Theobromine suppresses adipogenesis through enhancement of CCAAT-enhancer-binding protein β degradation by adenosine receptor A1

Takakazu Mitani [a], Shun Watanabe [b], Yasukiyo Yoshioka [c], Shigeru Katayama [b], Soichiro Nakamura [b], and Hitoshi Ashida [d*]

[a] Department of Interdisciplinary Genome Sciences and Cell Metabolism, Institute for Biomedical Sciences, Interdisciplinary Cluster for Cutting Edge Research, Shinshu University, 8304 Minamiminowa, Kamiina, Nagano, Japan

[b] Department of Bioscience and Biotechnology, Shinshu University, 8304 Minamiminowa, Kamiina, Nagano, Japan

[c] Organization of Advanced Science and Technology, Kobe University, Nada-ku, Kobe, Japan

[d] Department of Agrobioscience, Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 6578501, Japan

*Correspondence: Hitoshi Ashida, Ph.D., Department of Agrobioscience, Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan Tel.: +81-78-803-5878; Fax: +81-78-803-5878

E-mail: ashida@kobe-u.ac.jp

Abbreviations: **AC**, adenylate cyclase; **AR**, adenosine receptor; **CCPA**, 2-Chloro-N^6-cyclopentyladenosine; **C/EBP**, CCAAT/enhancer-binding-protein; **CRE**,

cAMP response elements; **CREB**, cAMP response element-binding protein; **FAS**, fatty acid synthase; **IBMX**, isobutylmethylxanthine; **PPAR**, peroxisome proliferator-activated receptor; **qPCR**, quantitative real-time PCR; **SENP2**, SUMO-specific protease 2; **SUMO**, small ubiquitin-like modifier.

Abstract

Theobromine, a methylxanthine derived from cacao beans, reportedly has various health-promoting properties but molecular mechanism by which effects of theobromine on adipocyte differentiation and adipogenesis remains unclear. In this study, we aimed to clarify the molecular mechanisms of the anti-adipogenic effect of theobromine in vitro and in vivo. ICR mice (4 week-old) were administered with theobromine (0.1 g/kg) for 7 days. Theobromine administration attenuated gains in body and epididymal adipose tissue weights in mice and suppressed expression of adipogenic-associated genes in mouse adipose tissue. In 3T3-L1 preadipocytes, theobromine caused degradation of C/EBPβ protein by the ubiquitin-proteasome pathway. Pull down assay showed that theobromine selectively interacts with adenosine receptor A1 (AR1), and AR1 knockdown inhibited theobromine-induced C/EBPβ degradation. Theobromine increased sumoylation of C/EBPβ at Lys133. Expression of the small ubiquitin-like modifier (SUMO)-specific protease 2 (SENP2) gene, coding for a desumoylation enzyme, was suppressed by theobromine. In vivo knockdown studies showed that AR1 knockdown in mice attenuated the anti-adipogenic effects of theobromine in younger mice. Theobromine suppresses adipocyte differentiation and induced C/EBPβ degradation by increasing its sumoylation. Furthermore, the inhibition of AR1 signaling is important for theobromine-induced C/EBPβ degradation.

Keywords: adenosine receptor; adipogenesis; CCAAT/enhancer-binding protein; sumoylation; theobromine

1. Introduction

Obesity is a serious health problem in both developed and developing countries and increases the incidence of many diseases, such as cardiovascular disease, hypertension and type 2 diabetes mellitus [1, 2]. The excessive numbers of adipocyte cells and increased adipocyte cell size in adipose tissues contribute to induction of obesity [3]. Adipocytes are differentiated from mesenchymal stem cells in adipose tissue. This adipocyte differentiation process has two phases, commitment and terminal differentiation. Mesenchymal stem cells are converted to committed preadipocytes, and then differentiated into mature adipocytes [4]. Therefore, suppression of adipocyte differentiation would be an effective strategy to prevent and treat obesity.

Adipocyte differentiation and adipogenesis are regulated by transcriptional factors including CCAAT/enhancer-binding protein (C/EBP) family members (C/EBPα, C/EBPβ and C/EBPδ) and peroxisome proliferator-activated receptor (PPAR) [5]. C/EBPβ and C/EBPδ can induce expression of PPAR and C/EBPα [6, 7]. PPARγ and C/EBPα can increase expression of such target genes as fatty acid synthase (FAS) and perilipin. The expression levels of C/EBPβ are significantly increased by the differentiation inducer isobutylmethylxanthine (IBMX). In early adipocyte differentiation, cAMP was reported to induce C/EBPβ expression through transcriptional factor cAMP response element-binding protein (CREB) [8]. The protein stability and transcriptional activity of C/EBPβ are regulated by post-translational modifications [9-10]. In particular, sumoylation was shown to decrease protein stability of C/EBPβ by promoting its degradation via the ubiquitin-proteasome pathway [11].

Adenosine acts as an endogenous ligand for adenosine receptors (ARs) in the

plasma membrane and is constitutively released from adipose tissues [12]. ARs are G protein-coupled receptors and AR signaling is an important regulator of proliferation and differentiation in mesenchymal stem cells [13]. ARs exist as four subtypes, AR1, AR2a, AR2b and AR3, and agonist-bound ARs regulate adenylyl cyclase activity, resulting in increased or decreased cAMP synthesis from ATP [14]. Exogenous ligands of AR either positively or negatively regulated adenylate cyclase activity in various cell types [15]. AR signaling has been implicated in development of many diseases, including type 2 diabetes, cardiovascular and nervous system disorders [16-18] and is, therefore, a potential therapeutic target for lifestyle-related diseases.

Theobromine is a methylxanthine found in cacao beans. Cacao beans, a very popular food worldwide, contain approximately 1% theobromine [19]. Theobromine has been implicated in the health benefits of cacao intake. For instance, theobromine intake increased apolipoprotein A-1 and HDL-cholesterol blood levels in humans [20] and suppressed diabetic kidney disease in rats [21]. However, the molecular mechanisms by which the effect of theobromine on adipocyte differentiation remain unclear. In this study, we report that theobromine promotes degradation of C/EBPβ through the inhibition of AR1 signaling, resulting in the attenuation of adipocyte differentiation in 3T3-L1. Furthermore, administration of theobromine suppresses adipose tissue weight gain in younger mice, whereas knockdown of AR1 canceled theobromine-suppressed adipose tissue weight gain.

2. Materials and Methods

2.1. Cell culture

Murine 3T3-L1 preadipocytes were cultured in DMEM supplemented with 10% bovine serum, 100 μg/ml streptomycin and 100 units/ml penicillin. The cells were maintained at 37°C in a 5% CO_2/95% air atmosphere at 98% humidity. Adipocyte differentiation was induced by treating confluent cells for the first 2 d with DMI cocktail (10 μg/ml insulin, 1 μM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX)) in DMEM with high glucose (4.5 g/l glucose), supplemented with 10% serum bovine serum and the same antibiotics. After the first 2 days, cells were induced to differentiate by adding 10 μg/ml insulin to the same medium.

2.2. Animal experiments

All animal experiments conformed to the protocols approved by the Institutional Animal Care and Use Committees and performed according to the Kobe University and Shinshu University animal experimentation regulations (Permission Nos. 26-03-03 and 280043, respectively) and the Guide for Care and Use of Laboratory animals (NIH Publications No. 8023, revised 1978). Male ICR mice were from Japan SLC (Shizuoka, Japan). Mice were housed under controlled conditions (temperature 23±2°C, alternating light-dark cycles with 12 h of light and 12 h of darkness) and had free access to food and water. The mice (4 week-old) were randomly divided into theobromine and vehicle groups (n = 6 per group). The groups received theobromine (0.1 g/kg) or vehicle alone, once daily by oral gavage, for 7 days.

For the *in vivo* siRNA experiment, we used a previously described method [22]. Mice were randomly divided into two groups (n = 5 per group). In one group, mice received

siRNA of control of AR1 (10 μM), transfected to the right and left epididymal adipose tissue, respectively, using Atelocollagen (AteloGene Local Use; Koken, Tokyo, Japan). Mice then received theobromine (0.1 g/kg) daily by oral gavage for 7 days. In the other group, siCont was transfected to the right epididymal adipose tissue and the left epididymal adipose tissue was sham-operated. These mice received vehicle by oral gavage daily for 7 days. At the end of the experiment, the mice were sacrificed under pentobarbital anesthesia and the liver and adipose tissue were harvested.

2.3. Primary preadipocyte cultures

Primary preadipocyte cultures were generated as previously described [23], with minor modifications. Briefly, primary preadipocytes were isolated from epididymal adipose tissue of male mice (ICR; 4 week-old). After digestion with collagenase II and centrifugation, preadipocytes were cultured in DMEM. When preadipocytes were fully confluent, they were treated with 0.5 mM IBMX for 8 h.

