Theophylline suppresses interleukin-6 expression by inhibiting glucocorticoid receptor signaling in pre-adipocytes

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Abbreviations: 11β-HSD, 11β-hydroxysteroid dehydrogenases; C/EBP, CCAAT-enhancer-binding protein; DEX, dexamethasone; FFAs, free fatty acids; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid response element; IBMX, 3-isobutyl-1-methylxanthine; IL-6, interleukin-6; LPS, lipopolysaccharide; qPCR, quantitative real-time PCR; TNF, tumor necrosis factor

ABSTRACT

Adipose tissues in obese individuals are characterized by a state of chronic low-grade inflammation. Pre-adipocytes and adipocytes in this state secrete pro-inflammatory adipokines, such as interleukin 6 (IL-6), which induce insulin resistance and hyperglycemia. Theophylline (1,3-dimethylxanthine) exerts anti-inflammatory effects, but its effects on pro-inflammatory adipokine secretion by pre-adipocytes and adipocytes have not been examined. In this study, we found that theophylline decreased IL-6 secretion by 3T3-L1 pre-adipocytes and mouse-derived primary pre-adipocytes. The synthetic glucocorticoid dexamethasone (DEX) induced IL-6 expression in 3T3-L1 pre-adipocytes, and this effect was suppressed by theophylline at the mRNA level. Knockdown of CCAAT/enhancer binding protein (C/EBP) δ inhibited DEX-induced IL-6 expression, and theophylline suppressed C/EBPδ expression. Furthermore, theophylline suppressed transcriptional activity of the glucocorticoid receptor (GR) through suppression of nuclear localization of GR. *In vivo*, glucocorticoid corticosterone treatment (100 μg/mL) increased fasting blood glucose and plasma IL-6 levels in C57BL/6N mice. Theophylline administration (0.1% diet) reduced corticosterone-increased fasting blood glucose, plasma IL-6 levels, and *Il6* gene expression in adipose tissues. These results show that theophylline administration attenuated glucocorticoid-induced hyperglycemia and IL-6 production by inhibiting GR activity. The present findings indicate the potential of theophylline as a candidate therapeutic agent to treat insulin resistance and hyperglycemia.

Keywords: Adipocyte; CCAAT-enhancer-binding proteins; glucocorticoid receptor; interleukin-6; theophylline

1. Introduction

Obesity, which is a risk factor for serious diseases such as insulin resistance, type 2 diabetes mellitus, and cardiovascular disease, is associated with an increase in adipocyte number and size. The accumulation of intracellular lipids during adipocyte differentiation increases adipocyte size [1]. Adipocyte differentiation is regulated by various transcription factors, such as CCAAT/enhancer binding protein (C/EBP)α, C/EBPβ, C/EBPδ, and peroxisome proliferator-activated receptor γ [2]. Adipose tissue in obese individuals is characterized by chronic low-grade systemic inflammation accompanied by elevated secretion of free fatty acids (FFAs) and various pro-inflammatory cytokines, such as interleukin 6 (IL-6), tumor necrosis factor α (TNFα), and plasminogen activator inhibitor-1, which are known as adipokines [3]. These pro-inflammatory adipokines are involved in the induction of insulin resistance, which contributes to hyperglycemia and type 2 diabetes mellitus [4]. Therefore, strategies aimed at decreasing the secretion of pro-inflammatory adipokines by adipocytes are crucial to the prevention and treatment of insulin resistance.

Adipose tissue contains various cells, including pre-adipocytes, mature adipocytes, and macrophages. The expression pattern of pro-inflammatory adipokines varies in these cells. TNFα is mostly produced by macrophages, whereas IL-6 is mainly produced by pre-adipocytes and mature adipocytes in adipose tissue [5]. Furthermore, IL-6 secretion is higher in pre-adipocytes than in mature adipocytes [6]. It has been reported that serum IL-6 levels and insulin resistance are strongly linked [7], and that IL-6 from adipocytes affects various tissues in autocrine and paracrine manners. In adipocyte model 3T3-L1 pre-adipocytes, IL-6 suppressed gene expression, inhibited adiponectin secretion, and acted as an insulin-sensitizing adipokine [8]. IL-6 activated SOC3, resulting in decreased insulin sensitivity in hepatocytes [9]. Furthermore, tail vein injection of IL-6-neutralizing antibodies improved insulin resistance in obese mice [10]. These findings indicated that adipose-derived IL-6 may be a target for prevention strategies against obesity-induced insulin resistance.

