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Prevention of Diet-Induced Hyperlipidemia and Obesity by Caffeic Acid in C57BL/6 Mice through Regulation of Hepatic Lipogenesis Gene Expression

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ABSTRACT: This study investigated the influence of phenolic caffeic acid on obesity in mice fed a high fat diet and its underlying mechanisms base on adipose and hepatic lipid lipogenesis. C57BL/6 mice were fed a normal diet or a HFD (20% fat, w/w) with or without caffeic acid (0.02% and 0.08%, w/w) for 6 weeks. The effects of caffeic acid on hyperlipidemia, hyperglycemia, visceral fat accumulation, and related enzyme activities in HFD-mice are examined. The supplementation of caffeic acid significantly lowered body weight, visceral fat mass, plasma GOT and GPT levels, FAS activity, and free fatty acid compared to the HFD group. Caffeic acid also lowered triglyceride and cholesterol concentrations in plasma and liver. Furthermore, we showed that caffeic acid efficiently inhibited cholesterol biosynthesis as evidenced by 3-hydroxy-3-methylglutaryl CoA reductase in the liver. Caffeic acid supplementation suppressed the activity of lipogenesis via sterol regulatory element-binding protein 1 c and its target enzyme fatty acid synthase. In addition, caffeic acid resulted in increased phosphorylation of AMP-activated protein kinase and decreased acetyl carboxylase, a downstream target of AMPK, which are related to fatty acid β-oxidation in the liver. In conclusion, these results indicate that caffeic acid exhibits a significant potential as an antiobesity agent by suppression of lipogenic enzymes and hepatic lipid accumulation.

KEYWORDS: *obesity, caffeic acid, AMP-activated protein kinase (AMPK), sterol regulatory element-binding protein 1 c (SREBP-1c), lipogenesis*

INTRODUCTION

Obesity is a chronic metabolic disorder caused by an imbalance of energy due to excess consumption of nutrients and inadequate physical activity.[1] The prevalence of obesity has been increasing sharply and has become a global concern of healthcare systems.[2] Excess energy intake accompanied with low energy expenditure induce lipid accumulation in both liver and adipose tissue leading to the development of metabolic disturbances.[3]

There are various strategies to control obesity or overweight, including dietary control, exercise, and medication. Antiobesity drugs, such as orlist and sibutramine, have modest clinical efficacy and serious side effects.[4] Thus, some botanicals might serve as a safe and natural way to manage body weight and effective alternative to synthetic drug.[4,5] There is growing interest in exploiting the potential of plants and naturally occurring materials for treating obesity.[6] Recent studies demonstrated that natural phenolic compounds including quercetin, resveratrol, and curcumin can treat obesity in an obese mouse model.[7−9]

Caffeic acid (3,4-dihydroxycinnamic acid) is the major dietary hydroxycinnamic acid and is abundant in nature.[10] Caffeic acid may esterify with chlorogenic acid and is widely available in plants, fruits, and vegetables.[11] It has a variety of potential pharmacological effects, such as anti-inflammatory, anticancer, and antiviral activities.[12,13] The phenolic caffeic acid may prevent atherosclerosis via inhibition of low-density lipoprotein (LDL) oxidation and reducing nuclear factor-κB (NF-κB) activity.[14,15] In addition, caffeic acid might be involved in antihyperglycemic activity through stimulation of insulin secretion and alleviation of insulin resistance in *db/db* mice.[16,17] This study investigated the protective effect of caffeic acid on HFD-induced obesity mice and the possible role in ameliorating the development of fatty liver.

MATERIALS AND METHODS

Animals and Experimental Design. All animal experimental protocols used in this study were approved by the Insitutional Animal Care and Use Committee of Chung Shan Medical University in Taiwan. Six-week-old male C57BL/6 mice were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) and fed a normal laboratory diet (Purina Lab Chow) for 1 week to stabilize metabolic conditions. Mice were exposed to a 12-h light/dark cycle, and the room was maintained at a constant temperature of 22 °C.

