Methylxanthine Derivative-Rich Cacao Extract Suppresses Differentiation of Adipocytes through Downregulation of PPARγ and C/EBPs

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Summary Cacao extract (CE) consumption has beneficial effects on human health, such as lowering the risk of obesity. However, the underlying molecular mechanism for the anti-obesity effect of CE remains incompletely understood. Here, we used a 50% aqueous alcohol extract of cacao mass, which is rich in methylxanthine derivatives (about 11%) and poor in flavan-3-ols (less than 1%), and assessed the suppression effects of this extract on adipocyte differentiation to investigate the anti-obesity mechanism. CE dose-dependently decreased fat accumulation in 3T3-L1 cells without affecting cell viability. CE also dose-dependently decreased the protein and gene expression levels of two adipogenesis-related transcription factors, peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs). Moreover, CE decreased protein expression levels of sterol regulatory element-binding protein 1 (SREBP1) and its downstream fatty acid synthase (FAS), which was accompanied by the retained localization of SREBP1 in the cytoplasm of 3T3-L1 cells. After ICR mice were fed a diet containing 1% CE for 1 wk, their white adipose tissue weight was lower, whereas their brown adipose tissue weight was higher compared with those of control animals. Additionally, the protein expression levels of PPARγ, C/EBPs, SREBP1, and FAS in the white adipose tissue of these mice were also lower than those in control animals. In contrast, diet supplementation with CE induced higher levels of phosphorylated AMP-activated protein kinase (AMPK) and its downstream acetyl-CoA carboxylase. In conclusion, methylxanthine derivative-rich CE decreases fat accumulation in adipocytes by downregulating the expression of the adipocyte differentiation master regulators through the activation of AMPK.

Key Words cacao, methylxanthine derivatives, PPARγ, C/EBPs, adipocyte differentiation

Obesity is linked to the increased onset of certain chronic diseases, such as diabetes and cardiovascular diseases (1–4). Under the condition of obesity, adipocytes accumulate abnormal or excessive fat. Since adipocyte differentiation is acutely involved in fat accumulation (5), controlling adipocyte differentiation is a promising strategy for the prevention of obesity.

During differentiation from fibroblast-like preadipocytes to mature adipocytes, peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs) are the master regulators or crucial determinants of adipocyte fate (6). AMP-activated protein kinase (AMPK) is a key modulator for maintaining both the cellular and whole-body energy balance (7). The activation of AMPK inhibits the differentiation of 3T3-L1 cells by downregulating the expression of PPARγ and C/EBPs (8). Moreover, activated AMPK interacts with sterol regulatory element binding protein 1 (SREBP1) and inhibits the expression of its target molecule, fatty acid synthase (FAS), leading to a reduction of lipogenesis and lipid accumulation (9–11), in addition to promoting phosphorylation of acetyl CoA carboxylase (ACC) and inhibiting its activity (12).

Certain food materials and phytochemicals have been reported to reduce the risk of obesity (13–16). Intake of cacao liquor or dark chocolate ameliorates and/or prevents obesity in humans (17, 18). Cacao-derived flavan-3-ol-rich extract has also been shown to prevent obesity in animal studies (19). Cacao liquor and its flavan-3-ols decrease the plasma cholesterol level (20). In addition to flavan-3-ols, cacao also contains methylxanthine-derivatives such as theobromine and caffeine, and these

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Abbreviations: ACC, acetyl CoA carboxylase; AMPK, AMP-activated protein kinase; C/EBPs, CCAAT/enhancer-binding proteins; DMEM, Dulbecco’s modified Eagle’s medium; DMI, dexamethasone, 3-isobutyl-1-methylxanthine, insulin; FAS, fatty acid synthase; PBS, phosphate-buffered saline; PPARγ, peroxisome proliferator-activated receptor gamma; qPCR, quantitative PCR; SREBP1, sterol regulatory element-binding protein 1.
compounds likewise perform functions that are beneficial to human health (21). A recent study reported that caffeine and catechins improve lipid metabolism synergistically through an AMPK-dependent action in mice fed a high-fat diet (22). These results indicate that cacao extract (CE) and its components possess anti-obesity effects. However, the underlying molecular mechanism for the anti-obesity effect of CE, particularly the effect of a methylxanthine derivative-rich CE, is not yet fully understood.

