Cocoa polyphenols suppress adipogenesis in vitro and obesity in vivo by targeting insulin receptor

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OBJECTIVE: To investigate the inhibitory effect of cocoa polyphenol extract (CPE) on adipogenesis and obesity along with its mechanism of action.

METHODS AND RESULTS: 3T3-L1 preadipocytes were cultured with isobutylmethylxanthine, dexamethasone and insulin (MDI), and male C57BL/6N mice (N = 44) were fed a high-fat diet (HFD) for 5 weeks with or without CPE. CPE at 100 or 200 μg ml⁻¹ inhibited MDI-induced lipid accumulation without diminishing cell viability. In particular, CPE reduced the protein expression levels of PPARγ and CEBPα, and blocked mitotic clonal expansion (MCE) of preadipocytes by reducing proliferating signaling pathways. This in turn attenuates lipid accumulation during the differentiation of 3T3-L1 preadipocytes. CPE effectively suppressed MDI-induced phosphorylation of extracellular signal-regulated kinase (ERK) and Akt, and their downstream signals. We then examined whether CPE regulates insulin receptor (IR), a common upstream regulator of ERK and Akt. We found that although CPE does not affect the protein expression level of IR, it significantly inhibits the activity of IR kinase via direct binding. Collectively, the results suggested that CPE, a direct inhibitor of IR kinase activity, inhibits cellular differentiation and lipid accumulation in 3T3-L1 preadipocytes. Consistently, CPE attenuated HFD-induced body weight gain and fat accumulation in obese mice fed with a HFD. We also found that HFD-induced increased fasting glucose levels remained unaffected by CPE.

CONCLUSION: This study demonstrates that CPE inhibits IR kinase activity and its proliferative downstream signaling markers, such as ERK and Akt, in 3T3-L1 preadipocytes, and also prevents the development of obesity in mice fed with a HFD.

INTRODUCTION

Obesity is a major health problem worldwide, increasing the risk of type 2 diabetes and other chronic diseases.1,2 Obesity is often caused by hyperthyroid (increase in cell size) and hyperplasia (increase in cell number) of adipocytes.3 While adult-onset obesity is typically due to adipocyte hypertrophy, adipocyte hyperplasia mainly occurs in children and morbidly obese adults.4,5 Therefore, both hyperthrophy and hyperplasia of adipocytes can be important targets in treating obesity.

Adipogenesis involves mitotic clonal expansion (MCE), early differentiation and terminal differentiation.6 MCE is a prerequisite for the terminal differentiation of preadipocytes.7,8 During MCE, growth-arrested cells synchronously re-enter the cell cycle, and the cell numbers are increased before the adipogenic genes are expressed.9 It has been suggested that the MCE facilitates DNA remodeling for gene expression during adipogenesis.6

Cell growth and protein synthesis in adipogenesis are closely associated with the Ras/mitogen-activated protein kinase (MAPK) pathway. This pathway involves tyrosine phosphorylation of insulin receptor substrate (IRS) proteins, which in turn interact with various proteins to activate Ras, stimulating Raf–MEK–ERK cascades.6 Activated extracellular signal-regulated kinase (ERK) translocates into the nucleus to initiate MCE in preadipocytes.10,11 Tyrosine phosphorylation of IRS-1 also induces phosphorylation of Akt,12 initiating MCE through activation of complex signaling cascades.11,13–17

Insulin receptor (IR) is a common upstream regulator of ERK and Akt signaling. Tyrosine autophosphorylation of IR is the earliest cellular response to insulin stimulation. Autophosphorylation of IR begins with phosphorylation of Tyr-1146 and either Tyr-1150 or Tyr-1151.18,19 Autophosphorylated IR subsequently phosphorylates the tyrosine residues of its substrates, such as IRS-1, which act as ‘docking sites’ for downstream proteins containing the Src-homology-2 domain. Many of these Src-homology-2 proteins are adapter molecules, such as PI3K and Grb2.19 Because insulin initiates proliferative signals at the onset of MCE,11,13–17 IR and its downstream signals, such as Shc-Raf-MEK-ERK and IRS-PI3K-Akt, have pivotal roles in adipogenesis.20 Accordingly, knockout of the adipose tissue-specific IR protected mice from obesity and obesity-related glucose intolerance,21 implying that the suppression of ERK and Akt activation may prevent adipogenesis, especially MCE.