At 7 weeks of age, C57BL/6 mice were divided into six groups (n = 10 in each group). Group I: a normal laboratory diet during the whole period. Group II: high fat diet (HFD, 20% lard oil and 2% cholesterol). Group III: HFD supplemented with 0.02% caffeic acid. Group IV: HFD supplemented with 0.08% caffeic acid. Group V: HFD

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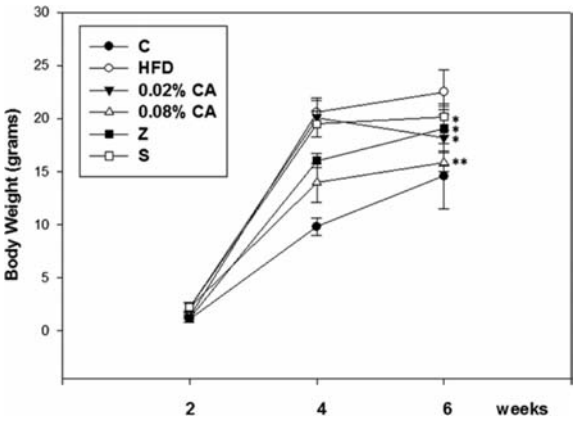

Figure 1. High-fat diet (HFD)-induced weight gain with caffeic acid supplementation. Weight gain in C57BL/6 mice (n = 10) fed a normal diet, HFD, HFD containing 0.02% caffeic acid, 0.08% caffeic acid, simvastatin (Z), and silymarin (S). Data are shown as the mean ± SD. n = 10 per group. * P < 0.05 compared with the HFD group. ** p < 0.01 compared with the HFD group.

supplemented with 1 mg/kg/body weight simvastatin (Z). Group VI: HFD supplemented with 100 mg/kg/body weight silymarin (S). Both simvastatin (an inhibitor of HMG-CoA reductase) and silymarin (an antioxidant in the treatment of liver disease) are positive controls. Body weight was measured every 2 weeks. After 6 weeks of feeding on the aforementioned diets, whole blood and livers were collected from mice that had fasted overnight and were then sacrificed.

Blood Sample Analysis. Blood samples were collected and immediately centrifuged at 1500g for 10 min at 4 °C. The serum samples from blood were then collected and stored at 4 °C. Serum levels of total cholesterol, triglyceride, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), free fatty acid (FAA), and ketone bodies were quantified by colorimetric methods and were measured using clinical chemistry reagent kits (HUMAN, Wiesbaden, Germany). Free fatty acids were assayed with a free fatty acid quantification kit (BioVision, San Francisco, USA; catalog: K612-100) according to the manufacturer's protocol. Briefly, the free fatty acids in serum samples were converted to their CoA derivatives, which were subsequently oxidized with the concomitant generation of color. Free fatty acids then can be easily quantified by a colorimetric (spectrophotometry at λ = 570 nm) method.