2.4. siRNA

The sequences for siRNA duplexes: siAR1#1 5′-CUCCUUGGGUGUGAAUAUUGA-3′ (Sigma-Aldrich, Saint Louis, MO). siAR1#2 and control siRNA were from Dharmacon (Chicago, IL) and Sigma-Aldrich, respectively. The duplexes (20 nM) were transiently transfected into 3T3-L1 preadipocytes using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA) and Opti-MEM (Life Technologies, Inc., Gaithersburg, MD) for 24 h, following the manufacturers' protocols.

2.5. Plasmids

Mouse C/EBP cDNA was amplified by nested PCR in two sequential steps and subcloned using Kpn I and Xba I sites in p3xFLAG-CMV (Sigma-Aldrich), yielding a C/EBPβ expression vector with three tandem N-terminal FLAG tags (p3×FLAG-C/EBPβ). The C/EBPβ(K133R) cDNA encoding a C/EBPβ mutant with an Arg for Lys substitution at position 133 was generated using C/EBPβ cDNA as a template. The N-terminal FLAG-tagged C/EBPβ(K133R) expression vector was constructed, and is termed p3×FLAG-C/EBPβ(K133R). Luciferase reporter vector pGL4.14-SENP2 (Wild-type) was constructed by introducing the promoter region (nucleotide sequence: -240 to -209) of mouse *Senp2* gene with CRE in pGL4.14 vector (Promega Corp., Madison, WI), and mutation of the *Senp2* promoter region of the vector pGL4.14-SENP2(Mutant form) was constructed according to Chung *et al* [24]. The CREB-responsive reporter vector (p6xCRE-Luc) was constructed by insertion of six tandem repeats of CREs in pGL4.14 vector (Promega Corp., Madison, WI). The insertion sequence of the CRE oligonucleotide is: 5′-CTGACGTCAACGGTGACGTCAACGGTGACGTCAGCTAGCTGACGTCAAC GGTGACGTCAACGGTGACGTCAA-3′.

2.6. Staining of intracellular lipid droplets

3T3-L1 adipocytes were induced toward adipocyte differentiation by treatment with DMI cocktail for 6 days. The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized and incubated with Sudan II for 1 h at

room temperature. After staining, cells were washed and the lipid droplets extracted with isopropanol containing 4% (v/v) Nonidet P-40. The extracted dye was measured in a Wallac spectrophotometer (ARVO, Perkin-Elmer Life Sciences, Boston, MA), at an absorbance of 490 nm.

2.7. Western blotting analysis

3T3-L1 preadipocytes were induced toward adipocyte differentiation by IBMX or DMI cocktail, in the presence or absence of theobromine, for 8 h or 6 days, respectively. For detection of exogenous C/EBPβ, 3T3-L1 preadipocytes were transiently transfected with FLAG-C/EBPβ or FLAG-C/EBPβ(K133R) expression vectors using Lipofectamine 2000 (Invitrogen) for 24 h, followed by incubation, with or without IBMX, for 8 h. The cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4 containing 150 mM NaCl, 0.5% Nonidet-P40, 10 mM sodium pyrophosphate, 2 mM EDTA, 1 mM phenylmethylsulfonylfluoride and 10 μg/ml leupeptin). The cell lysates were subjected to SDS-PAGE and analyzed by western blotting using the following rabbit polyclonal antibodies: anti-PPARγ (H-100), anti-C/EBPβ (C-19), anti-CREB (240; Santa Cruz Biotechnology, Santa Cruz, CA), anti-fatty acid synthesis (C20G5), anti-perilipin (D1D8), anti-ubiquitin (Cell Signaling Technology, Inc. Danvers, MA), anti-AR1 (Sigma-Aldrich), anti-SENP2 or anti-AR2a (Abcam, Cambridge, MA); other antibodies used were goat polyclonal anti-C/EBPα (N-19, Santa Cruz Biotechnology) and mouse monoclonal anti-β-actin (clone; C4) and anti-SUMO-1 (clone; D-11; Santa Cruz Biotechnology) antibodies. After incubation with primary antibodies, blots were washed and incubated with horseradish peroxidase-conjugated secondary antibodies and reacted

with Immunostar LD (Wako, Osaka, Japan).

2.8. Quantitative real-time PCR (qPCR)

Total RNA was extracted from 3T3-L1 preadipocytes using TRIzol (Invitrogen). cDNAs were synthesized using RevaTra Ace (TRT-101; Toyobo, Osaka, Japan) and subjected to qPCR using the primers (see Suppl. Table S1). qPCR was performed with SYBR PremixEx Taq II (Takara Bio., Shiga, Japan) and a two-step PCR method on a Thermal Cycler Dice real-time system (Takara Bio.). The relative mRNA levels for each gene were calculated using the $2^{-\Delta\Delta Ct}$ method [25, 26] and data were normalized to values for *Gapdh* as an endogenous control.

2.9. Reporter assay

3T3-L1 preadipocytes were transiently transfected with reporter vectors [pGL4.14-SENP2(Wild-type), pGL4.14-SENP2(Mutant form) or pCRE-Luc, and pRL-SV40 (control reporter vector; Promega)] using Lipofectamine 2000 for 24 h. After the medium was replaced with fresh medium, the cells were incubated with IBMX and theobromine (25 μM) for 8 h. Transfection efficiency was normalized to that of pRL-SV40. Firefly and Renilla luciferase activities were measured using the Dual Luciferase reporter assay kit and GloMax 20/20 Luminometer (Promega). Data are expressed as relative light units (RLU; firefly levels divided by Renilla levels).

2.10. Analysis of intracellular cAMP

3T3-L1 preadipocytes were incubated with IBMX cocktail in the presence or absence

of theobromine (25 μM) for 8 h. The cells were homogenized in 0.4 M perchloric acid using sonication, followed by centrifugation at 20,000 × g for 10 min. The supernatant was neutralized with 1 M acetic acid buffer, pH 8.0. The pellet was re-dissolved in 1 M acetic acid buffer, pH 8.0, by sonication and the protein content of each sample determined with the Bradford assay. Intracellular cAMP was analyzed as previously described [27] with slight modifications. Briefly, the analysis was performed with a triple quadrupole mass spectrometer (LCMS-8040, Shimadzu Corp., Kyoto, Japan). Chromatographic separation was performed using L-column2 ODS (1.5 × 150 mm; Chemical Evaluation and Research Institute, Tokyo, Japan). Mobile phase A was 0.1% formic acid in water and mobile phase B was 100% acetonitrile. The following gradient elution profile was applied at a flow rate of 0.2 ml/min: 0.00 min, 0% B; 1.00 min, 0% B; 2.00 min, 90% B; 4.40 min, 90% B; 4.60 min, 100% B; 9.60 min, 100% B; 9.70 min, 0% B; 14.70 min, 0% B.

2.11. Immunoprecipitation

3T3-L1 preadipocytes were pre-incubated with MG132 (10 μM) for 30 min, followed by incubation with IBMX in the presence or absence of theobromine (25 μM) for 8 h. The cells were lysed in denaturing cell extraction buffer (50 mM Tris-HCl, pH 7.5, containing 70 mM β-mercaptoethanol and 2% SDS) at 95°C for 10 min. The cell lysates were diluted 20-fold with dilution buffer (20mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% TritonX-100, 2.5 mM sodium pyrophosphate and protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan)) and centrifuged at 20,000 × g for 30 s. The supernatant was incubated with rabbit polyclonal anti-C/EBPβ IgG,

anti-FLAG IgG or control IgG at 4°C overnight, followed by incubation with 30 μl protein G-Sepharose resin (50% slurry; GE Healthcare, Waukesha, WI) at 4°C for 1 h. The resin was washed with lysis buffer three times and proteins bound to the resin were separated by SDS-PAGE and analyzed by western blotting.

2.12. Competition-binding assay

First, CNBr-activated Sepharose 4B resin (0.2 g freeze-dried powder; GE Healthcare) was activated by 1 mM HCl and incubated with adenosine (3 μmol) in coupling buffer (0.1 $NaHCO_3$ and 0.5 M NaCl) at room temperature for 2 h. The Sepharose resin was then washed three times with coupling buffer and incubated with 0.1 M Tris-HCl, pH 8.0, at room temperature for 2 h, followed by washing with coupling buffer. This resin was termed adenosine-affinity resin. Next, 3T3-L1 preadipocytes were lysed in lysis buffer, using sonication, and centrifuged at 20,000 × g at 4°C for 10 min. The supernatant (1 mg protein) was incubated with adenosine-affinity resin (100 μl; 50% slurry) in the presence or absence of adenosine (1 mM) or theobromine (1 mM) at 4°C for 1 h. The resin was washed with lysis buffer three times and bound proteins separated by SDS-PAGE and analyzed by western blotting.