Cocoa made from cacao (Theobroma cacao L.) contains a large amount of polyphenols,22 which are known to prevent several chronic diseases, including type 2 diabetes and cardiovascular diseases.23–27 It also has been reported that cocoa polyphenols regulate genes involved in fatty acid metabolism, thereby preventing obesity in mice.28 However, the mechanisms by
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which cocoa polyphenols regulate adipogenesis in 3T3-L1 preadipocytes and its direct target have not been elucidated. Hence, the purpose of this study was to examine the mechanisms by which cocoa polyphenol extract (CPE) mediates anti-adipogenic activity and to identify a direct target of CPE in 3T3-L1 preadipocytes, and also to confirm anti-obesity effect of CPE using diet-induced obese mouse model.

MATERIALS AND METHODS

Sample preparation

Cocoa polyphenols were extracted from 50 g of cocoa powder (Lotte Confectionary Co. Ltd, Seoul, Korea) with 50% (v/v) aqueous ethanol under reflux for 6 h. The extracted solution was filtered, loaded onto a column of styrene-based adsorption resin (60 × 450 mm; HP-20; Mitsubishi, Tokyo, Japan), washed with 20% (v/v) ethanol and eluted with 60% (v/v) ethanol. CPE was then concentrated under reduced pressure, frozen and dried.29

Chemicals

Isobutylmethylxanthine, dexamethasone, insulin, 1,2-propanediol and oil red O (ORO) powder were purchased from Sigma Chemical (St Louis, MO, USA); [32P] ATP and CNBr-Sepharose 4B were purchased from Amersham Biosciences (Piscataway, NJ, USA). 

Cell culture

3T3-L1 preadipocytes were purchased from ATCC (Manassas, VA, USA). Media and serum were obtained from Gibco (Grand Island, NY, USA). The 3T3-L1 preadipocytes were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% bovine calf serum until confluence. Confluent cells were incubated in DMEM supplemented with 10% fetal bovine serum (FBS), dexamethasone (1 μM), isobutylmethylxanthine (0.5 mM) and insulin (5 μg·ml⁻¹). After 2 days, the medium was replaced with DMEM containing 10% FBS and insulin (5 μg·ml⁻¹), and the cells were incubated for another 2 days. Until the preadipocytes were fully differentiated, they were cultured in DMEM containing 10% FBS, with the medium being replaced every 2 days.

MTT assay

3T3-L1 preadipocytes were seeded in a 24-well plate at a density of 2.5 × 10⁴ cells per well, then incubated until confluence. When confluent, control cells were treated with 10% FBS DMEM alone, whereas experimental cells were treated with 10% FBS DMEM supplemented with CPE.

ORO staining

3T3-L1 preadipocytes were seeded in a 24-well plate at a density of 2.5 × 10⁴ cells per well. After confluence, the cells were incubated in DMEM until they were differentiated. The mature adipocytes were fixed with 10% formalin. The fixed cells were then stained with ORO solution, and washed with phosphate-buffered saline. For quantification, the ORO stain was eluted from a given quantity of cells using isopropanol alcohol, and the absorbance was measured spectrophotometrically at 515 nm.

