Original Paper

Cinnamaldehyde Ameliorates Diet-Induced Obesity in Mice by Inducing Browning of White Adipose Tissue

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Key Words

Cinnamaldehyde • Anti-obesity • Mice • Fat mass • Adipose tissue browning

Abstract

Background/Aims: Obesity has become a major health concern with few effective medications. Cinnamaldehyde (CA) has been reported to exhibit anti-diabetic and anti-inflammatory properties. However, whether CA shows anti-obesity activity remains unknown. Therefore, the present study aimed to investigate the potential anti-obesity effects of CA on mice fed a high-fat diet (HFD) and to explore the possible mechanisms involved. **Methods:** Male C57BL/6J mice fed an HFD for 12 weeks were supplemented with CA (40 mg/kg/day) via gavage for an additional 8 weeks. Mice fed a standard diet were used as normal controls. **Results:** The results revealed that CA treatment decreased body weight, fat mass, food intake, and serum lipid, free fatty acid and leptin levels. CA administration also improved insulin sensitivity in HFD-induced obese mice. Additionally, CA inhibited the hypertrophy of adipose tissue and induced browning of white adipose tissue. Uncoupling protein 1 (UCP1) was expressed in white adipose tissue after the oral administration of CA. Furthermore, CA enhanced the expression of the peroxisome proliferator-activated receptor γ (PPAR γ), PR domain-containing 16 (PRDM16) and PPAR γ coactivator 1 α (PGC-1 α) proteins in both brown and white adipose tissues. **Conclusions:** The results suggest that CA exhibits therapeutic potency against obesity by inducing the browning of white adipose tissue in HFD-fed mice.

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Introduction

Obesity is becoming a serious global health concern. The number of obese people has rapidly increased recently due to unhealthy lifestyles, such as overeating, a lack of exercise, and unbalanced diets [1, 2]. More than 2.1 billion people (30% of the global population) are overweight or obese, and approximately half of the world's adult population will be overweight or obese by 2030 if the current growth trend continues [3]. Obesity is closely associated with the development of several diseases, including diabetes, hypertension, dyslipidemia, nonalcoholic steatohepatitis, coronary heart disease, and stroke [4-6]. According to previous reports, obesity is responsible for approximately 5% of deaths worldwide [3]. The high burden of obesity-related diseases has driven the search for novel and improved strategies to counter obesity [7, 8].

Current approaches for treating obesity include lifestyle changes, diet control, regular exercise, surgery and pharmacotherapy as an adjunct treatment. Unfortunately, anti-obesity drugs have consistently exhibited poor efficacy in the management of the patients with obesity, and numerous anti-obesity drugs have been withdrawn from the market based on US FDA warnings of serious side effects and the potential for drug abuse [9-11]. Therefore, interest in the identification of natural remedies to prevent and control obesity with few or no adverse reactions has grown. Recent studies have indicated that some diets and natural compounds, such as the Mediterranean diet and capsaicin, show counteractive effects on obesity [12, 13]. Cinnamaldehyde (CA), a pungent component of cinnamon (bark of *Cinnamomum cassia*), is widely used in drugs, food and spices. CA has achieved "Generally Regarded As Safe" status from the FDA. CA has been demonstrated to exhibit a wide range of pharmacological activities, including anti-bacterial, anti-inflammatory, and anti-tumor activities [14-16]. In a previous study, CA was shown to reduce fasting blood glucose and plasma lipid levels [17]. However, few reports have documented an anti-obesity effect of CA. Adipose tissues are important targets for anti-obesity drugs, and adipocyte differentiation is a pivotal process related to the development of obesity. Hence, the present study was designed to investigate the inhibitory effect of CA on body weight gain and the associated mechanisms by which it induces adipose tissue browning in obese mice fed a high-fat diet (HFD).

Materials and Methods

Reagents and antibodies

CA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Pioglitazone hydrochloride was purchased from Jiangsu Hengrui Medicine (Jiangsu, China). Antibodies against GAPDH, peroxisome proliferator-activated receptor γ (PPAR γ), PPAR γ coactivator 1 α (PGC-1 α), and uncoupling protein 1 (UCP1) were purchased from Abcam (Cat #: ab 8245, ab 209350, ab 54481, ab 10983, Cambridge, MA, USA). Antibodies against PR domain-containing 16 (PRDM16) were purchased from Santa Cruz Biotechnology (Cat #: sc-55697). All other reagents, except where specifically identified, were obtained from Beijing Sinopharm Chemical group (Beijing, China).