2.13. Histology of adipose tissues

Epididymal adipose tissues were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Paraffin sections, 10 μm thick, were dewaxed and stained with hematoxylin and eosin (H&E) to assess morphology. Tissue sections were imaged with a FSX100 digital microscopy (Olympus Optical Co. Ltd, Tokyo, Japan). The mean area

of adipocytes was calculated from three epididymal adipose tissue samples per group and 300 cells per mouse, using the measurement tool in ImageJ software (National Institutes of Health, Bethesda, MD).

2.14. Immunofluorescent microscopy

3T3-L1 preadipocytes were cultured in DMEM on round coverglasses. The confluent cells were incubated, with or without 25 μM theobromine, in the presence of IBMX for 8 h. The cells were fixed in 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS. Cells were treated with blocking solution containing 10% fetal bovine serum, 5% BSA and 0.1% sodium azide in PBS at room temperature for 1 h. Cell samples were incubated with primary antibodies (anti-SUMO-1 and anti-C/EBPβ) in PBS containing 3% BSA at 4°C overnight, followed by incubation with Alexa Fluor 488-conjugated secondary anti-rabbit IgG or Alexa Fluor 594-conjugated secondary anti-mouse IgG in PBS containing 3% BSA at room temperature for 1 h. Nuclei were stained with DAPI (1 μg/ml) at room temperature for 10 min, then visualized using a FSX100 fluorescence microscope.

2.15. ChIP

3T3-L1 preadipocytes were incubated with IBMX cocktail in the presence or absence of theobromine (25 μM) for 8 h. For cultured cells and tissues, ChIP assays were performed as described previously [28]. Briefly, cell lysates and tissue homogenates were incubated with rabbit polyclonal anti-CREB IgG or control rabbit IgG at 4°C overnight, followed by incubation with 30 μl protein G-Sepharose resin (50% slurry) at

4°C for an additional 1 h. Immunoprecipitated protein-DNA complexes were washed and eluted at 65°C for 6 h. The promoter region of the SENP2 gene was amplified by qPCR using the following primer set: forward primer 5′-CCTGTTGCTAGGCTTACAAGGAGC-3′ and reverse primer 5′-CTTCAGCCGTAGCCAGGATCAG-3′ [24]. The qPCR profiles consisted of the following program: 94ºC for 30 s, 65ºC for 15 s and 72ºC for 20 s. The relative amounts of each promoter region were calculated using the $2^{-\Delta\Delta Ct}$ method [25, 26], and data were normalized to values obtained for the input sample.

2.16. Statistical Analysis

Data were analyzed by Student's *t* test or by one- or two-way ANOVA with Tukey's post hoc testing. Statistical analysis was performed with JMP statistical software version 11.2.0 (SAS Institute. Cary, NC). Data are expressed as means ± SD and $p<0.05$ was considered statistically significant.

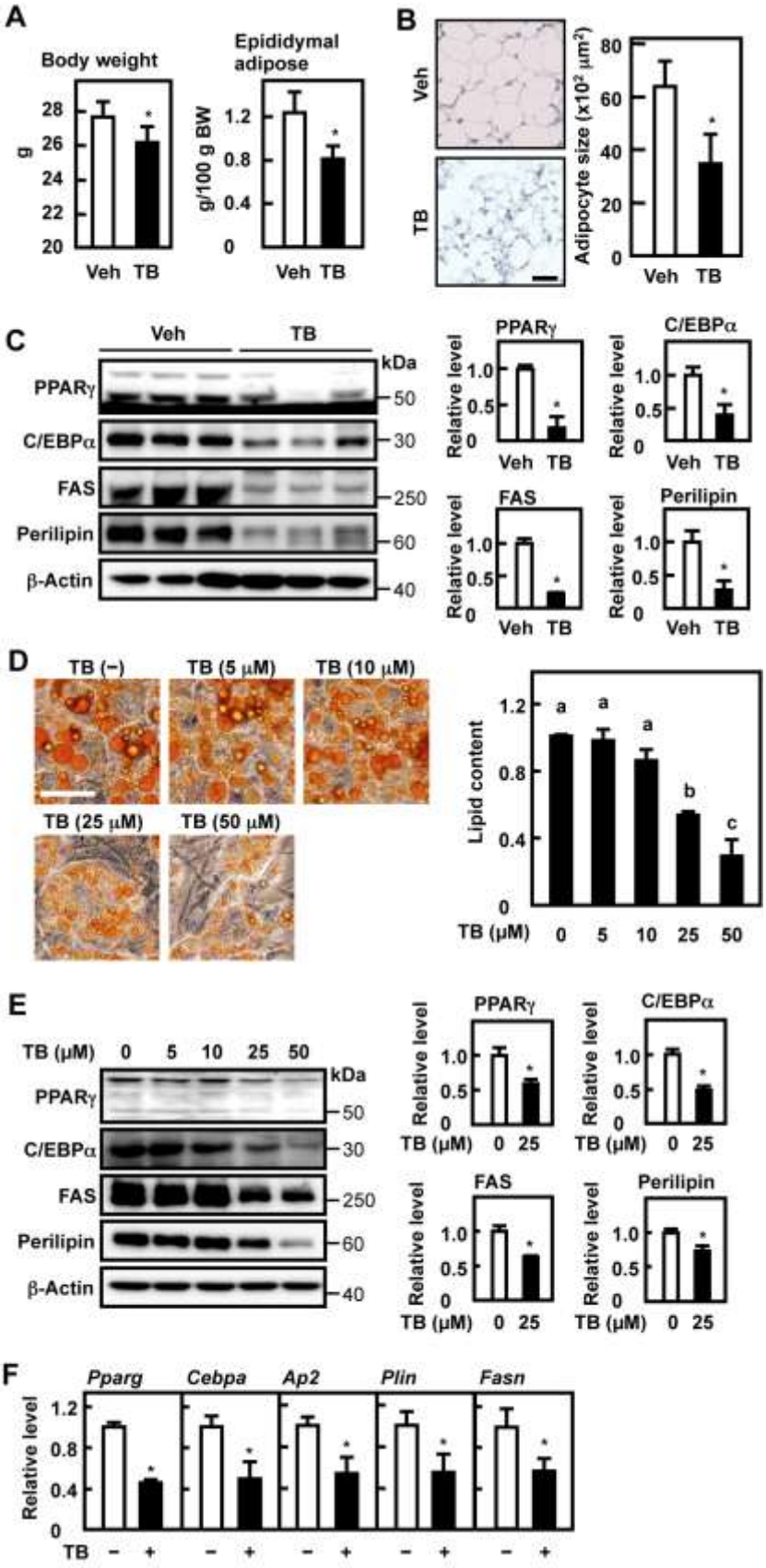
3. Results

3.1. Theobromine suppressed the adipose tissue weight gain and gene expression of adipogenesis-associated gene in younger generation

In adult organisms, adipocyte differentiation, adipogenesis, and lipolysis occur simultaneously in adipose tissues. Thus, we investigated effects of theobromine on and adipose tissue weight gain and adipogenesis using younger mice (4 week-old). The mice were orally administered theobromine (0.1 g/kg) for 7 days. Body weights were lower in the theobromine group than in the vehicle group. In addition, theobromine suppressed

gains in weight of epididymal and perirenal adipose tissues (Fig. 1A and Suppl. Fig. S1). In contrast, the liver weights were no different in the two groups. The mean adipocyte area was smaller in the theobromine group than in the vehicle group (Fig. 1B). Theobromine decreased expression of PPARγ, C/EBPα and adipogenic proteins, such as FAS and perilipin, in epididymal adipose tissue (Fig. 1C).

Next, we investigated effects of theobromine on adipogenesis in 3T3-L1 preadipocytes. Theobromine, at concentrations above 25 μM, decreased lipid accumulation in these cells (Fig. 1D). Cell viability was not affected by theobromine (Suppl. Fig. S2). Theobromine, at concentrations above 25 μM, suppressed protein expression of PPARγ, C/EBPα and adipogenic genes (Fig. 1E). The mRNA levels of these genes were also decreased by theobromine (Fig. 1F). These results indicated that theobromine had anti-adipogenic effects.