Flow cytometry using a fluorescence-activated cell sorter analysis

3T3-L1 preadipocytes were seeded in 6 cm dishes at a density of 1.5 × 10⁵ cells per dish and cultured until confluence. Confluent cells were incubated in DMEM with 10% FBS and isobutylmethylxanthine, dexamethasone and insulin (MDI) cocktail with or without CPE. The cells were then dissociated with trypsin and centrifuged (1000 r.p.m., 3 min). The pellets were resuspended in phosphate-buffered saline and reincubated. The pellets were then fixed by suspension in 70% (v/v) ethanol and maintained overnight at 4 °C. Subsequently, the fixed cells were centrifuged (1500 r.p.m., 3 min), resuspended in 600 μl phosphate-buffered saline containing 20 μg·ml⁻¹ of PI solution (Sigma) and 0.2 mg·ml⁻¹ of RNase (Amresco, Solon, OH, USA), and incubated in the dark (37 °C, 15 min). The fluorescence emitted from cells was measured using a FACs calibur flow cytometer (Becton–Dickinson, San Jose, CA, USA). A total of 10,000 cells per sample were analyzed as previously described.28

Western blot assay

Western blot analysis was performed as previously described.28 Briefly, 3T3-L1 preadipocytes were seeded in 6 cm dishes at a density of 1.5 × 10⁵ cells per dish and cultured for 2 days in DMEM with 10% bovine calf serum. When the cells reached confluence, the medium was changed to DMEM with 10% FBS and MDI cocktail with or without CPE. The protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. Cell lysates were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (GE Healthcare, Piscataway, NJ, USA). The membrane was blocked with 5% skim milk and incubated with specific primary antibody followed by HRP-conjugated secondary antibody. The protein bands were visualized using a chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

In vivo

Animals and diets

Male C57BL/6N mice (5-week-old) were purchased from Koatech (Pyeongtaek, Korea). After 1 week acclimation, mice were housed in climate-controlled quarters (23 ± 3 °C, 50 ± 10% humidity) with a 12-h light–dark cycle. Mice (N = 44) were divided into four different dietary groups (n = 11 each group): a normal diet, a high-fat diet (HFD) for 5 weeks. Animals were treated with either CPE, which was dissolved in 0.5% sodium carboxymethylcellulose, or vehicle (0.5% sodium carboxymethylcellulose),29 Body weight and food intake were monitored on a weekly basis.

Serum glucose and lipid contents

Blood was collected in serum separator tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged (3000 r.p.m., 20 min). Serum glucose was analyzed by standard clinical assays according to the manufacturer’s instructions (Asan, Hwasung, Korea). Enzymatic assays for serum triglyceride and total cholesterol were performed using kits purchased from Asan.

Statistical analysis

For in vitro study, data were expressed as means ± s.d., and the Student’s t-test was used for comparisons. A probability value of P < 0.05 or P < 0.01 was used as the criterion for statistical significance. In vivo data were expressed as means ± s.e.m. One-way analysis of variance with Newman–Keuls’s post-test was used to evaluate mean differences of group. Data were analyzed using GraphPad Prism (version 4.03; GraphPad Software, Inc., San Diego, CA, USA). Means with superscripts of different letters were significantly different at P < 0.05.
RESULTS
CPE inhibits MDI-induced adipogenesis in 3T3-L1 preadipocytes
To investigate the anti-adipogenic effect of CPE, cells were treated with MDI and simultaneously with different levels of CPE. Adipogenesis was assessed by ORO staining of lipid droplets. ORO staining result showed that CPE likely reduces MDI-mediated lipid accumulation in a dose-dependent manner (Figure 1a). Quantitative analysis of ORO staining indicated that MDI increases relative lipid contents by 3.45-fold in 3T3-L1 preadipocytes compared with undifferentiated controls (Figure 1b). MDI-induced greater lipid accumulation was reduced approximately 30% and 72% in the cells treated with 100 and 200 μg/ml of CPE, respectively (Figure 1b). To evaluate decreased lipid accumulation was not attributable to diminished cell viability, MTT assay was performed. CPE did not cause a considerable decline in cell viability (Figure 1c).