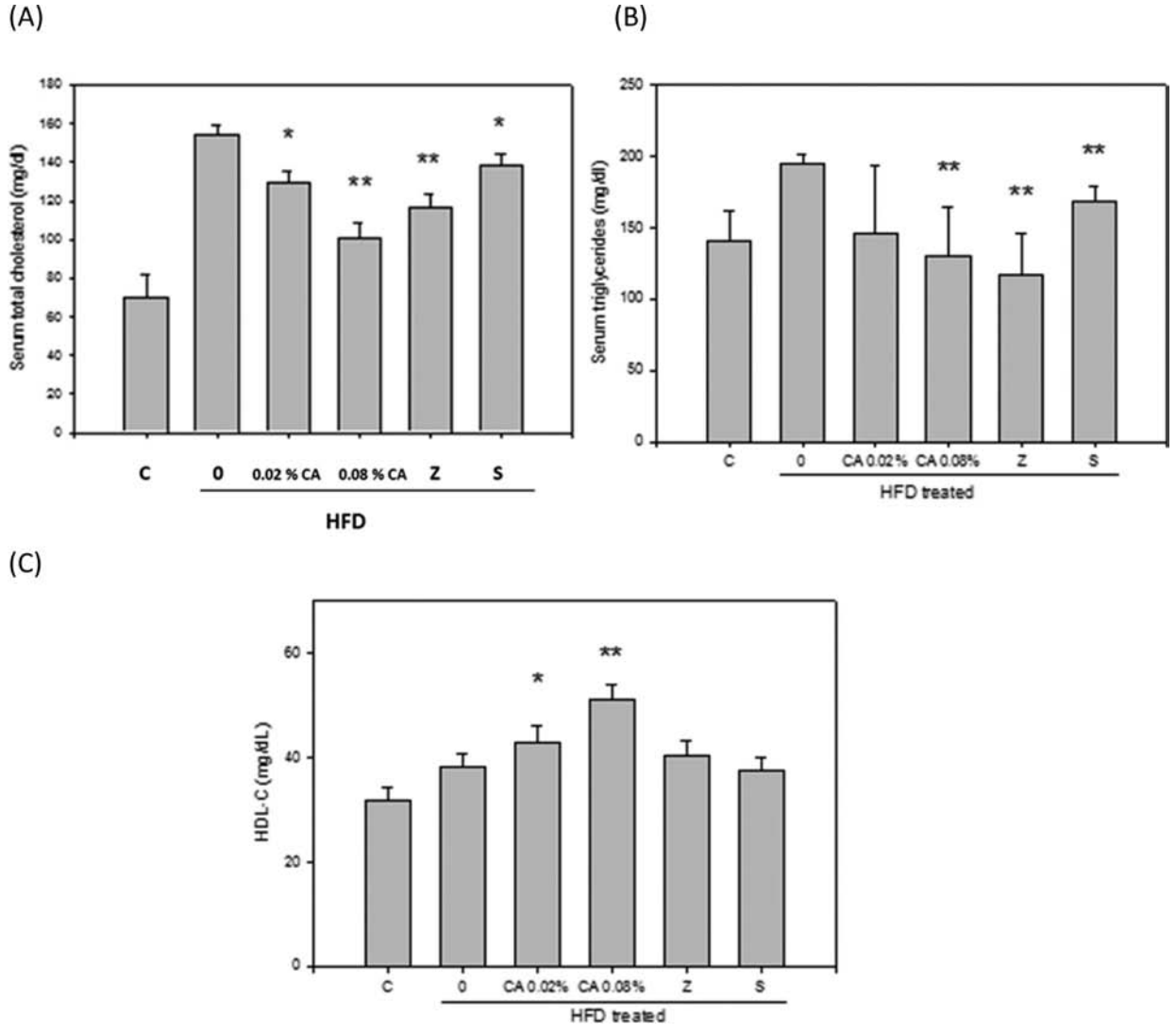

Figure 2. Effects of caffeic acid supplement on serum cholesterol and triglycerides in C57BL/6 mice fed HFD: (A) serum cholesterol, (B) triglycerides, (C) HDL-C in C57BL/6 mice (n = 10) fed HFD and HFD containing 0.02% caffeic acid, 0.08% caffic acid, simvastatin (Z), and silymarin (S) at six weeks of treatment. Corresponding levels in C57BL/6 mice fed a normal diet (C) were used as control. Data are shown as the mean ± SD. * P < 0.05 compared with the HFD group. ** p < 0.01 compared with the HFD group.

Determination of Total Cholesterol and TGs in the Liver. After removal from the animals, a portion of the fresh liver was collected for liver lipid extraction. Briefly, liver was homogenized with chloroform/methanol (2:1). Then chloroform (1.25 mL) and distilled water (1.25 mL) were added to the homogenate and mixed well. After centrifugation (1500*g* for 10 min), the lower clear organic phase solution was transferred into a new glass tube and lyophilized. The lyophilized powder was dissolved in chloroform/methanol (2:1) as the liver lipid extract and stored at −20 °C for 3 days. The liver TGs and liver cholesterol in the lipid extracts were measured by enzymatic colorimetric methods using commercial kits (HUMAN, Wiesbaden, Germany).

FAS Activity Assay. The FAS activity assay was performed as described by Goodridge et al.[18] The serum sample was used in the assays. FAS activity was measured by following the decrease in absorbance at 340 nm resulting from the oxidation of NADPH, which was dependent on added malonyl-CoA at 40 °C. Each cuvette contained 0.1 M potassium phosphate (pH 7.0), 3 mM EDTA, 0.1 mM NADPH, 25 mM acetyl-CoA, 1 mM DTT (all from Sigma-Aldrich, St.Louis, MO), and the sample. The reaction was initiated by adding malonyl-CoA to a final concentration of 0.1 mM. Doubly distilled H_2O was used for the blanks instead of the samples. Under these conditions, FAS activity was linear with respect to both time (for at least 10 min) and protein (0−200 mg/mL); 1 U of activity equals 1 nmol of palmitate formed per min (equivalent to the oxidation of 14 nmol of NADPH).

Preparation of Protein Extract of Liver Tissue. The protein from liver tissues was harvested in a cold RIPA (radioimmunoprecipitation assay) buffer (1% NP-40, 50 mM Tris-base, 0.1% SDS, 0.5% deoxycholic acid, and 150 mM NaCl [pH 7.5]) containing leupeptin (17 μg/mL) and sodium orthovanadate (10 μg/mL). The liver tissues were homogenized on ice for 3 min. All mixtures were then centrifuged at 12000*g* at 4 °C for 10 min, and the protein content of the supernatants was determined with Coomassie blue total protein reagent (Kenlor Industries, Santa Ana, CA) using bovine serum albumin as a standard.