A
Body weight
g
28
26
24
22
20
Veh TB
Epididymal adipose
g/100 g BW
1.2
0.8
0.4
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Veh TB
B
Veh
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Adipocyte size (x10² μm²)
80
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250
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Fig. 1. *Inhibitory effects of theobromine on adipogenesis in vivo and in vitro. (A) Body and epididymal adipose tissues weights for mice treated with either vehicle (Veh; white bars) or theobromine (TB; black bars) (n = 6 per group). Tissue weights were normalized to body weights (BW). (B) Hematoxylin and eosin stained paraffin sections of epididymal adipose tissues from mice (n = 3 per group). Scale bar indicates 50 mm (left panel). The data represent mean cross-sectional area/300 cells, in μm^2, of stained paraffin sections from epididymal adipose tissues (right panel). White bar shows vehicle group, and black bar shows TB group. Data are means ± SD (n = 3). * $p<0.05$ vs. Veh. (C) Western blots for PPARγ, C/EBPα, FAS and perilipin proteins in epididymal adipose tissues from mice administered either Veh or TB (n = 3 per group). (D) Lipid contents in 3T3-L1 preadipocytes after induction of differentiation with DMI, in the presence of the indicated concentrations of TB, for 6 days. Lipid droplets in the cells were stained (left panel; scale bar is 50 μm) and quantified (right panel). Data are means ± SD (n = 3). Significant differences ($p<0.05$) are indicated by corresponding letters. (E) Protein expression of PPARγ, C/EBPα, FAS and perilipin in 3T3-L1 preadipocytes after induction of differentiation with DMI, in the presence of the indicated concentrations of TB, for 6 days. (F) qPCR analysis of PPARγ (Pparg), C/EBPα (Cebpa), FAS (Fasn) and perilipin (Plin) gene expression in 3T3-L1 preadipocytes after induction of differentiation with DMI, in the presence (black bars) or absence (white bars) of TB (25 μM) for 6 days. Data were normalized to Gapdh levels. For western blotting, the intensity of each band was quantified by ImageJ 1.44, and the ratio of each band was normalized to the β-actin (a loading control) level. Error bars represent the mean ± SD (n = 3). * $p<0.05$ vs. in the absence of TB. Data shown in (D) to (F) are representative*

of triplicate independent experiments.

3.2. Theobromine promoted degradation of C/EBPβ protein via the ubiquitin-proteasome pathway

To determine the phase of differentiation at which theobromine exerted its anti-adipogenic effects, the compound was administered at different times during adipocyte differentiation, as indicated in the figure (Suppl. Fig. S3A). In groups 2 and 3, theobromine decreased lipid accumulation during adipocyte differentiation, whereas in other groups, it did not (Suppl. Fig. S3B). Furthermore, theobromine suppressed PPARγ and C/EBPα expression in group 2 and 3 (Suppl. Fig. S3C). These results indicated that the inhibitory effects of theobromine on adipogenesis occurred at an early phase of adipocyte differentiation. Theobromine (25 μM) decreased protein levels of C/EBPβ (0.61-fold), a transcription factor active in early phase adipocyte differentiation (Fig. 2A), but not its mRNA (Fig. 2B) in 3T3-L1 preadipocytes. To assess the mechanism of theobromine-induced C/EBPβ degradation, 3T3-L1 preadipocytes were differentiated in the presence of the proteasome inhibitor MG132. MG132 inhibited theobromine-induced degradation of C/EBPβ (Fig. 2C). Immunoprecipitation with anti-C/EBPβ IgG showed that theobromine enhanced the interaction of C/EBPβ with polyubiquitin (Fig. 2D). Furthermore, in a primary culture system, preadipocytes isolated from epididymal adipose tissues and cultured in differentiation medium, we confirmed that theobromine decreased protein expression of C/EBPβ (Fig. 2E). These results indicated that theobromine promoted C/EBPβ degradation via the ubiquitin-proteasome pathway.

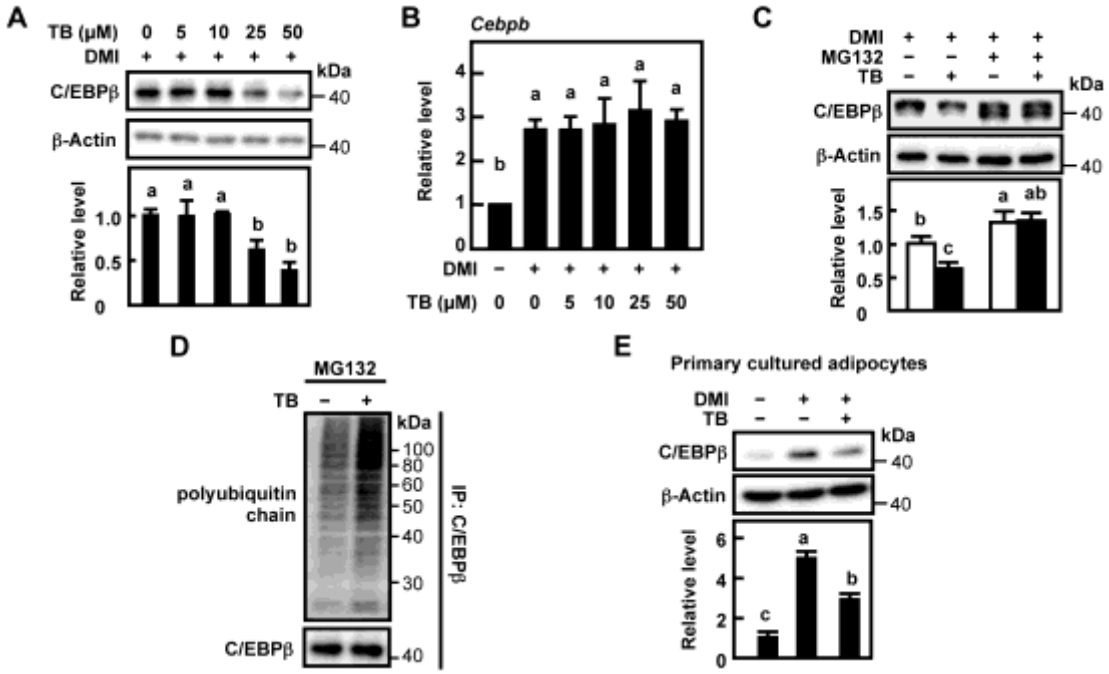


Fig. 2. *C/EBPβ expression patterns in the presence of theobromine. (A) Western blotting analysis of C/EBPβ protein in 3T3-L1 preadipocytes after induction of differentiation with DMI, in the presence of the indicated concentrations of theobromine (TB), for 8 h. (B) qPCR analysis of Cebpb expression in 3T3-L1 preadipocytes after induction of differentiation, in the presence or absence of TB at the indicated concentrations, for 8 h. Data were normalized to Gapdh levels. (C) Protein expression of C/EBPβ in 3T3-L1 preadipocytes after induction of differentiation, in the presence or absence of TB (25 μM) or MG132 (10 μM), for 8 h. (D) Immunoprecipitation (IP) assay using C/EBPβ IgG in 3T3-L1 preadipocytes after induction of differentiation, with or without TB (25 μM), in the presence of MG132 (10 μM). Immunoprecipitated proteins were analyzed by western blotting using C/EBPβ and ubiquitin antibodies. (E) Protein expression of C/EBPβ in primary adipocytes. Primary preadipocytes from mouse epididymal adipose tissue were cultured, with or without DMI and TB (25 μM), in differentiation medium for 8 h. For western blotting, the intensity of each band was*

quantified by ImageJ 1.44, and the ratio of each band was normalized to the β-actin (a loading control) level. All data, error bars represent the mean ± SD (n = 3). Significant differences ($p<0.05$) are indicated by the corresponding letters. Results shown are each representative of triplicate independent experiments.

3.3. AR1 was involved in theobromine-induced C/EBPβ degradation

Certain methylxanthines, such as caffeine and theophylline, are AR ligands [29]. Theobromine-induced C/EBPβ degradation was inhibited by AR1 (Fig. 3A). On the other hand, knockdown of AR2a did not cancel theobromine-induced C/EBPβ degradation (Suppl. Fig. S4). To assess whether theobromine acted as an AR1 ligand in our experiments, we performed competitive binding assays, using adenosine-immobilized Sepharose (adenosine-affinity resin) and theobromine. Free adenosine inhibited interaction of the adenosine-affinity resin with AR1 or AR2a in 3T3-L1 preadipocyte lysates (Fig. 3B). Theobromine blocked interaction of adenosine-affinity resin with AR1, but not with AR2a. We next investigated tissue distribution of the AR1 in mice. The *Adora1* gene (coding for AR1) was highly expressed in the epididymal and mesenteric adipose regions of white adipose tissues (Fig. 3C), indicating that expression of AR1 was tissue-selective. Theobromine did not have anti-adipogenic effects in 3T3-L1 preadipocytes with AR1 knocked down (Fig. 3D). Although insulin and dexamethasone minimally affected C/EBPβ levels, IBMX increased C/EBPβ protein levels and theobromine attenuated this effect (Suppl. Fig. S5). IBMX increased intracellular cAMP levels, leading to induction of adipocyte differentiation [30]. Theobromine suppressed IBMX-induced cAMP accumulation (Fig.

3E). AR1 knockdown prevented the theobromine-induced decrease in intracellular cAMP levels (Fig. 3F). These results indicated that theobromine suppressed adipocyte differentiation by decreasing intracellular cAMP levels, through AR1 stimulation.