Animals and experimental design

Forty male C57BL/6J mice were maintained under controlled temperature ($23 \pm 2^\circ\text{C}$) and humidity ($55 \pm 10\%$) conditions with a 12/12-h dark/light cycle. All animals had free access to food and water. At baseline, ten mice were fed a standard diet (10% kcal as fat, set as the normal control group), and the remaining 30 mice were fed an HFD (60% kcal as fat) for 12 weeks to promote obesity. The obesity model was considered successfully generated when the body weight of the mice had increased over that of the normal control group by more than 20%. All diets were manufactured by Mediscience Ltd. (Jingsu, China). Twenty-four obese mice in the HFD group were subsequently selected and randomly subdivided into three groups of eight mice each. The groups were administered pioglitazone (5 mg/kg/d), CA (40 mg/kg BW) or vehicle (using an equal volume of saline, set as the HFD model group) via gavage daily for 8 weeks.

During the experiment, food intake and body weight were monitored weekly. At the end of the study, the mice were anesthetized with pentobarbital sodium and sacrificed after overnight starvation. Blood samples were harvested from the abdominal vena cava, then incubated for 15 min at room temperature and centrifuged at 3,000 rpm for 15 min. The collected serum was stored until further analysis. Interscapular brown adipose tissue (iBAT) and epididymal white adipose tissue (eWAT) were removed and dissected. The samples were immediately frozen in liquid nitrogen and stored at -80 °C for subsequent analysis.

All procedures followed the conventional guidelines for the Care and Use of Laboratory Animals from the Committee for Animal Experiments of the National Center, and the experimental protocol was approved by the Animal Ethics Committee of Beijing University of Chinese Medicine.

Body composition assessment

The body composition of the mice was assessed via magnetic resonance imaging (EchoMRI-100 for mice, Echo Medical System, Houston, USA) every 4 weeks during the drug administration period (week 4 and 8). Total fat mass was measured, and the fat content was calculated (fat mass weight/body weight×100%).

Oral glucose tolerance test

An oral glucose tolerance test (OGTT) was conducted at weeks 4 and 8. Animals were fasted overnight, and the oral glucose load was then administered at a dose of 2 g/kg body weight. Glucose levels were measured in blood collected from the tail vein at 0, 30, 60, and 120 min after glucose administration. Glucose tolerance was evaluated by calculating the area under the curve (AUC).

Blood lipid analysis

Serum total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) concentrations were determined using chemical reagent kits (Nanjing Jiancheng Biology Engineering Institute; Nanjing, China) and an automated biochemical analyzer (Hitachi, Tokyo, Japan).

Measurements of free fatty acid and leptin levels

Plasma free fatty acid (FFA) and leptin levels were measured using ELISA kits (Clinimate NEFA, Tokyo, Japan and R&D Systems, USA) according to the manufacturer's guidelines.

Histological examination

At the end of the treatments, the iBAT and eWAT of each mouse were meticulously removed and stored for histological examination. Samples of the adipose tissues were preserved in a freshly prepared fixative solution for 48 h and then subjected to standard histological procedures, including paraffin embedding, sectioning at a 5 µm thickness and staining with hematoxylin and eosin (HE). Images were obtained using a light microscope. For the quantitative analysis of adipocytes, sections of eWAT were analyzed with an image analysis system.

Western blotting

All adipose tissues were harvested and lysed in RIPA buffer supplemented with a complete protease inhibitor cocktail, and the protein concentration was determined using a BCA Protein Assay kit. Then, 100 µg of each denatured protein sample was separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane. After blocking with 5% non-fat milk for 1.5 h at room temperature, the membranes were incubated with the appropriate primary antibodies overnight at 4 °C, followed by incubation with the corresponding HRP-labeled secondary antibody (1:5,000) for 1.5 h at room temperature on the second day. The resultant immune complexes were detected using an enhanced chemiluminescence kit. Protein bands were visualized via autoradiography, and intensities were analyzed using Quantity One (Bio-Rad Laboratories, Hercules, CA, USA). GAPDH was used as the loading control.

Statistical analysis

All values are presented as the mean ± standard errors of the mean (SEM). One-way ANOVA and subsequent Dunnett's multiple comparisons tests were conducted using GraphPad software (Prism 5.0, Inc., CA, USA). When data were compared between groups, P < 0.05 was considered significant.