Glucocorticoids (GCs), which are steroid hormones secreted by the adrenal cortex, have

been proposed to play both adipogenic and lipolytic roles in adipose tissue [11]. GC action depends not only on blood levels, but also on tissue-specific intracellular metabolic processes catalyzed by 11β-hydroxysteroid dehydrogenases (11β-HSDs). 11β-HSD converts inactive cortisone into active cortisol in humans, or into corticosterone, a major endogenous glucocorticoid, in rodents [12]. The expression of 11β-HSD1 in adipose tissues is elevated in obese human patients [13]. GCs bind to the glucocorticoid receptor (GR) in the cytoplasm. The ligand-bound GR then translocates into the nucleus and binds to glucocorticoid-response elements (GREs) on target gene promoters, resulting in increased expression of the target genes. GCs increased plasma levels of FFAs and expression levels of pro-inflammatory adipokines such as IL-6 and TNFα in white adipose tissue of mice [14, 15]. Furthermore, chronic exposure to GCs was associated with insulin resistance, diabetes, and hepatic steatosis in rodents [16]. Therefore, GC-GR signaling represents a potential therapeutic target against obesity-related insulin resistance and diabetes.

Theophylline (1,3-dimethylxanthine), a methylxanthine drug, was first extracted from tea leaves. Theophylline is widely used worldwide as a therapeutic agent for respiratory diseases [17]. In addition, recent reports have shown that theophylline ameliorated acetic-acid-induced ulcerative colitis by decreasing the levels of pro-inflammatory cytokines in the mouse colon [18]. However, theophylline effects on pro-inflammatory adipokine secretion in pre-adipocytes and adipocytes have not been investigated. In this study, we assessed the molecular effects of theophylline on the secretion and expression levels of IL-6 in 3T3-L1 pre-adipocytes. We demonstrated that theophylline suppressed IL-6 gene expression by inhibiting the transcriptional activity of GR in 3T3-L1 pre-adipocytes. Furthermore, we showed that administration of theophylline decreased GC-induced hyperglycemia and plasma IL-6 levels in mice.

2. Materials and Methods

2.1. Animal experiments

All animal experiments conformed to the protocols approved by the Institutional Animal Care and Use Committee of Shinshu University Animal Experimentation Regulations (Permission Number 280042) and the Guide for Care and Use of Laboratory animals (NIH Publications No. 8023, revised 1978). Five-week-old male C57BL/6N mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and housed under controlled temperature (20 ± 3 °C) with a 12 h light-dark cycle. Mice had free access to food and water. Six-week-old mice were randomly divided into three groups: vehicle group (Veh), corticosterone group (CORT), and theophylline group (TP) (n = 6 in each group). Veh group was fed High-Fat Diet 32 (CLEA Japan, Inc., Tokyo, Japan), and given free access to drinking water. CORT and TP groups were given drinking water containing 100 μg/mL corticosterone. CORT group was fed High-Fat Diet 32, and TP group was fed High-Fat Diet 32 containing 0.1% (w/w) theophylline. Six weeks after the start of the study, the mice were sacrificed under anesthesia, and their plasma and epididymal adipose tissues were harvested.

2.2. Cells and cell culture

Murine 3T3-L1 pre-adipocytes were purchased from JCRB Cell Bank (Osaka, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine serum, 100 μg/mL streptomycin, and 100 units/mL penicillin. The cells were maintained at 37 °C in the atmosphere of 95% air and 5% CO_2 and at 98% humidity. The cells were incubated with the synthetic glucocorticoid dexamethasone (DEX; 0.5 μM), 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM), and insulin (10 μg/mL) in DMEM containing high levels of glucose (4.5 g/L glucose) and 10% fetal bovine serum for 24 h.

2.3 Primary pre-adipocyte cultures

Primary pre-adipocyte cultures were generated as previously described [19], with minor

modifications. Briefly, primary pre-adipocytes were isolated from epididymal adipose tissue of male mice (ICR; 4-week-old). After digestion with collagenase II and centrifugation, pre-adipocytes were cultured in DMEM. When pre-adipocytes were fully confluent, they were treated with theophylline in the presence of 0.5 μM DEX for 24 h.