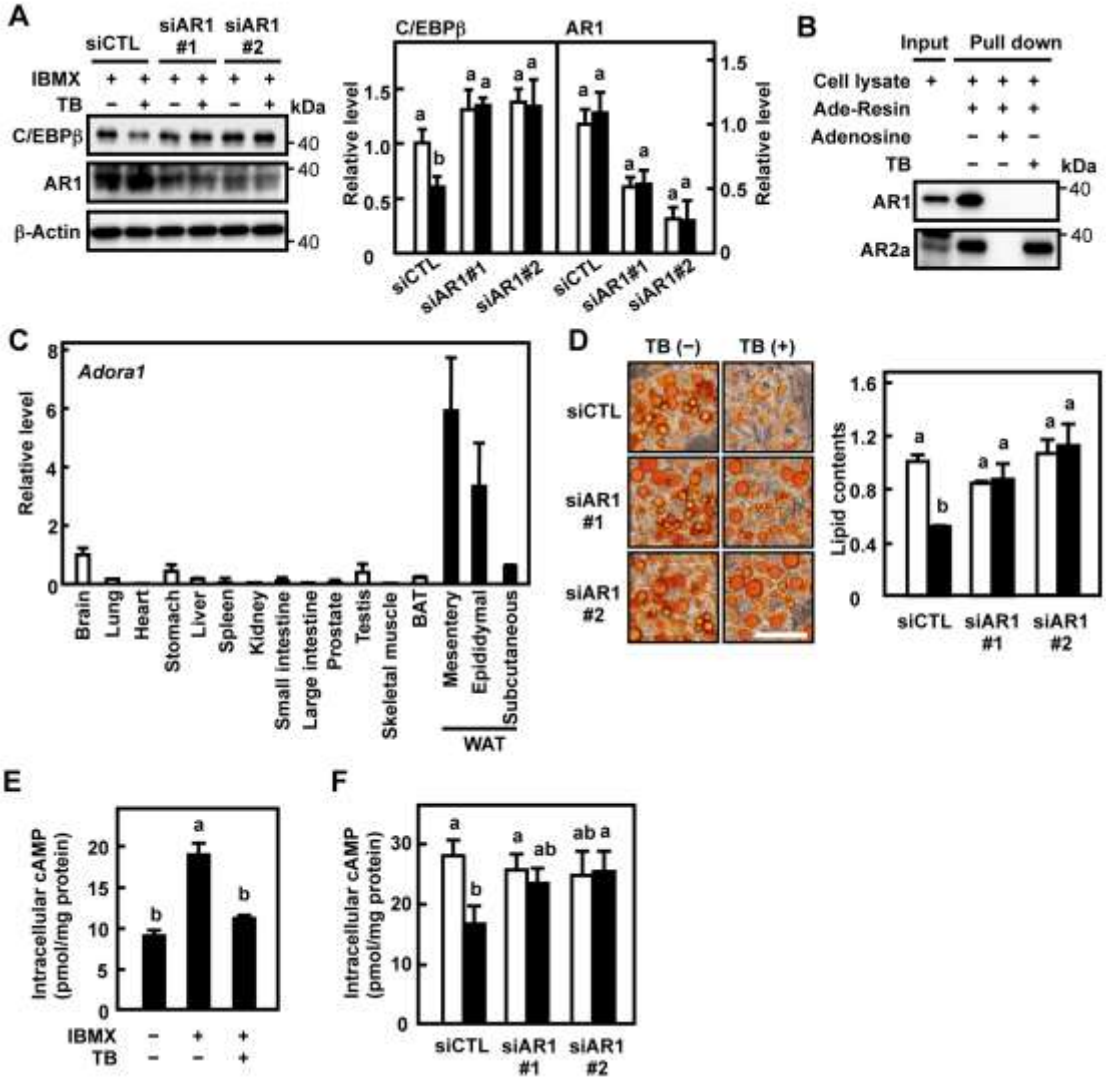


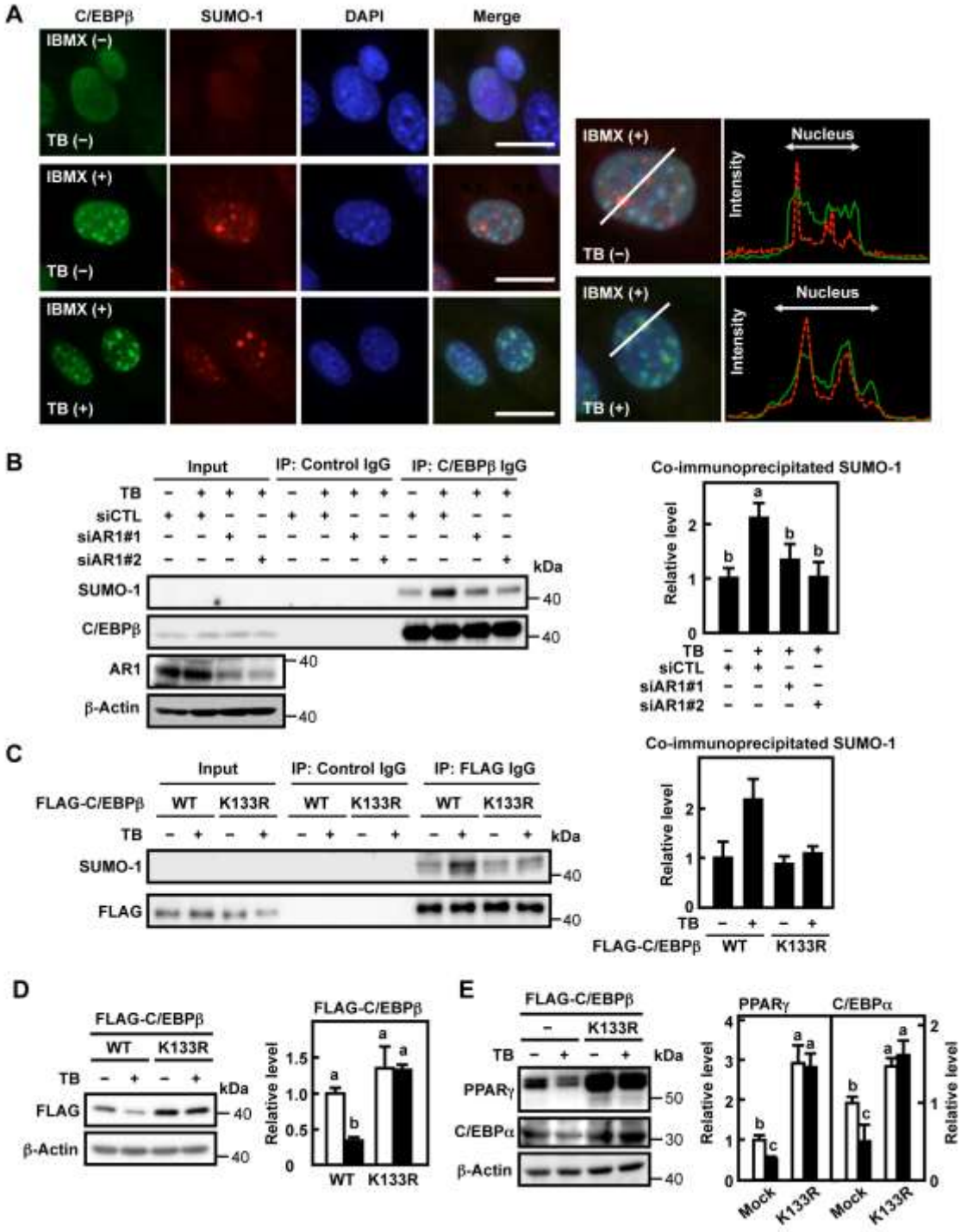
Fig. 3. *Involvement of AR1 in theobromine-induced suppression of adipogenesis. (A) 3T3-L1 preadipocytes were transfected with AR1 siRNA (siAR1#1 or siAR1#2) or control siRNA (siCTL). After transfection with siRNA, the cells were induced to differentiate with IBMX, in the presence or absence of 25 μM theobromine (TB), for 8 h. Cell lysates were analyzed by western blotting with anti-C/EBPβ and anti-AR1 antibodies. The intensity of each band was quantified by ImageJ 1.44, and the ratio of*

each band was normalized to the β-actin (a loading control) level. (B) Pulldown assay of AR1 in 3T3-L1 preadipocytes. The cell lysates were incubated with adenosine-affinity resin, in the presence or absence of adenosine (1 mM) or TB (1 mM), followed by western blotting with anti-AR1 and anti-AR2a antibodies. (C) mRNA expression of Adora1 (coded AR1) in various tissues of mice (n = 4). Adora1 levels were normalized to Gapdh levels in each tissue, and relative levels were based on levels in brain tissue. Black and white bars indicate white adipose tissues (WAT) and other tissues, respectively. BAT is brown adipose tissue. (D) Lipid content in 3T3-L1 preadipocytes transfected with siAR1#1, siAR1#2 or siCTL). After siRNA transfection, cells were induced to differentiate, in the presence (black bars) or absence (white bars) of TB (25 μM), for 6 days. Lipid droplets in the adipocytes were stained (left panel; scale bar is 50 μm) and quantified (right panel). (E) Quantification of intracellular cAMP in 3T3-L1 preadipocytes incubated, in the presence or absence of IBMX or TB (25 μM), for 8 h. (F) Quantification of intracellular cAMP in 3T3-L1 preadipocytes with AR1 knocked down, after induction of differentiation, in the presence (black bars) or absence (white bars) of TB (25 μM), for 8 h. For (A) and (D) to (F), data are means ± SD (n = 3). Significant differences ($p<0.05$) are indicated by the corresponding letters. Results shown are each representative of triplicate independent experiments.