PPARγ and C/EBPα are two master regulators of adipogenesis.31 The protein expression levels of these proteins were attenuated by 100 or 200 μg/ml of CPE (Figure 1d). Consistently, mRNA expression levels of PPARγ and C/EBPα, as well as other downstream genes, including aP2 and fatty acid synthase, were also significantly decreased by either level of CPE in 3T3-L1 preadipocytes treated with MDI (Figure 1e). Collectively, CPE inhibited adipogenesis by reducing expressions of adipogenesis-mediated proteins and/or genes during the cellular differentiation.

CPE blocks MDI-induced lipid accumulation during MCE in 3T3-L1 preadipocytes
To identify the key stage where CPE exerts its anti-adipogenic effect, CPE was treated at different stages of cellular differentiation as indicated in Figure 2a. Adipogenesis was assessed by ORO staining (Figure 2b). The lipid contents were quantified using spectrophotometer (Figure 2c). We found that CPE significantly diminished MDI-triggered lipid accumulation during 0–2, 0–4 and 0–8 days (Figure 2c). CPE effectively reduced lipid accumulation during days including its treatment between day 0 and 2, suggesting that CPE inhibits MDI-triggered adipogenesis by suppressing MCE.

CPE represses MDI-induced cell cycle progression during 3T3-L1 preadipocytes differentiation
We attempted to further examine whether CPE inhibits MCE and subsequent cell differentiation by suppressing cell cycle progression in 3T3-L1 preadipocytes. Fluorescence-activated cell sorter analysis results showed that MDI-induced cell cycle progression was completely blocked by 200 μg/ml of CPE (Figure 3a). The population of cells in each stage of the cell cycle was quantified (Figure 3b). Indeed, undifferentiated cells did not undergo cell cycle progression, staying in G1-S checkpoint (the basal state) from 16 to 24 h. Compared with the undifferentiated controls, a significant portion of MDI-treated 3T3-L1 preadipocytes appeared to be in the S phase at 16 h, and greater number of cells appeared to be in the S and G2 phases at 20 h. Meanwhile, the cells treated with both MDI and CPE did not undergo cell cycle progression, staying in G1-S checkpoint until 24 h. These results indicate that CPE repressed cell cycle progression during cellular differentiation, which may contribute to inhibit MDI-induced adipogenesis.

CPE suppresses MDI-induced ERK and Akt-mediated signaling pathways
As major regulators of cell proliferation,32,33 ERK and Akt contribute to initiate MCE during adipogenesis.8,11 Therefore, we aimed to investigate whether CPE downregulates ERK and Akt-mediated signaling pathways. CPE effectively suppressed MDI-induced phosphorylation of ERK and its downstream signals, including p90RSK, and its upstream signals, such as c-Raf and MEK (Figure 4a). The quantitative data indicated that the protein expressions of p-ERK from MDI treated with CPE at 100 and 200 μg/ml were 20.01% and 60.07% lowered, respectively,
compared with that in the controls without CPE treatment ($P < 0.05$; data not shown). CPE also suppressed Akt phosphorylation induced by MDI, and downstream markers of Akt, including mTOR and p70S6K (Figure 4b). In particular, the protein expressions of p-Akt from MDI-treated with CPE were decreased significantly ($P < 0.05$) up to 71.18%, compared with controls (data not shown). Collectively, these findings indicated that CPE inhibits ERK and Akt-mediated signaling pathways stimulated by MDI. This implies that CPE may have an important role in insulin-mediated signaling cascades, possibly by interacting with shared upstream regulators of ERK and Akt.

CPE attenuates IR kinase activity through direct binding

IR is a common upstream regulator of ERK and Akt during cellular differentiation. To elucidate the effect of CPE on IR function, we examined a direct interaction of CPE with IR. We found that the total tyrosine phosphorylation (PY99) of IR was not inhibited by CPE, nor was the activation of IR (autophosphorylation of Tyr1146) altered by CPE (Figure 5a). Assuming that any effect of CPE on the activity of IR would have to occur after its autophosphorylation, we examined the effect of CPE on IR kinase activity. CPE significantly attenuated the activity of the IR-active protein by $\sim 80\%$ (Figure 5b). To examine whether the CPE-mediated reductions in IR kinase activity occurs through a physical interaction between CPE and an IR protein, a pull-down assay was conducted. The IR-active protein bound to CPE–Sepharose 4B beads (Figure 5c, lane 3), but not to control Sepharose 4B beads (Figure 5c, lane 2). These results collectively suggest that CPE binds directly with IR to inhibit its kinase activity.