Western Blot Analysis. Equal amounts of protein samples were subjected to SDS−polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes (PerkinElmer). Membranes were blocked with 5% nonfat milk powder with 0.05% Tween-20 in TBS (Tris-buffered saline) for an hour and incubated with the primary antibody at 4 °C overnight. Monoclonal antibodies against AMPK, SREBP-1c, SREBP-2, ACC, and HMG-CoA reductase were purchased from Santa Cruz (Santa Cruz, CA, USA), and phosphor-AMPK and FAS were obtained from Cell Signaling Technology Inc. (Berverly, MA, USA).The membranes were then washed three times with 0.05% Tween-20 in TBS and incubated with the secondary antibody conjugated to horseradish peroxidase reagent. Bands were detected by enhanced chemiluminescence using ECL Western blotting detection reagents and exposed ECL hyperfilm in FUJFILM Las-3000. Protein quantity was determined by densitometry using FUJIFILM Multi Gauge, version 2.2, software.

Statistical Analysis. Results are reported as the mean ± standard deviation, and differences between values were analyzed by unpaired Student's *t* test by using SigmaPlot software (version 9.0; SYSTAT Software Inc., Point Richmond, CA). *P* values less than 0.05 were considered statistically significant.

RESULTS

Changes in Body Weight. The body weight of the HFD group increased throughout the experimental period, whereas that of the caffeic acid group decreased after 2 weeks. Thus, the body weights were significantly lower in the caffeic acid, simvastatin, and silymarin groups than in the HFD group at weeks 4 and 6 of the experimental period (Figure 1).

Effect of Caffeic Acid on Serum Lipid Levels in Mice. The serum cholesterol and TG levels in the HFD group were significantly higher than those in the control group. Compared to the HFD group, the serum levels of the caffeic acid (0.08%) group were decreased significantly by 34% for cholesterol and 32% for TG, respectively. Supplementation with simvastatin and silymarin also lowered the cholesterol and TG concentrations in the serum compared to the HFD group (Figure 2A,B). Similarly, serum GOT and GPT levels were increased in the HFD group compared to the control group. Six-weeks administration of caffeic acid (0.08%) reduced the (lipid) levels of GOT and GTP by 46% and 34% respectively (Figure 3), and resulted in 31% increase of HDL (Figure 2C). Therefore, these data indicated that caffeic acid might suppress the development of hyperlipidemia by regulating the serum level of lipids. However, the efficiency of caffeic acid in hyperlipidemia is better than that of simvastatin and silymarin.

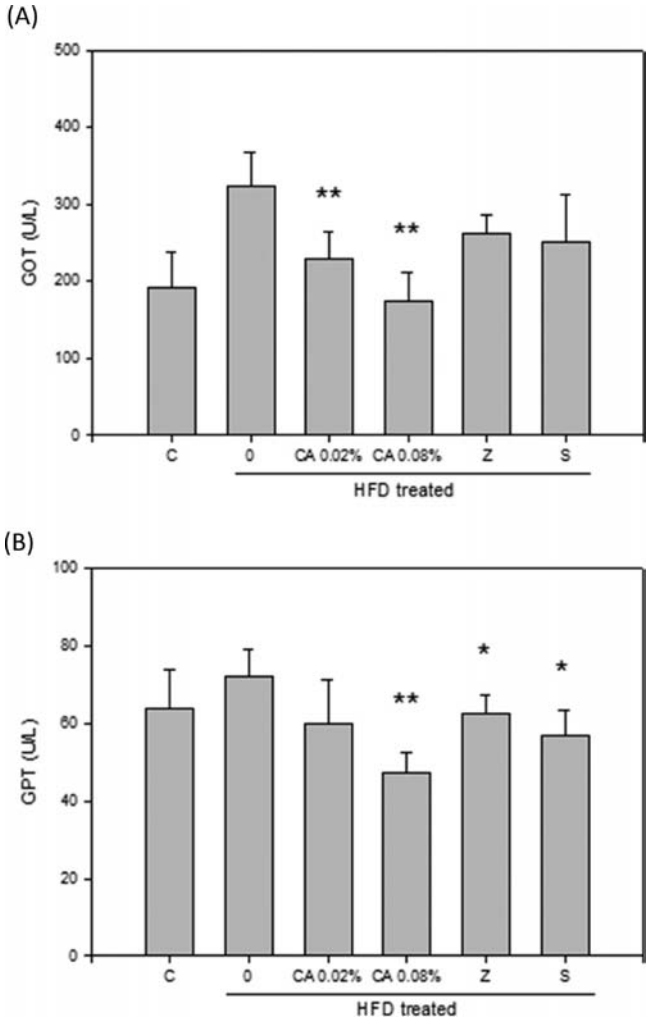

Figure 3. Effects of caffeic acid supplement on the serum GOT and GPT in C57BL/6 mice fed HFD: (A) serum GOT and (B) GPT in C57BL/6 mice fed HFD and HFD containing 0.02% caffeic acid, 0.08% caffic acid, simvastatin (Z), and silymarin (S). Corresponding levels in C57BL/6 mice fed a normal diet (C) were used as control. Data are shown as the mean ± SD. * $P < 0.05$ compared with the HFD group. ** $p < 0.01$ compared with the HFD group.