3.4. Theobromine increased C/EBPβ sumoylation through AR1

Co-localization and profile plots were constructed to demonstrate the subcellular distributions of C/EBPβ and SUMO-1. In the absence of theobromine, C/EBPβ and SUMO-1 were mainly localized to the nucleus (Fig. 4A left panels). However, in the

nucleus, these two proteins differed in their subcompartmental localizations (Fig. 4A right panel). In contrast, with theobromine treatment, C/EBPβ and SUMO-1 showed similar subcompartmental localizations in the nucleus. We investigated whether C/EBPβ was sumoylated in the presence of theobromine. Immunoprecipitation results showed that theobromine increased C/EBPβ sumoylation and that this was inhibited by AR1 knockdown (Fig. 4B). To identify the sumoylation site on C/EBPβ, we generated a C/EBPβ mutant with Lys133, the SUMO acceptor site, replaced with Arg (C/EBPβ(K133R)). Unlike the wildtype, this C/EBPβ mutant was not subject to sumoylation induced by theobromine (Fig. 4C). Theobromine did not affect protein levels of C/EBPβ(K133R) (Fig. 4D). Furthermore, when C/EBPβ(K133R)-overexpressed 3T3-L1 preadipocytes were differentiated for 6 day, protein level of endogenous PPARγ and C/EBPα was increased even in the presence of theobromine (Fig. 4E). These results indicated that theobromine decreased protein levels of C/EBPβ through increasing its sumoylation at Lys133.



1 ***Fig. 4.*** *Inhibition by theobromine of C/EBPβ sumoylation. (A) Immunofluorescence*
2 *analysis of C/EBPβ (green) and SUMO-1 (red) in 3T3-L1 preadipocytes incubated, in*
3 *the presence or absence of IBMX or 25 μM theobromine (TB), for 8 h. Nuclei were*
4 *stained with DAPI (blue) (left panels). Profile plots were generated to demonstrate*
5 *intracellular distributions of C/EBPβ (green lines) and SUMO-1 (red dashed lines)*

fluorescence (right panels). Scale bar indicates 100 μm. (B) Immunoprecipitation assay (IP) using control IgG or anti-C/EBPβ IgG in 3T3-L1 preadipocytes transfected with AR1 siRNA (siAR1#1 or siAR1#2) or control siRNA (siCTL), after induction of differentiation with IBMX and MG132 (10 μM), in the presence or absence of TB (25 μM), for 8 h. Co-immunoprecipitated proteins were analyzed by western blotting using C/EBPβ and SUMO-1 antibodies. (C) Sumoylation of C/EBPβ in 3T3-L1 preadipocytes. FLAG-C/EBPβ wild-type (WT) or FLAG-C/EBPβ(K133R) were expressed in 3T3-L1. The transfected cells were incubated with IBMX and MG132 (10 μM), in the presence or absence of TB (25 μM), for 8 h. FLAG-C/EBPβ proteins were co-immunoprecipitated with anti-FLAG IgG, followed by western blotting analysis. (D) Detection of exogenous C/EBPβ in 3T3-L1 preadipocytes transfected with FLAG-C/EBPβ wildtype (WT) or FLAG-C/EBPβ(K133R). Transfected 3T3-L1 preadipocytes incubated with IBMX, in the presence or absence of TB (25 μM), for 8 h, followed by western blotting analysis. (E) Protein levels of PPARγ and C/EBPα in FLAG-C/EBPβ (K133R)-expressed 3T3-L1 cells. After transfection with FLAG-C/EBPβ (K133R) vector, the cells were induced to differentiate in the presence or absence of 25 μM theobromine (TB) for 6 day. For western blotting, the intensity of each band was quantified by ImageJ 1.44, and the ratio of each band was normalized to the co-immunoprecipitated C/EBPβ (B), co-immunoprecipitated FLAG-C/EBPβ (C) or β-actin (D and E) level. For (B) to (E), data are means ± SD (n = 3). Significant differences ($p<0.05$) are indicated by the corresponding letters. Results shown are each representative of three independent triplicate experiments.

3.5. Theobromine decreased SENP2 gene expression through suppression of CREB activity

SENP2 gene expression was induced by IBMX and theobromine suppressed this in a dose-dependent manner (Fig. 5A). AR1 knockdown restored theobromine-decreased SENP2 mRNA and SENP2 protein expression in the presence of IBMX (Fig. 5B and 5C). We examined the effect of theobromine on the promoter activity of SENP2 using a luciferase reporter vector, pGL4.14-SENP2(wild-type), which contains promoter region of *Senp2* gene [24]. IBMX increased promoter activity of SENP2(wild-type), and theobromine suppressed IBMX-induced promoter activity of SENP2(wild-type) (Fig. 5D). *Senp2* gene is regulated by CREB, and there are cAMP response elements (CRE) in promoter region of *Senp2* gene [24]. To determine whether the CRE is important for theobromine-mediated suppression of SENP2 promoter activity, we constructed pGL4.14-SENP2(Mutant form), which mutated CRE sequence in pGL4.14-SENP2 (wild-type). IBMX and theobromine did not affect the promoter activity of SENP2(Mutant form). Theobromine suppressed IBMX-induced formation of the active, phosphorylated form of CREB (Fig. 5E). We examined whether theobromine suppressed the transcriptional activity of CREB using a luciferase reporter assay. Theobromine suppressed IBMX-induced CREB transactivation and AR1 knockdown prevented this effect (Fig. 5F). In addition, we determined whether theobromine acts as an AR1 agonist or antagonist. Two AR1 selective agonists, N^6-Cyclopentyladenosine and 2-Chloro-N^6-cyclopentyladenosine, enhanced CREB transactivation in 3T3-L1 preadipocytes, both the presence and absence of IBMX (Suppl. Fig. S6), indicating that theobromine might be an antagonist of AR1. Results from ChIP assays showed that

IBMX increased CREB binding to the CRE on the promoter region of the SENP2 gene and that this binding was suppressed by theobromine (Fig. 5G). When AR1 was knocked down in 3T3-L1 preadipocytes, theobromine did not affect binding of CREB to CRE (Fig. 5H). Data from ChIP assays using primary adipocytes from epididymal adipose tissues also showed that theobromine decreased CREB binding to CRE (Fig. 5I). These results indicated that theobromine suppressed SENP2 expression by inhibiting CREB activation.