2.4. siRNA

Double-stranded siRNAs for C/EBPδ were chemically synthesized (Sigma-Aldrich, Saint Louis, MO). Target sequences for siRNA duplexes were as follows: siC/EBPδ#1, 5′-CGACTTCAGCGCCTACATT-3′ and siC/EBPδ#2, 5′-CGCAGACAGTGGTGAGCTT-3′. The duplexes (20 nM) were transiently transfected into 3T3-L1 pre-adipocytes using Lipofectamine RNAiMAX reagent (Invitrogen; Carlsbad, CA) and Opti-MEM (Thermo Fisher Scientific, Lafayette, CO) for 24 h, according to the manufacturer's protocol.

2.5 Plasmids

The consensus sequence of androgen-responsive element (ARE) is commonly recognized by GR [20]. Accordingly, we used the pARE2 [21] reporter assay vector to determine transcriptional activity of GR.

2.6. Measurement of secreted IL-6

The levels of IL-6 secreted by 3T3-L1 pre-adipocytes and plasma IL-6 levels in mice were determined by enzyme-linked immunosorbent assays (ELISA). A rat monoclonal anti-IL-6 antibody (catalog number MAB406, 1:10,000 dilution; R&D Systems, Minneapolis, MN) was coated on 96-well plates at 4 °C overnight. The plates were washed with PBS and incubated with the sample (100 μL) at 37 °C for 2 h. Washed plates were incubated with a biotinylated goat polyclonal anti-IL-6 antibody (Cat# BAF406, 1:1,000 dilution; R&D Systems) at 37 °C for 2 h, followed by further incubation with horseradish peroxidase-conjugated streptavidin (N100;

1:10,000 dilution; Thermo Fisher Scientific, Waltham, MA) at 37 °C for 1 h. The plates were washed and 3,3′,5,5′-tetramethylbenzidine (100 μL; Sigma-Aldrich) was added to each well. Ten minutes later, the reaction was stopped using 2N H_2SO_4. Absorbance at 450 nm was measured in the plates by using a multi-plate reader (Bio-Rad, Richmond, CA).

2.7. Western blotting

3T3-L1 pre-adipocytes were incubated with theophylline in the presence of 0.5 μM DEX for 2 h or 24 h. SDS-PAGE and western blotting were performed as described previously [22]. Cell lysates were analyzed by western blotting using the following rabbit polyclonal antibodies: anti-C/EBPδ (Cat# sc-9315) and anti-C/EBPβ (Cat# sc-150; Santa Cruz Biotechnology, Santa Cruz, CA), anti-GR (Cat# 12041S; Cell Signaling, Danvers, MA), anti-IL6 (Cat# ab6672), and anti-Histone H3 (Cat# ab1791; Abcam, Cambridge, UK). In addition, a mouse monoclonal anti-β-actin antibody (Cat# sc-47778; Santa Cruz Biotechnology) was used. The immunoreactive proteins were reacted with Immunostar LD (Wako, Osaka, Japan) and visualized using Ez-Capture MG (ATTO Co., Tokyo, Japan).

2.8. Quantitative real-time PCR (qPCR)

Total RNA was extracted from 3T3-L1 pre-adipocytes using TRIzol (Invitrogen). cDNAs were synthesized using RevaTra Ace and subjected to qPCR using sets of specific primers (see Table S1 for the sequences). qPCR was performed with SYBR PremixEx Taq II (Takara Bio, Shiga, Japan) using a two-step PCR method on a Thermal Cycler Dice real-time system (Takara Bio.). The relative expression levels of each gene were calculated using the $2^{-\Delta\Delta Ct}$ (CT, cycle threshold) method, and data were normalized to the expression level of *Gapdh*, which was used as endogenous control.

2.9. Luciferase reporter assay

The luciferase reporter assay was performed as described previously [23]. 3T3-L1 pre-adipocytes were transiently transfected with the reporter vectors pARE2-TATA-Luc and pRL-SV40 (control reporter vector; Promega) using Lipofectamine 2000 for 24 h. After the medium was replaced, cells were incubated with 0.5 μM DEX and theophylline. Transfection efficiency was normalized by luminescence levels in pRL-SV40-transfected cells. Firefly and Renilla luciferase activities were measured using a Dual Luciferase reporter assay kit and GloMax 20/20 Luminometer (Promega). Data were expressed as relative light units (RLU; firefly levels divided by Renilla levels).

2.10. Immunostaining

Immunostaining was performed as described previously [22]. In brief, fixed cell samples were incubated with a rabbit polyclonal anti-GR antibody in phosphate buffered saline containing 3% bovine serum albumin at 4 °C overnight, followed by the incubation with Alexa Fluor 488-conjugated secondary anti-rabbit IgG at room temperature for 1 h. The nuclei were stained with Hoechst 33258 (1 μg/mL; Dojindo Lab, Kumamoto, Japan) at room temperature for 10 min, followed by inspection using an EVOS FL Auto microscope (Thermo Fisher Scientific).