CPE ameliorates obesity, but does not regulate hyperglycemia in obese mice fed a HFD

To further investigate anti-obesity effects of CPE, mice were fed a HFD with or without CPE for 5 weeks. Photographic data showed that the administration of CPE causes less obese phenotype, which might be associated with decreased fat accumulation (Figure 6a). We found that HFD induced greater obesity ($30.60 \pm 0.65 \text{ g}$) compared with control mice ($24.52 \pm 0.41 \text{ g}$; $P < 0.05$). The average body weight of HFD-fed mice was about 19.9% higher than that of control mice (Figure 6b). Administration of CPE at 40 and 200 mg kg$^{-1}$ BW significantly reduced HFD-induced body weight gain by 5.8% and 12.6%, respectively ($P < 0.05$).

There were no significant differences ($P > 0.05$) in daily food intake (g per day) between HFD-fed mice supplemented with CPE at 0 mg kg$^{-1}$ BW ($2.301 \pm 0.003$) and 40 mg kg$^{-1}$ BW
Daily calorie intake was not different ($P > 0.05$) between HFD-fed controls and mice fed a HFD with CPE at $40 \text{ mg kg}^{-1} \text{BW}$ (Figure 6c left panel). These data suggest that the decreased body weight gain by low levels of CPE was not due to a decreased food or calorie intake. Treatment with high dose of CPE ($200 \text{ mg kg}^{-1} \text{ BW}$), however, significantly reduced food and calorie intakes compared with obese controls ($P < 0.05$). The food efficiency ratio (FER) was calculated by dividing weight gain by food intake, and the values were normalized against the group receiving a normal diet. FER of the HFD-fed group was $3.5$ times higher than that of control group, and greater FER was significantly decreased by CPE treatment by up to $27\%$ ($P < 0.05$; Figure 6c right panel). In addition, CPE significantly reduced epididymal fat and liver masses in HFD-fed mice ($P < 0.05$; Figure 6d). Taken together, CPE attenuated HFD-induced body weight gain and lipid accumulation, which was likely associated with decreased food efficiency by CPE.

Serum glucose, triglyceride and total cholesterol levels were measured using standardized assay kits. Although either level of CPE did not alter increased fasting glucose levels following HFD feeding (Figure 6e left panel), higher levels of CPE ($200 \text{ mg kg}^{-1} \text{ BW}$) significantly ($P < 0.05$) attenuated circulating triglyceride levels in HFD-fed mice (Figure 6e middle panel). Total cholesterol levels were affected by neither diet nor CPE treatment (Figure 6e right panel).

Figure 3. Effects of CPE on MDI-induced cell cycle progression in 3T3-L1 preadipocytes. (a) CPE inhibited MDI-induced cell cycle progression. The data are representative of three independent experiments that give similar results. (b) The population of cells in each stage of the cell cycle was quantified.
CPE inhibited adipogenesis in 3T3-L1 preadipocytes and body weight gain in obese mice fed with a HFD. CPE reduced the expressions of various adipogenesis-related proteins and/or genes and inhibited MCE during the differentiation of 3T3-L1 preadipocytes. This was likely associated with the repression of MDI-induced cell cycle progression. CPE also suppressed ERK and Akt-mediated signaling cascades, which trigger proliferation of preadipocytes, and this inhibitory effect was attributable to the inhibition of IR kinase by direct binding of CPE to active IR. In vitro study was further supported by the in vivo evidence indicating that CPE reduces body weight gain in HFD-fed mice.