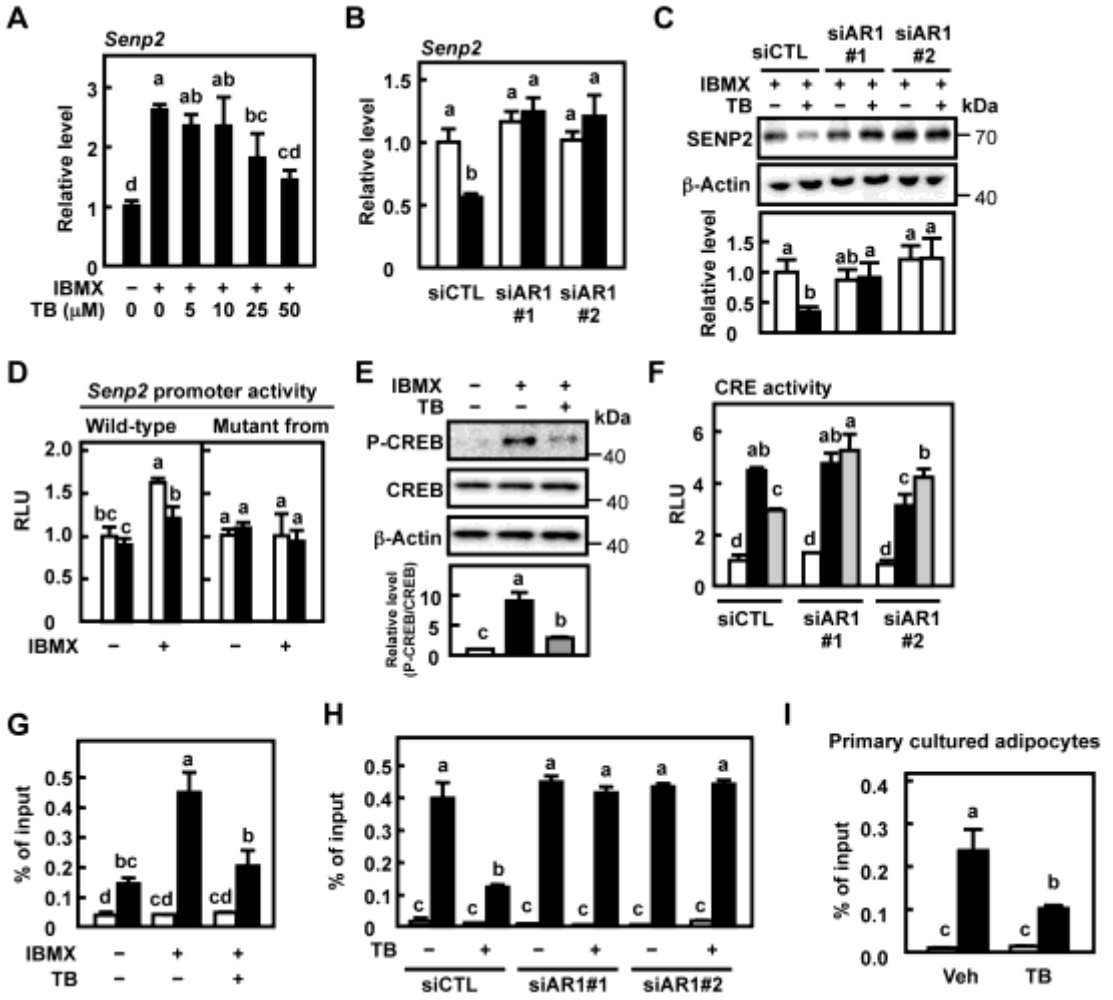


Fig. 5. *Association of CREB and CRE on the SENP2 gene in the presence of theobromine. (A) qPCR analysis of SENP2 gene expression in 3T3-L1 preadipocytes after induction of differentiation with IBMX, in the presence of indicated concentrations*

of theobromine (TB), for 8 h. Data were normalized to Gapdh levels. (B) Quantification of SENP2 gene expression in 3T3-L1 preadipocytes transfected with AR1 siRNA (siAR1#1 or siAR1#2) or control siRNA (siCTL), after induction of differentiation with IBMX, in the presence (black bars) or absence (white bars) of TB (25 μM), for 8 h. Data were normalized to Gapdh levels. (C) 3T3-L1 preadipocytes were transfected with AR1 siRNA (siAR1#1 or siAR1#2) or control siRNA (siCTL). After transfection with siRNA, the cells were induced to differentiate with IBMX, in the presence or absence of 25 μM theobromine (TB), for 8 h. Cell lysates were analyzed by western blotting with anti-SENP2 antibody. (D) Senp2 promoter activity in 3T3-L1 preadipocytes were transiently transfected with pGL4.14-Senp2(Wild-type) or pGL4.14-Senp2(Mutant form) vector, followed by induction of differentiation with (black bars) or without (white bars) TB (25 μM) in the presence or absence of IBMX for 8 h. Luciferase activities are expressed as relative light units (RLU). (E) Levels of CREB phosphorylation in 3T3-L1 preadipocytes after induction of differentiation with IBMX, in the presence or absence of TB (25 μM), for 8 h. Total CREB and β-actin were used as loading controls. (F) Transcriptional activity of CREB in 3T3-L1 preadipocytes injected with siAR1#1, siAR1#2 or siCTL. siRNA-injected 3T3-L1 preadipocytes were transiently transfected with a pCRE-Luc vector, followed by induction of differentiation in the presence (black bars) or absence (white bars) of IBMX or in the presence of IBMX and TB (25 μM) (grey bars), for 8 h. Luciferase activities are expressed as RLU. (G) ChIP using anti-CREB IgG or control rabbit IgG in 3T3-L1 preadipocytes after induction of differentiation with IBMX, in the presence (black bars) or absence (white bars) of TB (25 μM), for 8 h. Co-immunoprecipitated CREB-DNA complexes were analyzed by

qPCR. (H) 3T3-L1 preadipocytes were transfected wit siAR1#1, siAR1#2 or siCTL, followed by induction of differentiation with IBMX, in the presence (black bars) or absence (white bars) of TB (25 μM), for 8 h. CREB-DNA complexes were immunoprecipitated using anti-CREB IgG, then analyzed by qPCR. (I) Primary preadipocytes from epididymal adipose tissues in mice were incubated, with TB (25 μM; black bars) or without TB (white bars), in the presence of IBMX for 8 h. The cells were crosslinked and soluble chromatin was immunoprecipitated with anti-CREB IgG or control rabbit IgG. Co-immunoprecipitated CREB-DNA complexes were analyzed by qPCR. All data are means ± SD (n = 3). For western blotting, the intensity of each band was quantified by ImageJ 1.44, and the ratio of each band was normalized to the β-actin (C) or total CREB (E) levels. Significant differences ($p<0.05$) are indicated by the corresponding letters. Results shown are each representative of three independent triplicate experiments.

3.6. AR1 knockdown in mice prevented the theobromine-induced decreased fat mass in epididymal adipose tissues

To assess whether theobromine would decrease fat accumulation through AR1 signaling *in vivo*, we transfected AR1 siRNA into the epididymal adipose tissue of male mice. Although theobromine decreased fat mass in epididymal adipose tissue transfected with control siRNA, this effect was attenuated with AR1 siRNA (Fig. 6A). There was no difference between mice receiving a sham operation and those transfected with control siRNA. Immunohistochemical showed theobromine decreased adipocytes size in control siRNA-transfected adipose tissues, and knockdown of AR1 canceled

theobromine-decreased adipocyte size (Fig. 6B). Furthermore, we investigated the expression level of adipogenic proteins in AR1-knockdwon adipose tissues. This AR1 knockdown also decreased the effects of theobromine on protein expression of PPARγ, C/EBPα and FAS (Fig. 6C). These *in vivo* results strongly supported the *in vitro* data, both showing that theobromine suppressed adipogenesis by attenuating AR1 signaling.

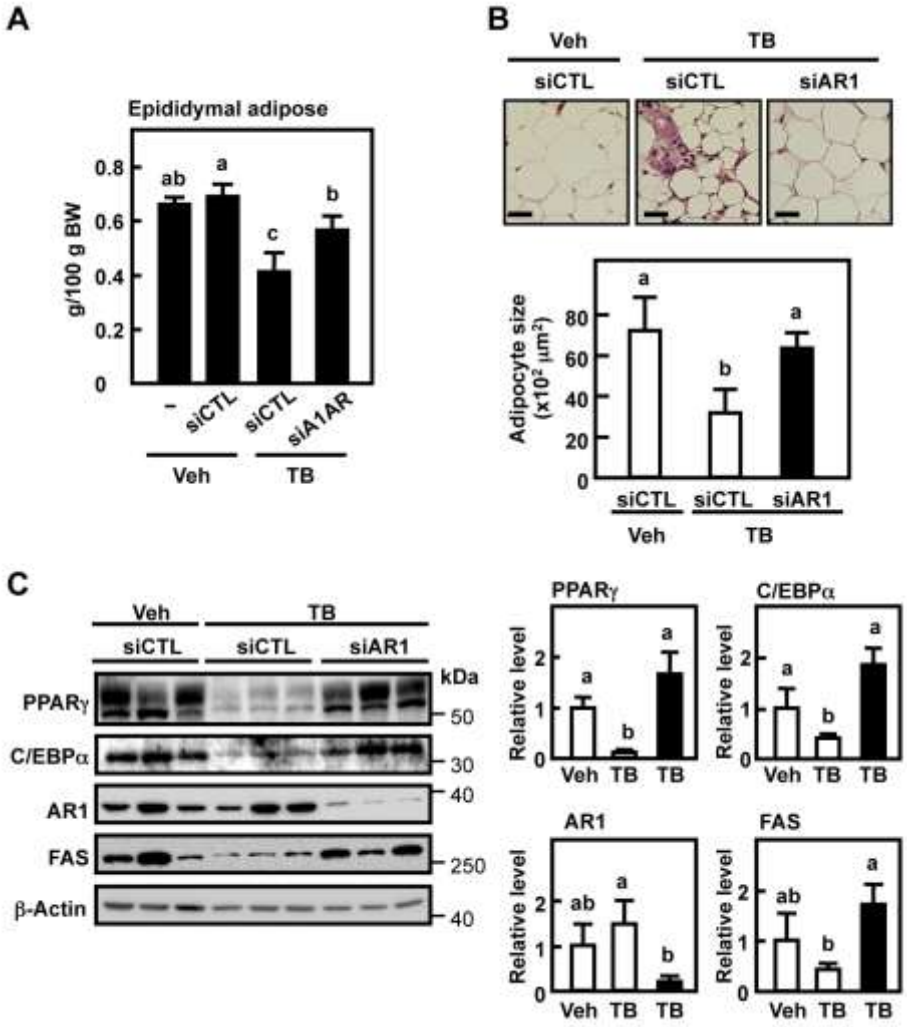


Fig. 6. *Involvement of AR1 in the theobromine-induced decrease in fat mass of mouse epididymal adipose tissue. (A) Left epididymal adipose tissues in mice in the vehicle group were injected with control siRNA (siCTL). Control and AR1 siRNAs were injected into right and left epididymal adipose tissues, respectively, in mice in the theobromine*