In this study, we investigated that expression of PPARγ and C/EBPs and of their downstream adiposity-related factors, SREBP1 and FAS, in 3T3-L1 adipocytes after treatment with a methylxanthine-rich CE. To confirm the observed anti-obesity effect of this extract, we fed mice a diet supplemented with CE for 7 d, and their expression levels of PPARγ, C/EBPs, SREBP1, and FAS were assessed. Moreover, we also examined the phosphorylation of AMPK as an upstream factor involved in the expression of PPARγ and C/EBPs.

**MATERIALS AND METHODS**

**Materials.** Methylxanthine derivative-rich CE was kindly gifted from Glico Co., Ltd., Osaka, Japan. Briefly, cacao mass produced in the Republic of Ghana (3.6 kg) was defatted with hexane, and the residue (1.6 kg) was extracted with 50% (v/v) aqueous ethanol at 80°C for 4 h. The obtained extract was concentrated in vacuo and freeze-dried. The CE yield was 286 g (7.9% cacao mass), and the CE contained 10.0% theobromine, 0.71% caffeine, 0.41% (−)-epicatechin, 0.24% (+)-catechin, 0.19% procyanidin B2, 0.13% procyanidin C1, and trace amounts of cinnamottannin A2 and other unidentified compounds.

Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Calf serum and fetal bovine serum (FBS) were obtained from Gibco BRL (Gaithersburg, MD) and Biological Industries (Kibbutz Beit Haemek, Israel), respectively. Antibodies against β-actin, PPARγ, C/EBPα, C/EBPβ, C/EBPδ, and SREBP1, horseradish peroxidase-conjugated anti-rabbit IgG, and anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antibodies against p-AMPK, AMPK, p-ACC, and ACC were purchased from Cell Signaling Technology (Beverly, MA). Anti-rabbit Alexa 488-conjugated antibody was purchased from Molecular Probes (Eugene, OR).

**Cell culture.** 3T3-L1 preadipocytes were maintained in DMEM supplemented with 10% calf serum, 100 μg/mL streptomycin, and 100 units/mL of penicillin. Adipocyte differentiation was induced as described previously (23). Briefly, 1 d after reaching confluence, the cells were treated with a DMI (10 μg/mL insulin, 1 μmol/L dexamethasone, and 0.5 mmol/L 3-isobutyl-1-methylxanthine) cocktail in DMEM-high glucose (4.5 g/L glucose) supplemented with 10% FBS and the above antibiotics for 2 d. During differentiation, the cells were treated with 10 μg/mL insulin every 2 d.

**Sudan II staining.** Intracellular lipid accumulation was stained with Sudan II. Adipocyte differentiation was induced in 3T3-L1 cells via treatment with a DMI cocktail for 6 d. Determination of lipid accumulation in 3T3-L1 cells was performed by Sudan II staining as described previously (24).

**Cell viability assay.** Cell viability was determined by crystal violet staining assays, as described previously (24). Briefly, 3T3-L1 cells were incubated with the indicated concentrations of CE in the presence of DMI for 72 h. The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature and stained with 0.2% (w/v) crystal violet in 2% (v/v) ethanol for 10 min at room temperature. The cells were washed, and the dye was extracted with 0.5% (w/v) SDS in 50% (v/v) ethanol. The absorbance was measured at 570 nm with a reference wavelength at 630 nm.