Serum Biochemical Parameters in Mice Administrated HFD and Caffeic Acid. The HFD group had significantly increased levels of serum fasting glucose compared with the control group. However, the fasting glucose levels of caffeic acid-, simvastatin-, and silymarin-fed mice were reduced ($p < 0.05$) compared with those of the HFD group (Figure 4A).

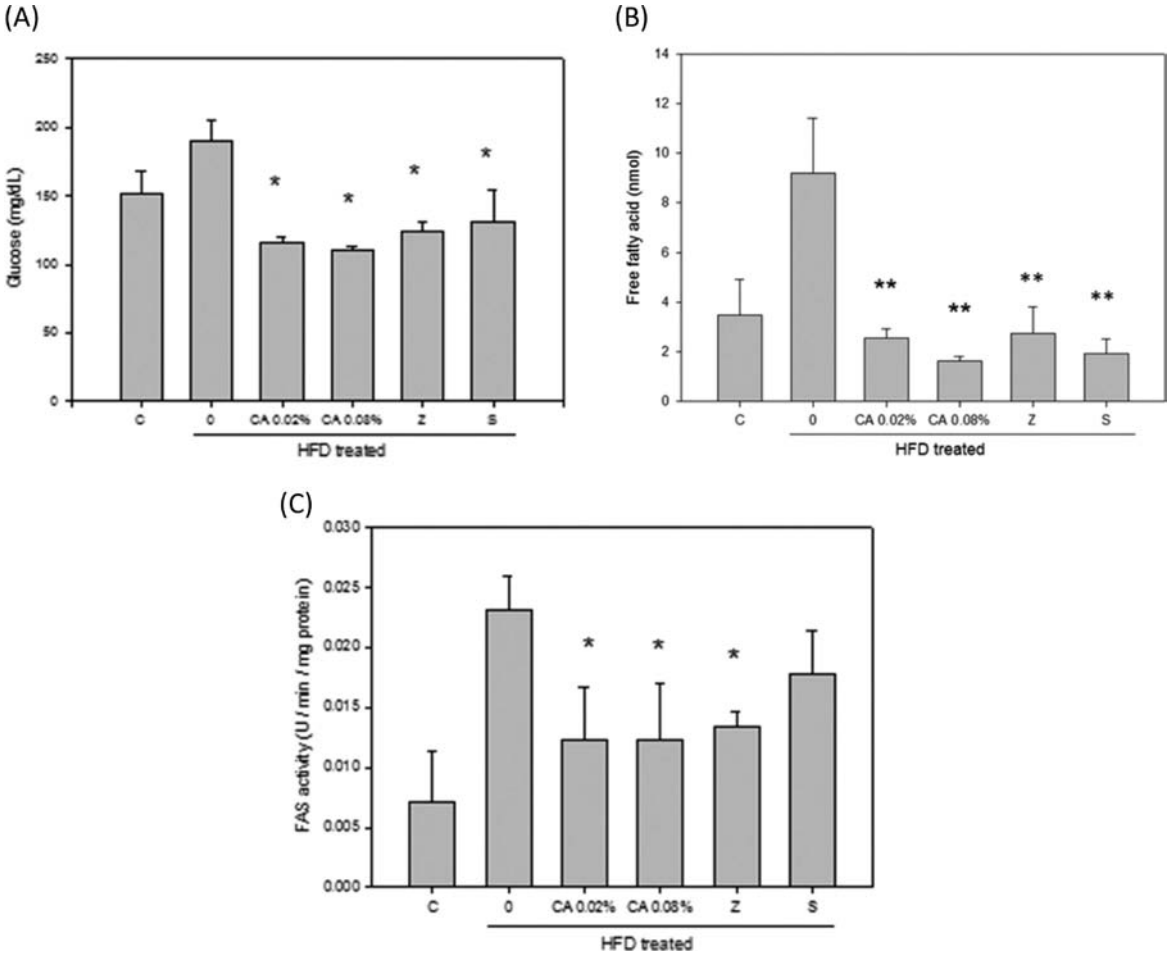

Figure 4. Effects of caffeic acid on serum biochemical parameters in mice administered HFD: (A) serum glucose, (B) FAA, and (C) FAS activity in C57BL/6 mice fed HFD and HFD containing 0.02% caffeic acid, 0.08% caffic acid, simvastatin (Z), and silymarin (S) at six weeks of treatment. Corresponding levels in C57BL/6 mice fed a normal diet (C) were used as control. Data are shown as the mean ± SD. * $P < 0.05$ compared with the HFD group. ** $p < 0.01$ compared with the HFD group.