group. Mice were treated with or without theobromine for 7 days. The ratio of epididymal adipose tissue/body weight was calculated for each sample. Data are means (n = 5 per group). (B) Hematoxylin and eosin stained paraffin sections of epididymal adipose tissues from mice (n = 3 per group). Scale bar indicates 50 mm (upper panel). The data represent mean cross-sectional area/300 cells, in μm^2, of stained paraffin sections from epididymal adipose tissues (bottom panel). Error bars represent the mean ± SD (n = 3). (C) Western blots for PPARγ, C/EBPα, FAS and AR1 proteins in siRNA-transfected epididymal adipose tissues in mice receiving Veh or TB. The intensity of each band was quantified by ImageJ 1.44, and the ratio of each band was normalized to β-actin (a loading control) level. The data are presented as means ± SD (n = 3 per group). Significant differences ($p<0.05$) are indicated by corresponding letters.

4. DISCUSSION

Obesity is associated with adverse metabolic consequences, such as type 2 diabetes and cardiovascular diseases. Recent studies reported that various phytochemicals had anti-adipogenic effects, including inhibition of adipocyte differentiation and adipogenesis [31-33]. We elucidated molecular mechanism by which theobromine suppresses adipocyte differentiation *in vitro* and *in vivo*. Theobromine induced degradation of C/EBPβ by suppressing AR1 signaling, resulting in attenuation of adipocyte differentiation.

Theobromine increased sumoylation of wildtype C/EBPβ, but not of C/EBPβ (K133R) (Fig. 4E). The function of C/EBPβ can be regulated by multiple post-translational modifications, including phosphorylation, acetylation, ubiquitination

and sumoylation [10, 11, 34]. Sumoylation was shown to affect multiple aspects of protein function, including transcriptional activity, subcellular localization and protein stability [35]. Sumoylation at Lys173 of human C/EBPβ was linked to its transcriptional activity [36] and C/EBPβ sumoylated at Lys133 showed increased degradation through the ubiquitin-proteasome pathway in mice [11]. Similarly, our results indicated that, in mice, theobromine induced C/EBPβ degradation by increasing its sumoylation at Lys133. Furthermore, our data showed that theobromine suppressed IBMX-induced SENP2 expression (Fig. 5C). Balances in sumoylation are regulated by SUMO E3 ligase and de-sumoylation protease. Expression of SUMO E3 ligase was decreased during adipocyte differentiation while, conversely, that of SENP2 was increased at the early phase of the process [11]. SENP2 knockdown increased C/EBPβ ubiquitination and promoted its degradation in 3T3-L1 preadipocytes [24]. Furthermore, SENP2 overexpression increased fatty acid synthesis in skeletal muscle [37]. Taken together, our results indicated that theobromine promoted C/EBPβ degradation by increasing its sumoylation, through suppression of SENP2 expression.

Theobromine-induced C/EBPβ degradation was prevented by knockdown of AR1, but not of AR2a. AR2a expression was higher in brown adipocytes than in white adipocytes, whereas AR1 was primarily expressed in white adipocytes in both mice and humans [38]. Adenosine increased adipogenesis through AR1 signaling in white adipocytes [39, 40]. Adipogenic differentiation resulted in increased AR1 expression in mesenchymal stem cells and activation of this receptor was associated with further increases in adipogenesis [41]. Beyond its role in adipocytes, AR1 was implicated in adipogenesis in other tissues. For example, AR1 overexpression increased mRNA levels

of an adipocyte marker and led to lipid accumulation in a murine osteoblast precursor cell line, 7F2 [42]. These results indicated that suppression of AR1 signaling attenuated adipogenesis in both adipocytes and preadipocytes. Interestingly, our data showed that theobromine administration suppressed weight gain in epididymal, but not in mesentery, adipose tissue (Fig. 1A). As one possible reason for these differences in the two adipose tissues, adenosine receptor signaling is believed to have different potencies in different cells. Thus, AR1 signaling activation would have opposing effects in preadipocytes, compared with in mature adipocytes [15, 43]. The effects of theobromine on C/EBPβ degradation were consistent in 3T3-L1 preadipocytes and primary preadipocytes from epididymal adipose tissue (Fig. 2E and 5I), suggesting that AR1 affected adipocyte differentiation in both 3T3-L1 preadipocytes and epididymal adipocytes. These results indicated that theobromine exerted its anti-adipogenic effects through suppression of AR1 signaling.

Theobromine decreased intracellular cAMP pools and the transcriptional activity of CREB. These effects were prevented by AR1 knockdown. As a second messenger, cAMP is important for intracellular signal transduction in many tissues. Increasing intracellular cAMP levels initiated, and decreased levels inhibited, adipocyte differentiation [44]. cAMP activated protein kinase A and CREB, and the activated CREB induced expression of the SENP2 gene [24, 45]. Our data showed that AR1 agonists, selectively, enhanced CREB transcriptional activity (Suppl. Fig. S6), indicating that AR1 signaling increased intracellular cAMP pools in adipocytes. However, many studies reported that AR1 signaling suppressed adenylate cyclase (AC) activity, through activation of Gi protein, resulting in decreased intracellular cAMP

pools [46]. Thus, these reports indicated that inhibition of AR1 signaling would increase intracellular cAMP pools, contrary to our findings (Fig. 3F). As a potential explanation, although Gi activation inhibited the activity of AC type I in the hippocampus, AC type II was constitutively activated by Gi protein [47]. Therefore, our data suggested that theobromine-bound AR1 decreased intracellular cAMP pools by activating AC type II, although further research would be needed to confirm this.

Theobromine inhibited the interaction of adenosine with AR1, but not with AR2a. Caffeine and theophylline, both methylxanthines, all acted as nonselective antagonists for adenosine receptors [29]. Likewise theobromine, caffeine and theophylline showed similar effects on central nervous system and locomotor activity [48]. However, recent and growing evidence indicated that theobromine had different psychoactive actions and blood pressure effects than did caffeine and theophylline [49]. Furthermore, theobromine significantly inhibited carrageenan-induced lung inflammation, through suppression of poly(ADP-ribose)polymerase-1, while the effects of caffeine on this process were very weak [50]. We propose that these differences might have been caused by different alkyl group positions in theobromine, compared with in the other methylxanthines. Our results indicated that theobromine acted as a selective AR1 ligand in adipocytes.

Our data showed that theobromine, at concentrations above 25 μM, exhibited anti-adipogenic effects. Jang *et al* reported that theobromine decreased adipogenesis in 3T3-L1 preadipocytes, through activation of AMP-activated protein kinase and ERK signaling, at 150 μg/ml (877 μM) [51]. In human intervention studies, plasma concentrations of theobromine were increased to 28.75 μM in subjects receiving 850 mg

theobromine per day for 4 weeks [20]. In addition, peak plasma concentrations of theobromine were approximately 50 μM in humans after intervention [52], suggesting that physiological plasma concentrations of theobromine were lower than 50 μM. The effects demonstrated in our study were caused by theobromine at physiological concentrations.

ARs have been considered potential therapeutic targets in some diseases, including cardiac and inflammatory disorders [14, 46]. Drug discovery approaches led to development of selective ligands for each of the AR subtypes [53]. Our results indicated that theobromine acted as a selective AR1 ligand and, consequently, attenuated adipocyte differentiation through C/EBPβ degradation. Thus, theobromine, as an anti-adipogenic phytochemical, might be new therapeutic agents to suppress obesity.

Authors' contribution

T.M. and H.A. conceived and designed the experiments and discussed the results. T.M., S.W. and Y.Y performed the experiments. T.M., S.W., Y.Y., S.K., S.N. and H.A. analyzed the data. T.M. and H.A. wrote the manuscript with the help of all other authors. All authors approved the final version of the manuscript.

Conflict of interest statement

The authors declare that they have no conflicts of interest with the contents of this article.

Acknowledgements

This work was supported by JSPS KAKENHI Grant Number JP16K18701 (to T.M.).

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