Results

CA reduced body weight and food intake in HFD-fed mice

Compared with mice fed a standard diet, mice fed an HFD exhibited an obvious increase in body weight. Both pioglitazone and CA decreased body weight beginning at 6 weeks (Fig. 1A). At the end of the experiment, the mice fed an HFD gained more weight than the mice fed a standard diet, and both CA and pioglitazone administration tended to decrease HFD-induced body weight gain. However, compared with the mice in the HFD control group, only the CA-treated group exhibited a significant difference (Fig. 1B).

Furthermore, food intake was concomitantly reduced in the CA-treated group mice (Fig. 1C). Food intake was not significantly different between the HFD-fed mice with or without CA supplementation until the fifth week. The mice fed an HFD consumed more calories than the mice fed a standard diet. The results indicate that CA supplementation triggered a negative energy balance by suppressing energy intake.

CA altered the body composition of HFD-fed mice

In the fourth week, the fat mass of the mice fed an HFD had increased by approximately twofold compared with the normal control group. The CA and pioglitazone treatments tended to decrease the fat mass of the mice, but the difference was not significant compared with the HFD group (Fig. 1D).

At the end of the experiment, the fat mass of the mice fed an HFD was increased approximately threefold compared with the normal control group, whereas CA administration obviously reversed this trend, as demonstrated by a 32.1% decrease in fat mass relative to the HFD control group. Again, the fat mass of the mice fed an HFD and treated with pioglitazone was significantly reduced (approximately 36.0%) compared with the HFD control group

Fig. 1. Cinnamaldehyde reduced body weight, food intake and fat mass of the HFD-exposed mice. (A) Body weight changes in the mice. (B) Body weight gains were decreased by cinnamaldehyde. (C) Daily food intake of the mice. (D) Differences in fat masses/body weights between groups at week 4. (E) Cinnamaldehyde reduced fat masses/body weights at the end of the experiment. Data are expressed as the mean $\pm$ SEM of eight mice from each group. * $P < 0.05$, ** $P < 0.01$ vs. normal control group. # $P < 0.05$, ## $P < 0.01$ vs. HFD control group.

(Fig. 1E). The results suggest that CA-treated mice gained more lean body mass than fat body mass.

Effect of CA on OGTT in HFD-fed mice

As shown in the OGTT results presented in Fig. 2, no significant differences in glucose levels or the AUC were observed among the groups at week 4 (Fig. 2A and B). However, at the end of the treatment period, the glucose levels of the mice fed an HFD were significantly increased at 30, 60 and 120 min after oral glucose administration compared with the mice fed a standard diet. The increase in blood glucose levels in the HFD model group was significantly reduced by CA and pioglitazone treatments (Fig. 2C). In addition, HFD induced a significant increase in the AUC, which was decreased by CA and pioglitazone treatments (Fig. 2D). Therefore, CA administration alleviated insulin resistance in obese mice in a time-dependent manner.

Effect of CA on the blood lipid profile in HFD-fed mice

Serum TC, TG and LDL-C levels were significantly increased in the mice fed an HFD compared with the mice fed a standard diet. CA treatment significantly suppressed the increases in serum TC, TG and LDL-C levels by 24.9%, 57.0%, and 50.9%, respectively (Fig. 3A-C). The serum HDL-C levels of the CA-treated group were increased by 44.9% compared with the HFD model group (Fig. 3D). The obtained results suggest that CA treatment beneficially modulates the blood lipid profile.

Fig. 2. Cinnamaldehyde improved OGTT in HFD-exposed mice. (A) and (C) show the results of OGTT for each group at the fourth and eighth weeks. (B) and (D) show comparisons of the areas under the OGTT curves (AUCs) among groups at the fourth and eighth weeks. Data are expressed as the mean $\pm$ SEM of eight mice from each group. * $P < 0.05$, ** $P < 0.01$ vs. normal control group. # $P < 0.05$, ## $P < 0.01$ vs. HFD control group.

CA decreased serum FFA and leptin contents in HFD-fed mice

As shown in Fig. 3E, FFA and leptin levels were higher in the mice fed an HFD than in mice fed a standard diet. In addition, serum FFA levels were significantly reduced by CA and pioglitazone treatments (10.1% and 10.4%, respectively). Similarly, serum leptin levels were decreased by approximately 28.7% and 42.6% in mice fed an HFD that were treated with CA and pioglitazone, respectively, compared with the HFD control group (Fig. 3F).