2.11. Subcellular fractionation

Confluent 3T3-L1 pre-adipocytes were incubated with theophylline, RU486 (1 μM), or xanthine (100 nM) in the presence of DEX for 30 min. The cells were lysed in hypotonic buffer containing 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Hepes-NaOH (pH 7.5) and protease cocktail (Nacalai Tesque, Kyoto, Japan). Subcellular fractionation was performed as described previously [21]. Proteins in each fraction were analyzed by western blotting.

2.12. Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed as described previously [23]. In brief, 3T3-L1 pre-adipocytes were incubated with 100 nM theophylline and 0.5 μM DEX for 30 min. The promoter region of the *Cebpd* gene was amplified by qPCR using a set of specific primers (see supplemental Table S1 for sequences). qPCR profiles were obtained after running the following program: 94 °C for 30 s, 65 °C for 15 s, 72 °C for 20 s. The relative amounts of each promoter region were calculated using the $2^{-\Delta\Delta Ct}$ method, and the data were normalized to that of the input sample.

2.13. Pull-down assay

First, EAH Sepharose 4B (625 μL; 80% slurry; GE healthcare, UK) was washed by 0.5 M NaCl three times and incubated with theophylline-7-acetic acid (1.6 mg) in coupling buffer (100 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, pH4.5, 50% ethylene glycol, and 0.25 M NaCl) 4 °C overnight. The Sepharose resin was washed three times with wash buffer 1 (50% ethylene glycol, and 0.25 M NaCl, pH 4.5), wash buffer 2 (0.1 M sodium acetate, pH 4.0), and wash buffer 3 (0.1 M Tris-HCl, pH 8.0, containing 0.5 mol/L NaCl), respectively. The resin was termed theophylline-affinity resin. Next, 3T3-L1 cell lysates (0.5 mg protein) was incubated with theophylline-affinity resin (100 μL; 50% slurry) in the presence or absence of 1 mM theophylline at 4 °C for 2 h. The resin was washed with lysis buffer three times and bound proteins separated by SDS-PAGE and analyzed by western blotting.

2.14. Statistical analysis

Data were analyzed using the Student's *t* test or one-way analysis of variance (ANOVA), with Turkey's post hoc test, if appropriate. Statistical analysis was performed using JMP statistical software version 11.2.0 (SAS Institute. Cary, NC). Data are expressed as the mean ± standard deviation (S.D.). All statistical analyses were conducted with a significance level of $\alpha = 0.05$ ($P < 0.05$).

3. Results

3.1. Theophylline decreases IL-6 secretion by 3T3-L1 pre-adipocytes

We examined the effect of theophylline on IL-6 secretion in 3T3-L1 pre-adipocytes. IL-6 secretion levels by 3T3-L1 pre-adipocytes were increased by the treatment with a differentiation-inducing reagent that contained DEX, IBMX, and insulin. At concentrations above 100 nM, theophylline decreased the reagent-induced secretion of IL-6 (Fig. 1A). In addition, theophylline concentration-dependently suppressed the reagent-induced increase in IL-6 protein (Fig. 1B). To identify the active component of the differentiation-inducing reagent that was responsible for inducing IL-6 expression, 3T3-L1 pre-adipocytes were treated with DEX, IBMX, or insulin. IL-6 protein level was induced by DEX, but not by IBMX or insulin (Fig. 1C). Furthermore, theophylline suppressed DEX-induced increase in IL-6 expression at protein and mRNA levels (Fig. 1D and 1E). These results indicated that theophylline suppressed DEX-induced IL-6 expression at the transcriptional level in 3T3-L1 pre-adipocytes.

3.2. Theophylline decreases IL-6 secretion by primary pre-adipocytes

We examined the effect of theophylline on the levels of secreted and intracellular IL-6 in primary pre-adipocytes from male and female mice. DEX increased the level of secreted IL-6 in male and female pre-adipocytes fourfold and twofold, respectively, whereas theophylline counteracted this effect of DEX (Fig. 2A). In addition, theophylline suppressed protein level of IL-6 in both male and female primary pre-adipocytes (Fig. 2B). These results indicated that theophylline suppressed DEX-induced IL-6 expression not only in mouse 3T3-L1 pre-adipocytes, but also in primary pre-adipocytes.