Plasma free fatty acid (FFA) concentration was significantly lowered by caffeic acid- ($p < 0.01$), simvastalin- ($p < 0.05$), and silymarin- ($p < 0.05$) fed mice compared with the HFD-fed mice (Figure 4B). The expression of FAS, an important fatty acid enzyme for fatty acid synthesis, was also significantly higher in the HFD group than in the control group. The caffeic acid-fed group showed significantly reduced FAS activity ($P < 0.05$), as compared to the HFD group (Figure 4C).

Effect of Caffeic Acid on Lipid Content in the Liver and Adiposity. Supplementation with caffeic acid, simvastatin, and silymarin significantly lowered the cholesterol and triglyceride concentration in the liver compared to the HFD group (Figure 5A,B). To test whether this body weight reduction was caused by the decrease in adiposity, animal fat pads were weighed. Mice fed the HFD displayed increased gonadal fat pad mass compared to the mice fed the normal diet. When compared with HFD mice, caffeic acid-fed mice had significantly decreased gonadal fat pad mass ($P < 0.05$), and simvastatin- as well as silymarin-fed mice had slightly decreased gonadal fat pad mass (Figure 5C). Furthermore, the ratios of various adipose tissues (epididymal, retroperitoneal, mesenteric fat tissue) and body weight were dramatically reduced in the caffeic acid-, simvastatin-, and silymarin-fed mice compared with the HFD-fed mice ($P < 0.05$) (Figure 5D).

Effect of Caffeic Acid on Protein Expression in Liver Tissue. To test whether the reduction of fat mass in caffeic acid-treated mice is accompanied by changes in lipogenesis, Western bolts were performed. As shown in Figure 6, the expression of SREBP-1c, a major transcription factor involved in the activation of lipogenic genes, and that of its representative target protein, FAS, were remarkably decreased in caffeic acid-treated mice compared with HFD mice. We also showed that caffeic acid administration efficiently inhibited fatty acid and cholesterol biosynthesis. As evidenced by suppressing the activity of HMG-CoA reductase and SREBP-2, next, we investigated the expression of protein responsible for fatty acid β-oxidation in liver tissue. Expression of the phosphorylation of AMPK was higher and its immediate substrate, ACC, was lower in caffeic acid-treated mice than in HFD-treated mice (Figure 6).

DISCUSSION

Obesity is a chronic metabolic disorder that is characterized by enlarged fat mass and elevated lipid accumulation in blood.[19,20] High-fat feeding has commonly been used to induce visceral obesity in rodent animal models because the pathogenesis of obesity is similar to that in humans.[21,22] We examined the effects of caffeic acid on HFD-induced fat accumulation in the adipose tissue of C57BL/6 mice. Body weight gain, adipose