**Immunofluorescence microscopy.** 3T3-L1 cells were differentiated via treatment with DMI in the presence or absence of CE at 100 μg/mL for 6 d. The cells were fixed with 4% paraformaldehyde in PBS for 20 min and permeabilized with 0.1% (w/v) Triton X-100 in PBS for 5 min at room temperature. The cells were incubated with rabbit polyclonal anti-SREBP1 antibody at 4°C overnight, followed by incubation with Alexa 488-conjugated anti-rabbit antibody. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) at 1 μg/mL. Fluorescent images were acquired with an Olympus FFX100 fluorescence microscope (Olympus, Tokyo, Japan).

**Quantitative PCR (qPCR).** Total RNA was extracted from 3T3-L1 cells using TRIzol (Invitrogen), and cDNA was synthesized using reverse transcriptase. The resulting cDNA was subjected to qPCR using the following primers: Gapdh (forward primer 5′-ACAACCTTTGGCATTGTGGAAA-3′ and reverse primer 5′-GATGCAGGAGTGATGTCTG-3′); Pparγ (forward primer 5′-ACGTGCACGCTACTGATGTA-3′ and reverse primer 5′-AGAGAACAGCTTGTCAGG-3′); and Cebpα (forward primer 5′-GAAGCTTGAAGCACAATCGATC-3′ and reverse primer 5′-GATCAGGAGACTACGATGTA-3′). qPCR was performed via a two-step PCR method on a Thermal Cycler Dice real-time system (Takara Bio Inc., Shiga, Japan). Ct values were transformed into relative quantification data by the 2−ΔΔCt method, and data were normalized to Gapdh as an endogenous control.

**Western blot analysis.** Cell lysate preparation and Western blotting were performed as described in our previous reports (16, 24). Specific immune complexes were detected with the ATTO Light-Capture II Western Blotting Detection System. The density of specific bands was calculated using ImageJ image analysis software (National Institutes of Health, Bethesda, MD).

**Animal treatment.** All animal experiments were approved by the Institutional Animal Care and Use Committee (Permission #27-05-09) and were performed according to the Guidelines for Animal Experiments set by Kobe University. Male ICR mice (4 wk old, n=10) were obtained from Japan SLIC, Inc. (Shizuoka, Japan) and kept in a temperature-controlled room (23±2°C) with a 12:12-h light/dark cycle (lights were
Fig. 1. Effect of CE on lipid accumulation in 3T3-L1 adipocytes. (A) Sudan II staining of lipid droplets in 3T3-L1 cells. The cells were incubated with CE at various concentrations in the presence or absence of DMI for 6 d, and lipid droplets were stained with Sudan II (upper panels). The stained lipid droplets in the cells were quantified (bottom panel). Data are presented as the mean±SE \((n=3)\), and the lipid content is shown based on a sample from cells cultured in the absence of DMI and CE. (B) Cell viability of 3T3-L1 cells following treatment with CE. After 3T3-L1 cells were incubated with the indicated concentrations of CE in the presence of DMI for 72 h, the cell viability was determined by crystal violet staining assays. Data are presented as the mean±SE \((n=3)\). Different letters indicate statistically significant differences \((p<0.05)\).

Fig. 2. Effect of CE on the protein and mRNA expression of PPARγ and C/EBPα in 3T3-L1 adipocytes. 3T3-L1 cells were incubated with the indicated concentrations of CE in the presence of DMI for 6 d. (A, B) Protein (A) or mRNA (B) expression of PPARγ \((Pparg)\) and C/EBPα \((Cebpa)\) was measured by Western blotting and qPCR, respectively. For protein expression, β-actin was used as a loading control. The intensity of each band was quantified by ImageJ 1.44, and the ratio of each target band level was normalized to the β-actin level. For mRNA expression, the value of Gapdh was used as an internal control. Data are presented as the mean±SE \((n=3)\), and relative values are shown based on a sample from cells cultured in the absence of DMI and CE. Different letters indicate statistically significant differences \((p<0.05)\).
turned on at 9:00 am). The mice had free access to tap water and an AIN-93 M laboratory-purified diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and were acclimatized for 7 d before use in experiments. The provided food was changed every other day. The mice were then randomly divided into two groups of five and fed a diet containing 0% or 1% CE and the tap water for another 7 d.