Effect of CA on adipose tissue morphology in HFD-fed mice

As shown by the HE staining results presented in Fig. 4, the morphology of iBAT and eWAT in the mice was altered by the HFD. However, CA and pioglitazone supplementation attenuated these changes. Specifically, in iBAT, multilocular brown adipocytes with larger lipid droplets were observed in the HFD model group, indicating more intense lipid metabolism. However, HFD-fed mice treated with CA and pioglitazone exhibited few lipid droplets within multilocular brown adipocytes, implying high metabolic activity (Fig. 4A and 4B). The sizes and numbers of adipocytes in eWAT were quantified in a fixed area (600,000 μm^2). The results demonstrated that the HFD-induced adipocyte hypertrophy and hyperplasia in eWAT. Furthermore, the administration of CA or pioglitazone prevented adipocyte hypertrophy and hyperplasia in HFD-fed mice, as demonstrated by a decrease in the average adipocyte diameter (Fig. 4C and 4D). The results suggest that CA administration

Fig. 3. Cinnamaldehyde decreased blood lipid profile and FFA and leptin levels in HFD exposed mice. (A) TG, triglyceride; (B) TC, total cholesterol; (C) LDL-C, low-density lipoprotein cholesterol; (D) HDL-C, high-density lipoprotein cholesterol; (E) FFA, free fatty acid; (F) Leptin. Values are expressed as the mean $\pm$ SEM of eight mice from each group. * $P < 0.05$, ** $P < 0.01$ vs. normal control group. # $P < 0.05$, ## $P < 0.01$ vs. HFD control group.

attenuates HFD-induced adipocyte hypertrophy and hyperplasia in eWAT and promote metabolic activity in iBAT in mice.

CA regulates the expression of the UCP1, PPAR γ , PGC-1 α and PRDM16 proteins in iBAT and eWAT in HFD-fed mice

As expected, the PPAR γ and PRDM16 proteins were down-regulated in iBAT from HFD-fed mice compared with their expression in iBAT from animals fed a standard diet. CA and

pioglitazone supplementation increased the levels of the PPAR γ protein compared with the HFD-fed group. In addition to the agonistic effects on PPAR γ , the expression of its target proteins PRDM16 and PGC-1 α was also significantly increased. However, we did not observe any changes in UCP1 protein expression in iBAT between the groups (Fig. 5A).

The UCP1 protein was not detected in eWAT from mice fed a standard diet or the HFD control group. However, both CA and pioglitazone induced UCP1 protein expression, suggesting that these treatments induced the appearance of brown-like adipocytes in eWAT. In addition, the CA treatment induced a significant increase in the relative expression levels

Fig. 4. Cinnamaldehyde altered the morphology of adipose tissues. (A) and (B) are representative images (200 $\times$) of HE-stained sections of adipose tissues from the iBAT and eWAT of the mice in the normal control group (1), HFD control group (2), pioglitazone treatment group (3) and cinnamaldehyde treatment group (4). Scale bars = 50 μ m. (C) Numbers of adipocytes and (D) percent of adipocyte diameters in the fixed area (600,000 μ m²) in eWAT.

of the PGC-1 α , PPAR γ and PRDM16 proteins compared with the saline-treated mice fed an HFD (Fig. 5B).

Discussion

Emerging evidence supports the notion that natural products can play a positive role in the management of obesity [18, 19]. Cinnamon, which is used as an herbal drug and spice, has been employed to treat obesity and diabetes for thousands of years in traditional Chinese medicine [20]. CA, the main volatile oil constituent of cinnamon, exhibits glucose-lowering activity [21]. Furthermore, CA positively alters body composition *in vivo* [22, 23]

Fig. 5. Cinnamaldehyde regulates expression of the relevant proteins. The representative western blotting results demonstrated that cinnamaldehyde treatment attenuated HFD-induced obesity through regulation of the expressions of PPAR γ , PGC-1 α , UCP1, and PRDM16 in iBAT and eWAT in mice. (1), normal control group; (2), HFD control group; (3), pioglitazone treatment group; (4), cinnamaldehyde treatment group. PPAR γ , peroxisome proliferator-activated receptor γ ; PGC-1 α , PPAR γ coactivator 1 α ; UCP1, uncoupling protein 1; PRDM16, PR domain-containing 16. Values are expressed as the mean $\pm$ SE of three mice from each group. * $P < 0.05$ vs. normal control group. # $P < 0.05$ vs. HFD control group.