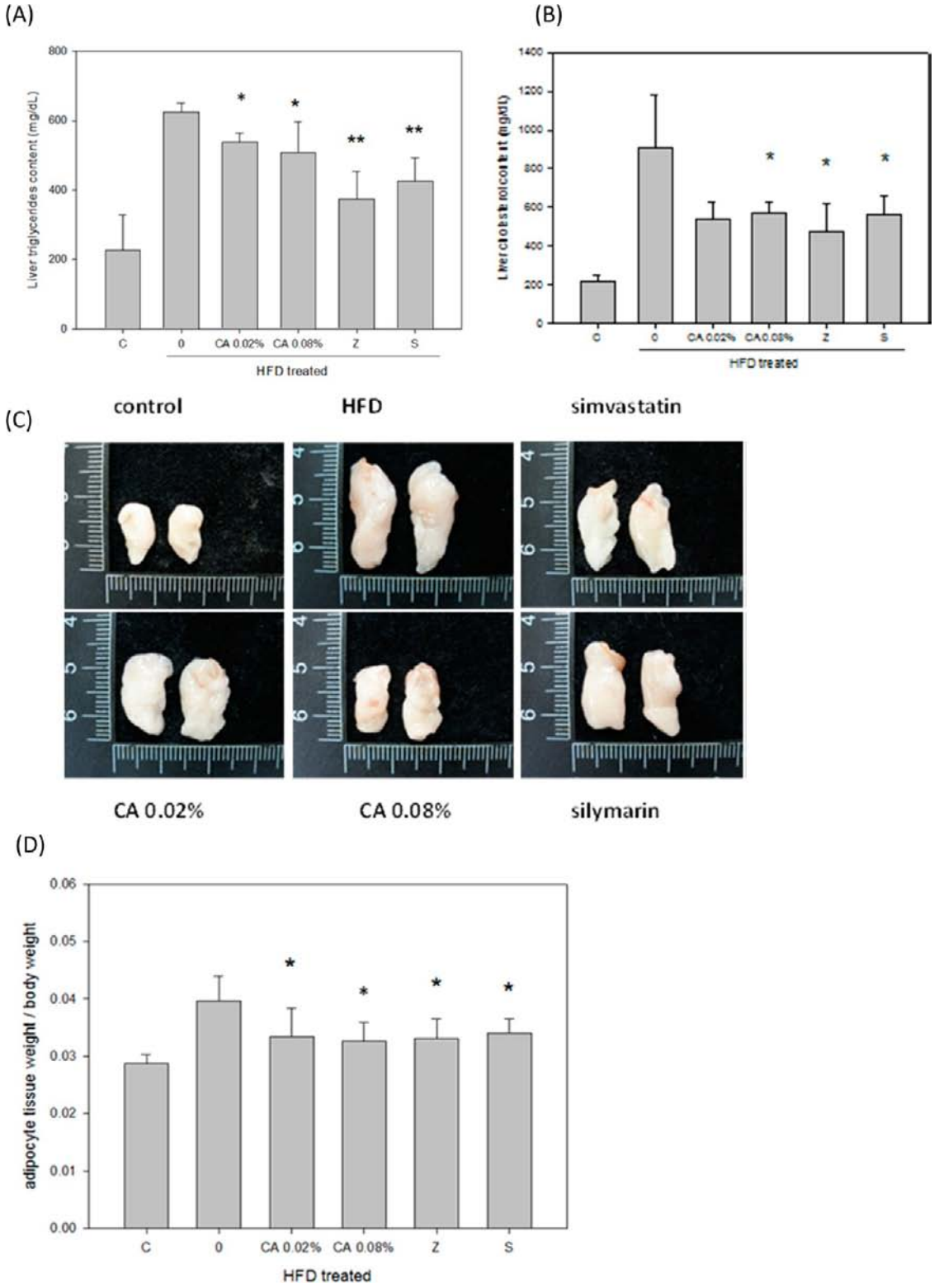

Figure 5. Effect of caffeic acid on lipid content in the liver and adiposity: (A) liver triglycerides content, (B) liver cholesterol content, (C) gonadal fat pad mass, and (D) body fat index (total adipose tissue/body weight) in C57BL/6 mice fed HFD and HFD containing 0.02% caffeic acid, 0.08% caffic acid, simvastatin (Z), and silymarin (S) at six weeks of treatment. Data are shown as the mean ± SD. * $P < 0.05$ compared with the HFD group. ** $p < 0.01$ compared with the HFD group.

tissue weight, cholesterol, and TG serum levels were significantly lowered in the caffeic acid-treated group compared to the HFD group. One of the most common characteristics among people with obesity is the development of fatty liver.[23,24] The quantifications of TG and cholesterol revealed that the amount of lipids in the liver of caffeic acid-treated mice was lowered compared with that of the HFD group (Figure 5A,B). Serum GOT and GPT levels are clinically important indicators

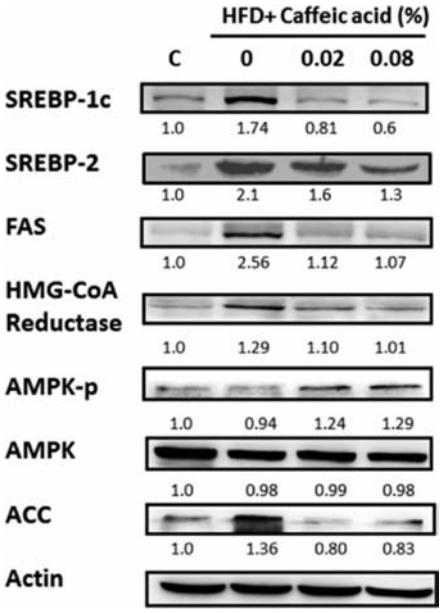

Figure 6. Effects of caffeic acid on protein expression in liver tissue. C57BL/6 mice fed HFD and HFD containing 0.02% caffeic acid and 0.08% caffic acid for 6 weeks. Protein expressions of SREBP-1c, SREBP-2, FAS, HMG-CoA reductase, AMPK-p, AMPK, and ACC were analyzed by Western blot. The results from three repeated and separate experiments were similar.