At the end of the experiment, the mice were sacrificed at 9:00 after a 15-h fast. Exsanguination via cardiac puncture was performed under anesthesia using sevoflurane as an inhalational anesthetic and sodium pentobarbital as an analgesic. Blood was collected in a heparinized tube. Plasma was then prepared by centrifugation at 800 × g for 10 min at 4 °C and subjected to measurements of glucose, total cholesterol, and triacylglycerol levels using corresponding commercial kits (Lab assay™ Glucose Wako kit, Cholesterol-E test, and Triacylglycerol test, respectively, all from Wako Pure Chemical Industries, Ltd.). The plasma adiponectin level was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Mouse/Rat High Molecular Weight Adiponectin ELISA Kit from Shibayagi, Gunma, Japan). The liver, white adipose tissue (mesenteric, epididymal, perirenal, and subcutaneous adipose tissues), and brown adipose tissue were collected, washed with 1.15% (w/v) KCl, weighed, immediately frozen using liquid nitrogen, and kept at −80 °C until use. Mesenteric white adipose tissue was used for the measurement of protein expression of adipogenesis- and lipid metabolism-related factors by Western blot analysis.

**Statistical analysis.** All data are presented as the means±SE (n=3 for in vitro cell culture experiments and n=5 for in vivo animal experiments). Statistical significance was analyzed by one-way ANOVAs with a Turkey’s post-hoc test for in vitro cell culture experiments or by Student’s t-tests for in vivo animal experiments. Statistical analyses were performed with JMP statistical software version 11.2.0 (SAS Institute, Cary, NC). Differences with a p<0.05 were considered statistically significant.

**RESULTS**

To examine the effect of CE on adipogenesis in 3T3-L1 cells, the cells were differentiated via treatment with DMI in the presence of CE for 6 d. Intracellular lipid accumulation was visualized by staining with Sudan II (Fig. 1A, top panels). DMI treatment induced a significantly higher lipid content in 3T3-L1 cells compared with undifferentiated controls [DMI(−)], and the DMI-induced lipid accumulation was suppressed by CE in a concentration-dependent manner (Fig. 1A, bottom panel). To evaluate whether the lower lipid accumulation was due to a reduction in cell viability, a crystal violet staining assay was performed. The results show that CE had no influence on the cell viability of 3T3-L1 cells at the indicated concentrations (Fig. 1B). These results suggest that CE suppresses lipid accumulation during adipocyte differentiation without affecting cell viability.

To clarify the underlying mechanism responsible for the CE-induced suppression of lipid accumulation in adipocytes, we investigated whether CE suppressed the expression of PPARγ and C/EBPα, which are the master regulators of adipogenesis (5, 6). As expected, DMI treatment induced higher expression levels of PPARγ and C/EBPα were measured by Western blotting. β-Actin was used as a loading control. The intensity of each band was quantified by ImageJ 1.44, and the ratio of each target band level was normalized to the β-actin level. Data are presented as the mean±SE (n=3), and the relative values are shown based on a sample from cells cultured in the absence of DMI and CE. Different letters indicate statistically significant differences (p<0.05).

![Fig. 3. Effect of CE on the protein expression levels of C/EBPβ and C/EBPδ in 3T3-L1 adipocytes. 3T3-L1 cells were incubated with the indicated concentrations of CE in the presence of DMI for 24 h. Protein expressions of C/EBPβ and C/EBPδ were measured by Western blotting. β-Actin was used as a loading control. The intensity of each band was quantified with ImageJ 1.44, and the ratio of each target band level was normalized to the β-actin level. Data are presented as the mean±SE (n=3), and the relative values are shown based on a sample from cells cultured in the absence of DMI and CE. Different letters indicate statistically significant differences (p<0.05).](image-url)
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DMI for 24 h, significantly higher protein expression levels of C/EBPα/H9252 and C/EBPα/H9254 were observed compared with those of control cells (Fig. 3). CE decreased the DMI-induced expression of these proteins in a concentration-dependent manner, and a statistically significant decrease was observed at 50 and 100 μg/mL of CE for C/EBPα/H9252 and C/EBPα/H9254, respectively. These results indicate that the reduced expression of PPARG/H9253 and C/EBPs is involved in the CE-induced suppression of lipid accumulation in adipocytes.