and regulates adipocyte differentiation *in vitro* [24]. Consistent with previous studies [14, 25], our results demonstrated that CA significantly suppressed body weight gain, food intake and fat accumulation in HFD-induced obese mice. Furthermore, eight weeks of CA administration reduced the blood lipid profile and FFA and leptin levels. As reported in previous studies, leptin resistance may be one of the major causes of obesity [26]. The effect of CA in decreasing leptin contents may be responsible for body weight loss. In addition, elevated FFA levels are important for insulin resistance in obesity [27]. In the present study, insulin resistance in the obese mice was relieved by CA treatment, suggesting that CA potentially improves insulin sensitivity by regulating FFA levels.

Obesity arises from an imbalance between energy intake and expenditure. Obesity is closely related to the hypertrophy and hyperplasia of white adipocytes. Two major types of adipose tissue exist in mammals: WAT and BAT. WAT generally accounts for as much as 20% of the body weight of normal adult humans and primarily acts as a storage site for triglycerides, conserving excess calories for use in times of scarcity. Conversely, BAT is specialized to dissipate chemical energy as heat [28, 29]. The unique ability of BAT makes it a metabolically beneficial phenotype for obesity and obesity-related diseases [30]. Due to the similarity of the structures of the two adipose tissue types, they can mutually trans-differentiate into the other type. Based on accumulating evidence, morphological transition from the WAT phenotype to the BAT phenotype, a process known as “browning”, may facilitate the treatment of obesity [31]. The conversion of WAT to BAT is initiated by various conditions and agents, such as cold exposure [32] and the PPAR agonist rosiglitazone [33, 34]. Hence, pioglitazone was chosen as a positive control drug. The activation and/or expansion of BAT reduces body weight and increases insulin sensitivity. In the present study, CA attenuated HFD-induced adipocyte hypertrophy and hyperplasia in eWAT and iBAT and increased the number of multilocular brown adipocytes in eWAT, triggering the browning of eWAT [35].

Then, we further measured the expression of relevant proteins to investigate the molecular mechanisms correlated with the morphological changes occurring in adipose tissues.

The “beige” fat cells generated from the browning process switch from an energy storage state to an energy dissipation state and express specific molecular markers, such as UCP1 [36, 37]. UCP1, which constitutes the most specific difference in the biochemical characteristics of white adipocytes and brown adipocytes, is an essential molecule for metabolic thermogenesis. UCP1 is only expressed in the mitochondria in brown adipocytes [38]. As expected, the expression of the UCP1 protein was increased in iBAT and emerged in eWAT. Moreover, recent studies have identified several other dominant transcriptional regulators of brown adipocyte development and function, including PPAR γ , cAMP response element-binding protein (CREB), PGC-1 α , and PRDM16 [39, 40]. PPAR γ is expressed abundantly and at equal levels in white fat and brown fat and is required for the development of both cell types [41]. However, activation of PPAR γ by synthetic ligands induces a brown fat-like gene program in WAT [42]. Increased PPAR γ expression in WAT reduces the number of mature, large adipocytes and increases the number of small adipocytes, which improves the metabolism of this tissue [43]. Therefore, PPAR γ is recognized as a master regulator of adipogenesis. However, PPAR γ activation is not adequate or sufficient for white-to-brown adipocyte transition. Instead, PRDM16, which directly interacts with PPAR γ and coactivates the transcriptional activity of PPAR γ , is also required to activate a thermogenic brown fat gene program [42]. In addition, PGC-1 α is of particular importance, given its roles in up-regulating UCP1 expression and the uncoupling of mitochondrial fat combustion from ATP production [44, 45]. Consistent with these reports, in the present study, we showed that CA supplementation up-regulates the expression of the PPAR γ , PRDM16 and PGC-1 α proteins to promote the conversion of WAT to BAT.

In summary, CA treatment reduced body weight, body fat and blood lipid levels by inducing the browning of WAT and suppressing the whitening of brown adipose tissue in HFD-fed mice. Based on these findings, we concluded that CA is an efficient and safe alternative to anti-obesity agents. Therefore, CA has the potential to play a role in the therapeutic treatment of obesity and related disorders in the future, although the underlying mechanism requires further investigation.

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Disclosure Statement

None.

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