Figure 4. Effects of CPE on MDI-induced ERK and Akt signaling pathways of 3T3-L1 preadipocytes. (a) CPE downregulated MDI-induced ERK signaling pathway. (b) CPE repressed the MDI-induced Akt signaling pathway. The data are representative of three independent experiments that give similar results.

Figure 5. Effects of CPE on IR kinase activity. (a) Autophosphorylation of IR was not inhibited by CPE. CPE did not alter total tyrosine phosphorylation (PY99) and phosphorylation of the activation site of IR (Tyr1146). (b) CPE inhibited active IR kinase activity. The result was determined from three independent experiments. The data are presented as a percent of control, expressed as means ± s.d. Significant difference between the blank and the active IR kinase (**P < 0.01). Significant differences between the active kinase incubated without and with CPE (***P < 0.05). (c) CPE bound directly to IR. Lane 1, active IR (50 ng); lane 2, negative control; lane 3, active IR bound to CPE-Sepharose 4B beads.

Decreased body weight gain was likely due to the decreased epididymal fat and liver masses in mice fed with a HFD. Interestingly, our serum data indicated that CPE does not alter HFD-induced increased fasting glucose levels.

Insulin signaling in adipose tissue has a pivotal role in lipid storage as well as glucose homeostasis. In the presence of insulin, the IR autophosphorylates and subsequently phosphorylates IRS family, thereby activating two main signaling pathways, including Ras-MAPK and PI3K-Akt pathways. Ras functions as a molecular switch, stimulating a serine kinase cascade through the stepwise activation of Raf kinase, MEK and ERK. Activated ERK translocates into the nucleus, initiating MCE in preadipocytes. Tyrosine phosphorylation of IRS-1 also induces phosphorylation of Akt, activated by binding between the regulatory subunit of PI3K and phosphotyrosine residues on IRS-1. Consequently, the PI3K-Akt signaling pathway participates in initiating MCE through complex signaling cascades. Therefore, the inhibitory effect of CPE on IR kinase activity and its downstream ERK and Akt signaling pathways appears to mediate its anti-adipogenic effect.

Meanwhile, PI3K-Akt pathway is also responsible for a wide range of metabolic actions. Thus, blocking IR kinase activity...
could be detrimental to health. Previous studies have reported that mutations in the IR gene may contribute to insulin resistance in a subpopulation with non-insulin-dependent diabetes mellitus. Furthermore, IR tyrosine kinase activity is defective in the skeletal muscle of obese and diabetic humans, and liver-specific IR knockout mice exhibit severe insulin resistance and...
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Another study is the first attempt to examine the anti-obesity effect of CPE in obese mice fed with an HFD. Currently, the mechanisms by which CPE protects against HFD-induced obesity and type 2 diabetes have not been clearly understood. One of the possibilities to explain anti-obesity activity of cocoa polyphenols is its inhibition of lipid digestion and absorption. Recent study indicates that the inhibition of pancreatic lipase activity is one of the targets to prevent obesity via attenuating fat digestion. It is probable that the decreased body fat and mass by CPE may be associated partly with decreased lipid digestion and/or absorption.

In summary, this study provides the first evidence that CPE inhibits early stage of adipogenesis in 3T3-L1 by reducing IR kinase activity via direct binding. CPE also exhibits anti-obesity activity in diet-induced obese mice, which is attributable to decreased adipose tissue mass. CPE unaltered HFD-induced fasting glucose levels following HFD treatment, which may be associated with different insulin signaling responses between tissues to CPE. This suggests a potential of CPE to prevent obesity without causing a notable deterioration of glucose metabolism by targeting IR in tissue-specific manner.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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REFERENCES
10 Ptasny D, Park B, Davis K, Farmer S. Activation of MEK/ERK signaling promotes adipogenesis by enhancing peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPβ expression during the differentiation of 3T3-L1 preadipocytes. J Biol Chem 2002; 277: 46226.