We next examined expression of the downstream factors of PPARG and C/EBPs. SREBP1 is a transcription factor that regulates the expression of lipogenic genes, such as FAS and low-density lipoprotein receptor (11). As shown in Fig. 4A, DMI treatment induced markedly higher protein expression levels of SREBP1 and FAS compared with those of controls. CE-treatment significantly prevented the DMI-induced expression of these proteins at concentrations above 50 μg/mL (Fig. 4A). SREBP1 is activated through the protease-processing pathway, and activated SREBP1 enters the nucleus and induces the expression of its target genes, including FAS (11).

Male ICR mice were administered a diet containing 0% (Control) or 1% CE in their tap water for 7 d. Data are presented as the mean±SE (n=5), *p<0.05, **p<0.01.

Table 1. Body and adipose tissue weight of mice with or without CE supplementation.

|                | Control | 1% CE |
|----------------|---------|-------|
| Body weight (g)   | 28.0±0.3 | 24.5±0.2** |
| Tissue weight (g/100 g BW) |         |       |
| Liver             | 5.89±0.21 | 5.56±0.33 |
| Total white adipose tissue | 3.18±0.17 | 2.22±0.11** |
| Mesenteric        | 0.38±0.08 | 0.23±0.02 |
| Epididymal        | 1.01±0.06 | 0.74±0.03** |
| Perirenal         | 0.26±0.01 | 0.15±0.01** |
| Subcutaneous      | 1.38±0.12 | 0.98±0.12* |
| Brown adipose tissue | 0.45±0.01 | 0.57±0.03** |

Male ICR mice were administered a diet containing 0% (Control) or 1% CE in their tap water for 7 d. Data are presented as the mean±SE (n=5), *p<0.05, **p<0.01.

Table 2. Plasma glucose, lipid, and adiponectin levels of mice with or without CE supplementation.

|                  | Control | 1% CE |
|------------------|---------|-------|
| Blood glucose (mg/dL) | 121±4   | 111±5 |
| Plasma cholesterol (mg/dL) | 101±10 | 88±7  |
| Plasma triacylglycerol (mg/dL) | 124±7  | 68±5**|
| Plasma adiponectin (ng/mL)  | 73±6   | 106±8** |

We next examined expression of the downstream factors of PPARG and C/EBPs. SREBP1 is a transcription factor that regulates the expression of lipogenic genes, such as FAS and low-density lipoprotein receptor (11). As shown in Fig. 4A, DMI treatment induced markedly higher protein expression levels of SREBP1 and FAS compared with those of controls. CE-treatment significantly prevented the DMI-induced expression of these proteins at concentrations above 50 μg/mL (Fig. 4A).

SREBP1 is activated through the protease-processing pathway, and activated SREBP1 enters the nucleus and induces the expression of its target genes, including FAS (11). We further analyzed the localization of SREBP1 in 3T3-L1 cells by immunofluorescence microscopy. In the absence of CE, SREBP1 is localized in both the cytoplasm and nucleus (Fig. 4B, top panels). However, in the presence of 100 μg/mL CE, SREBP1 was mainly local-
ized in the cytoplasm (Fig. 4B, bottom panels). From these results, we confirm that the CE-induced reduction in PPARγ and C/EBPs expression levels results in the suppression of SREBP1 and FAS expression.