as a result of tissue damage by disease conditions. In the HFD group, the activity of liver function markers, including serum GOT and GPT, was significantly elevated related to those in the control group and were improved by caffeic acid supplementation (Figure 3). Taken together, these results indicated that CA might effectively improve various lipid-related parameters in the liver and the efficiency of caffeic acid is better than that of simvastatin and silymarin.

Visceral adipose tissue releases a large amount of free FFA and cytokines in the vein that can be delivered to the liver and interact with hepatocytes.[25] Hepatic esterification of FFA to triglycerides leads to the formation of fatty liver that is accelerated by an increased lipogenesis as a result of hyperinsulinemia and decreased FFA oxidation.[26] In this study, caffeic acid significantly lowered the fasting blood glucose and FFA compared with HFD mice, which is in agreement with previous studies done by others.[27] The possible mechanism by which caffeic acid mediates its antidiabetic action may be due to enhanced transport of blood glucose to adipose tissue. Adipose GLUT4 overexpression is known to eliminate insulin resistance and pancreatic defects in *db/db* mice.[28] Studies showed that caffeic acid enhanced the GLUT 4 protein expression in adipose tissue.[16,29]

AMPK is known to play a major role in glucose and lipid metabolism and to control metabolic disorder such as diabetes, obesity, and cancer.[30] Studies showed that AMPK activation is associated with metabolic organs including the liver, skeletal muscle, pancreas, and adipose tissue; it is widely recognized as a useful target for treatment of metabolic disorders such as dyslipidemia.[31,32] In the present study, caffeic acid recovered the phosphorylation of AMPK, which has been reduced in C57BL/6 mice fed the HFD. Furthermore, AMPK activation increases fatty acid oxidation by reducing malonyl-CoA through the inhibition of ACC (Figure 6), and this process upregulated CPT-1a expression.[33] The activity of HMG-CoA reductase, the rate-limiting enzyme of cholesterol synthesis, and SREBP-2 was significantly lowered by administration of caffeic acid compared with obese mice. These results suggest that caffeic acid promotes activation of AMPK and mediates lipid lowering effect via inhibition of cholesterol biosynthesis in HFD-induced mice.

The role of SREBP-1c has been clearly established in the liver. It is a key regulator of adipose tissue lipogenesis, which controls the expression of fatty acid biosynthetic genes such FAS and acetyl CoA carboxylase (ACC).[34] In this study, SREBP-1c level decreased significantly following caffeic acid administration. In addition, FAS expression clearly decreased in the caffeic acid group compared to the HFD group (Figure 6). The results are in agreement with our previous study on dietary supplementation with phenolic compounds from plants in HDF-induced obese mice.[35] Increasing HDL concentration potentially repressed development of atherosclerosis and protected endothelial cells from cytotoxic effects of oxidized LDL.[36] Phenolic compounds are known to elevate HDL concentration.[37] Therefore, in this study, we found that administration of caffeic acid raised HDL levels in high-fat animals.

Caffeic acid and its esterified form of chlorogenic acid (5-*O*-caffeoylquinic acid) are generally the most abundant phenolic acids and represent among 75−100% of the total hydroxycinnamic acid content of fruit.[38] Caffeic acid intake alone was 206 mg/day adult, and the major source was coffee, which supplied 92% of caffeic acid.[38] In this study, the dose of 0.08% caffeic acid in human daily food consumption (500 g/dry weight) is about 400 mg. Studies have noted a high variability in polyphenol intake ranging from 6 to 987 mg/day in Germany.[39] Therefore, we speculate that the intake of caffeic acid from coffee may be helpful in the alleviation of fatty liver.

In conclusion, we showed that administration of caffeic acid to mice with HFD-induced obesity reduced body weight gain, adipose tissue weight, and serum cholesterol and TG, thereby inhibiting lipogenesis. In addition, caffeic acid increased β-oxidation by activation of AMPK in the liver. Taken together, our findings demonstrate that caffeic acid improved HFD-induced obesity through β-oxidation and lipolysis in liver tissue.

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Author Contributions

§C.-C.L. and T.-T.O. contributed equally to this work and therefore share first authorship.

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Notes

The authors declare no competing financial interest.

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