed as mean ± SEM (n = 7). *P< 0.05, **P< 0.01, ***P< 0.001 indicate statistical significance between HFD-Veh, high-fat diet with water administration and HFD-THI, high-fat diet with THI administration groups.

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 PPARɣ, 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

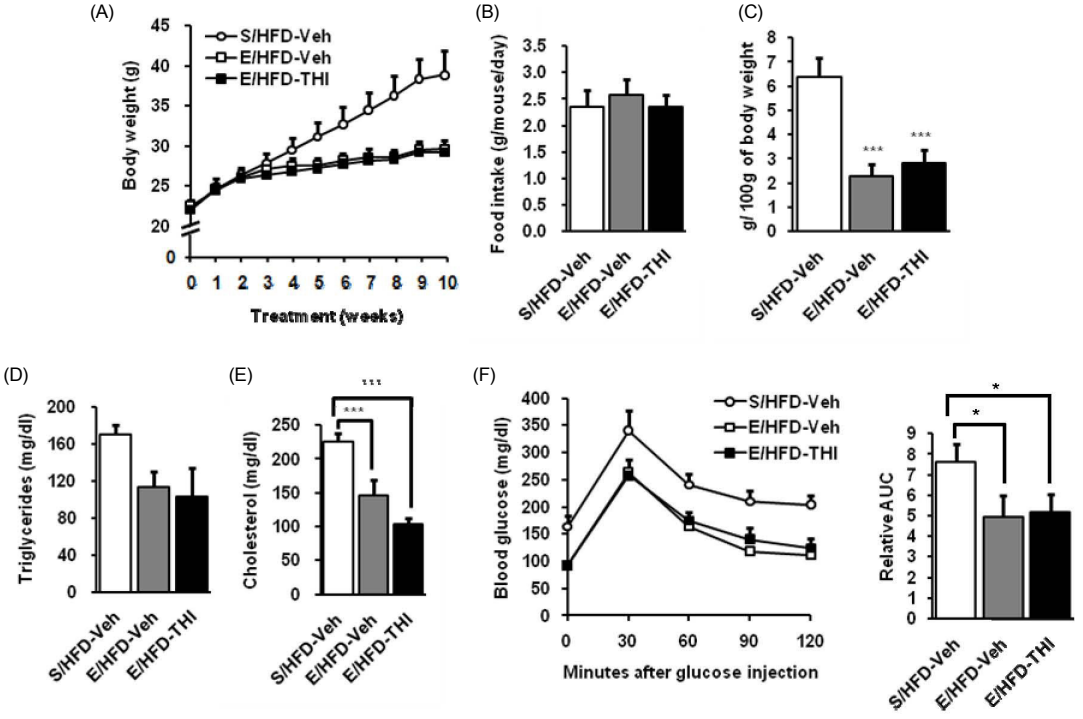

Fig. 6. THI administration with swimming exercise synergistically decreases total serum cholesterol levels. (A) Body weight changes were measured 10 weeks after high-fat diet (S/HFD-Veh, sedentary high-fat diet with water administration), swimming exercise (E/HFD-Veh, exercising high-fat diet with water administration), and combination of THI administration and swimming exercise (E/HFD-Veh, exercising high-fat diet with THI administration) (n = 7 for each group). (B) Food intake per mouse per day was measured. (C) Epididymal white adipose tissue weight was measured and expressed as g/100 g of body weight. ***P< 0.001 indicates statistical significance of each group versus the S/HFD-Veh group. (D) Serum triglycerides levels were measured (n = 5). (E) Total serum cholesterol levels were measured (n = 5). ***P< 0.001 indicates statistical significance between indicated groups. (F) Oral glucose tolerance test was performed (n = 7, left), and relative areas under curves (AUC) were calculated. All values are given as mean ± SEM.

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 PPARɣ, 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, capsaicin, baicalein, and procyanidins are all reported to inhibit adipogenesis [14]. Among these, genistein, EGCG, and capsaicin 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 PPARɣ [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 PPARɣ and C/EBPα, major transcription factors, as shown in Fig. 4C. PPARɣ, 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 PPARɣ alone has been shown to induce adipogenesis in fibroblasts [18]. PPARɣ is also known to induce the expression of C/EBPα by binding to its promoter region [19], and the THI-induced reduction of C/EBPα expression may be the result of decreased PPARɣ 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, PPARɣ 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 anxiolytic 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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