Lastly, we performed in vivo experiments to confirm the results obtained from the in vitro cell-culture experiments. Diet supplementation with 1% CE for 7 d resulted in less body weight gain and lower total white adipose tissue weights in male ICR mice compared with control mice (Table 1), without altering the amount of food intake (control: 3.68±0.34 g/d/head vs. CE: 3.33±0.37 g/d/head). In contrast, brown adipose tissue weight was slightly, but significantly, higher in the mice that had consumed CE. Although the plasma glucose and total cholesterol levels remained similar between the two groups, the plasma triacylglycerol level following CE supplementation was only about 50% of that in the control animals (Table 2). Interestingly, after
CE supplementation, the mice also had a higher level (1.45-fold) of plasma adiponectin compared with control animals.

Although the mesenteric white adipose tissue weight was not significantly lower in animals following CE supplementation, this tissue produces the highest levels of monocyte chemoattractant protein-1 in obese mice (25), which indicates that, among all types of white adipose tissue, mesenteric white adipose tissue plays the most important role in obesity. Thus, mesenteric white adipose tissue was used in the ensuing experiments. The protein expression of PPARγ and C/EBPs was measured in mesenteric white adipose tissue. As shown in Fig. 5, supplementation with 1% CE for 7 d resulted in significantly lower levels of PPARγ, C/EBPα, and C/EBPβ protein expression compared with controls. However, the level of C/EBPβ expression following CE supplementation remained similar to that in control mice. Supplementation with CE also produced lower levels of protein expression of SREBP1 and FAS, which are the downstream factors for PPARγ and C/EBPs (Fig. 6). AMPK is known to inhibit adipocyte differentiation as an upstream factor of PPARγ and C/EBPs (8). CE supplementation induced significantly higher levels of AMPK phosphorylation and of the downstream ACC in mesenteric white adipose tissue compared with controls (Fig. 7). From these in vivo results, we conclude that CE intake suppresses the expression of adipocyte differentiation markers via AMPK activation.

**DISCUSSION**

Since obesity is involved in the increased onset of many diseases, much attention has been focused on targeting food components that may help prevent obesity. For obesity prevention by food components, the following strategies appear promising: inhibition of adipocyte differentiation (5), modulation of lipid metabolism (inhibition of lipogenesis and promotion of lipolysis) (26), and promotion of energy expenditure, including the formation of beige adipocytes (27). Various food materials and food-derived phytochemicals have been reported to inhibit adipocyte differentiation (23, 24, 28, 29). In this study, we found that CE containing abundant methylxanthine derivatives decreased both lipid accumulation in 3T3-L1 cells (Fig. 1) and adipose tissue weight in mice (Table 1). Reduced expression levels of PPARγ and C/EBPs were found to be involved in the anti-adipogenic mechanism of CE (Figs. 2, 3, and 5). Additionally, because phosphorylation of AMPK has been reported to inhibit expression of PPARγ and C/EBPs (8), we examined AMPK phosphorylation in vivo (Fig. 7), and we found that this is also involved in the anti-adipogenic mechanism of CE. Thus, CE possesses anti-adipogenic effect via reducing the expressions of PPARγ and C/EBPs. The results of our in vivo experiments are consistent with those from our in vitro experiments, indicating that the CE mechanism observed in vitro likely contributes to the prevention of obesity in vivo.

PPARγ and C/EBPs play pivotal roles in adipocyte differentiation and adipogenesis (7, 30, 31). PPARγ forms a heterodimer with retinoic acid X-receptor (RXR) (32) and regulates the transcription of adipocyte-specific genes (33). C/EBPα functions as another principal player in adipogenesis and is most abundant in mature adipocytes (34). C/EBPβ and C/EBPδ are known to induce the expression of PPARγ and C/EBPα (35, 36).
In this study, CE did not suppress protein expression of C/EBPβ in the mesenteric white adipose tissue of mice (Fig. 5), even though it significantly suppressed C/EBPβ in 3T3-L1 adipocytes (Fig. 3). Our previous report demonstrated that Ashitaba calcones, 4-hydroxyderricin and xanthoangelol, downregulated the expression of C/EBPα and PPARγ accompanied by a decrease in the expression of C/EBPβ but not in that of C/EBPδ (23). These results suggest that C/EBPδ is not critical for the induction of PPARγ and C/EBPα expression.