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glucose intolerance.37 In this regard, CPE that acts as an IR kinase inhibitor can possibly trigger insulin resistance. However, we could not determine whether CPE improves insulin resistance in the present study as we only measured serum glucose levels, which cannot be used as a sole marker of insulin resistance. Despite the difficulties to define insulin resistance, we observed that CPE does not affect HFD-induced fasting hyperglycemia in obese mice. This is seemingly inconsistent with existing evidence suggesting that polyphenol rich cocoa has anti-diabetic activity in healthy subjects18 as well as in streptozotocin-induced diabetic rats.33

The discrepancy between our results and existing evidence can be firstly explained by tissue-specific effect of CPE on insulin action. Our data showed that CPE tends to attenuate HFD-mediated increased ERK phosphorylation in epididymal fat pad (Supplementary Figure 2). Although no dose-dependent trend of CPE was observed in epididymal fat, the protein expression levels of Akt phosphorylation were likely decreased in the liver and muscle. Assuming that CPE affects Akt-mediated insulin signaling cascades in several tissues of obese mice, its tissue-dependent effects may cause various metabolic consequences as IR functions differ between tissues. Although adipose tissue-specific knockout of IR prevents obesity and obesity-related glucose intolerance,21 liver-specific deletion of IRS-2 does not impair hepatic glucose and lipid metabolism in mice, and muscle-specific knockout of IR does not induce insulin resistance, but produces features of metabolic syndrome.36 Taken together, CPE regulates insulin signaling in tissue-specific manner, which may cause compensatory mechanism, leading to unaltered hyperglycemia following HFD feeding. Further studies are required to examine the different metabolic effects of CPE between the tissues using accurate molecular and physiological biomarkers of insulin resistance.

Phosphorylation of IRS in serine resides in response to insulin or other stimuli (for example, cytokines and fatty acids) causes insulin resistance and diabetes.32 ERK, p70S6K and c-jun N-terminal kinase (JNK), which are downstream signaling of IRS, increase serine phosphorylation of IRS via a negative feedback mechanism.41–43 Our data showed that CPE suppresses phosphorylation of JNK (Supplementary Figure 1) as well as ERK and p70S6K in 3T3-L1 preadipocytes. The negative feedback loop between IRS-serine phosphorylation and JNK stimulated by CPE possibly contribute to attenuate adipogenesis and obesity without triggering insulin resistance.

Finally, although activation of IR by insulin is a major inducer of adipogenesis, insulin-like growth factor (IGF)-1 and IGF-1 receptor (IGF1R) are other critical factors to initiate insulin signaling by activating Akt-mediated signaling cascades.44 Thus, it may be possible that CPE interacts with IGF1R, leading to the inhibition of MCE without diminishing insulin action. Further studies are required to investigate whether CPE protects against the development of insulin resistance by increasing IGF1R activity, regardless of the inhibitory effect of CPE on IR.

Our study is the first attempt to examine the anti-obesity effect of CPE in obese mice fed with an HFD. Currently, the mechanisms by which CPE protects against HFD-induced obesity and type 2 diabetes have not been clearly understood. One of the possibilities to explain anti-obesity activity of cocoa polyphenols is its inhibition of lipid digestion and absorption. Recent study indicates that the inhibition of pancreatic lipase activity is one of the targets to prevent obesity via attenuating fat digestion.45 In fact, cocoa polyphenols have been reported to inhibit pancreatic lipase activity.46 Cocoa procyanidins also have shown to inhibit fat digestion and absorption by reducing the activities of enzymes, including α-amylase, pancreatic lipase and secreted phospholipase A2.47 Thus, it is probable that the decreased body fat and mass by CPE may be associated partly with decreased lipid digestion and/or absorption.

In summary, this study provides the first evidence that CPE inhibits early stage of adipogenesis in 3T3-L1 by reducing IR


Supplementary Information accompanies the paper on International Journal of Obesity website (http://www.nature.com/ijo)