As an upstream factor for C/EBPβ, the activation of AMPK is likely also involved in the mechanism for CE induced effects. It was previously reported that AMPK activation inhibited the differentiation of 3T3-L1 cells by downregulating the expression of C/EBPs and PPARγ (8). Our earlier report also demonstrated that an AMPK inhibitor compound C prevented the Ashitaba calcones-induced downregulation of C/EBPβ, C/EBPα, and PPARγ (23). Moreover, activated AMPK interacts with SREBP1 and inhibits the expression of its target molecule FAS, leading to a reduction of lipogenesis and to lipid accumulation (9–11). The activation of AMPK may contribute to the increased level of adiponectin in plasma, given that an AMPK activator is able to promote adiponectin multimerization in 3T3-L1 adipocytes (37). However, the target molecule of CE is still unclear, and further study is needed to clarify this issue.

The anti-obesity effects of CE are well-documented. For example, the intake of cacao liquor such as dark chocolate decreases BMI in humans (17, 18), and cacao liquor procyanidins ameliorate lipid metabolism in mice (19). Many researchers have focused on cacao polyphenols, particularly flavan-3-ols, as the active compounds. However, the polyphenol content in the CE used here was less than 1%, whereas this extract contained abundant methylxanthines, such as 10.0% theobromine and 0.71% caffeine. Based on the above composition, 28 μmol/L theobromine and 1.8 μmol/L caffeine exist in the minimum concentration of CE (50 μg/mL) that is effective for the inhibition of lipid accumulation.

Recently, Jang et al. (38) reported that theobromine reduced adipogenesis in 3T3-L1 cells through the suppression of AMPK and ERK signaling at a concentration of 150 μg/mL (=877 μmol/L). A human study demonstrated that plasma concentrations of theobromine increase to 28.75 μmol/L after consumption of 850 mg of theobromine for 4 wk (39). Another report showed that the maximum plasma concentration of theobromine in humans is approximately 50 μmol/L (40). Our recent data show that theobromine above concentrations of 25 μmol/L exhibits an anti-adipogenic effect accompanied by lower expression of PPARγ and C/EBPs in 3T3-L1 adipocytes (41). Thus, theobromine is a strong candidate for the effective compound in CE.

Caffeine suppresses the intracellular lipid accumulation of 3T3-L1 adipocytes after full differentiation (42). Furthermore, coffee containing caffeine inhibits adipocyte differentiation through the inactivation of PPARγ (43). Recently, Kim et al. (44) demonstrated that caffeine at 1 mmol/L inhibits the expression of C/EBPβ, C/EBPα, and PPARγ during 3T3-L1 preadipocyte differentiation through the AKT/glycogen synthase kinase 3β pathway. In contrast, our results demonstrate that caffeine indirectly suppresses lipid accumulation in 3T3-L1 adipocytes by decreasing secretion of inflammatory cytokines from Caco-2 cells, even though direct treatment of 3T3-L1 cells with 50 mmol/L caffeine did not affect lipid accumulation (41). These results indicate that caffeine at a physiological concentration does not affect adipocyte differentiation, but it is possible for this compound to inhibit adipocyte differentiation at higher, non-physiological concentrations. In the present study, CE inhibited adipocyte differentiation not only in 3T3-L1 adipocytes but also in the adipose tissue of mice. Furthermore, the caffeine concentration in CE is too low to possess an anti-adipogenic effect in 3T3-L1 cells. Thus, caffeine must not be the effective compound in CE.

In conclusion, methylxanthine-rich CE inhibits adipocyte differentiation through an AMPK-induced reduction in the expression of PPARγ and C/EBPs. Thus, methylxanthine-rich CE is an attractive novel food material with which to suppress obesity. To clarify the detailed mechanism of this effect, experiments are in progress using a methylxanthine compound.

Author Contributions
YY and TM contributed equally to the study.

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