3.3. C/EBPδ is involved in the expression of IL-6

The promoter region of the *Il6* gene contains a CCAAT box motif, which is the binding site of C/EBPs [24]. We found that DEX increased C/EBPδ protein level in 3T3-L1 pre-adipocytes (Fig. 3A). Furthermore, to determine whether DEX-induced C/EBPδ involved in IL-6 expression, 3T3-L1 pre-adipocytes were transiently transfected with two siRNAs with different sequences to avoid off-target effects. C/EBPδ siRNA reduced DEX-induced protein expression of C/EBPδ (Fig. 3A), and knockdown of C/EBPδ attenuated DEX-induced IL-6 secretion and protein expression (Fig. 3B and 3C). These results suggested that DEX up-regulated *Il6* expression through C/EBPδ in 3T3-L1 pre-adipocytes.

3.4. Theophylline decreases C/EBPδ expression at the mRNA level

We examined the effect of theophylline on C/EBPδ expression in 3T3-L1 pre-adipocytes. Theophylline decreased DEX-induced increase in C/EBPδ protein level in a concentration-dependent manner (Fig. 4A). However, DEX and theophylline did not affect C/EBPβ protein level. Although the proteasome inhibitor MG132 increased C/EBPδ protein levels in the absence of theophylline, this compound did not restore C/EBPδ protein level, reduced by theophylline, to normal levels (Fig. 4B). Furthermore, we observed that theophylline suppressed DEX-induced gene expression of *Cebpd* (Fig. 4C). These results indicated that theophylline suppressed DEX-induced IL-6 expression by decreasing C/EBPδ expression in 3T3-L1 pre-adipocytes.

3.5. Theophylline suppresses the transcriptional activity of GR

DEX is a GR agonist, therefore it was expected that the GR antagonist RU486 inhibited DEX-induced increase in the levels of secreted and intracellular IL-6 (Fig. 5A). We then examined whether theophylline suppressed the transcriptional activity of GR using a luciferase reporter assay for GR transactivation. Theophylline suppressed DEX-enhanced GR transactivation in a concentration-dependent manner (Fig. 5B). However, theophylline did not

decrease GR protein levels (Fig. 5C). We assumed that theophylline regulated the binding of GR to glucocorticoid-responsive genes. Multi-genome analysis using the MAPPER database [25, 26] showed that the promoter region of *Cebpd* contained two GRE candidates: one located at −2110 to −2096, and the other at −1301 to −1289 of *Cebpd*. We performed the ChIP assay using 3T-L1 cell lysates and three sets of PCR primers, as shown in Fig. 5D (left panel). When the ChIP assay was performed using the primer sets P2 and P3, but not set P1, DEX increased the relative level of interaction between GR and DNA (Fig. 5D, right panel). Theophylline decreased DEX-induced interaction between GR and DNA. These results indicated that theophylline suppressed DEX-induced binding of GR to DNA.

3.6. Theophylline decreases the nuclear localization of GR

We investigated the effect of theophylline on the intracellular localization of GR in 3T3-L1 pre-adipocytes using immunofluorescence microscopy. GR was distributed throughout the cell in the absence of DEX, and DEX increased the nuclear localization of GR. In contrast, treatment with theophylline resulted in decreased nuclear localization of GR in the presence of DEX (Fig. 6A). In addition, cellular fractionation showed that RU486 decreased DEX-induced nuclear accumulation of GR (Fig. 6B). Likewise, theophylline decreased its nuclear accumulation. However, xanthine did not suppress nuclear import of GR in the presence of DEX. These results indicated that theophylline suppressed GR transactivation by decreasing nuclear accumulation of GR.

3.7. Theophylline interacts with GR in 3T3-L1 pre-adipocytes

3T3-L1 pre-adipocytes were treated with six xanthine derivatives, including theophylline, to determine their effect on GR transactivation (Fig. 7A). Xanthine has three methylation sites at positions 1, 3, and 7. Theophylline-7-acetic acid and 1-methylxanthine, as well as theophylline, suppressed DEX-enhanced GR transactivation in 3T3-L1 pre-adipocytes (Fig. 7B). In contrast,

xanthine, 3,7-dimethylxanthine and 1,3,7-trimethylxanthine did not affect DEX-enhanced GR transactivation. When the effects of xanthine derivatives on protein levels of C/EBPδ and levels of secreted IL-6 were examined, similar results were obtained (Fig. 7C and 7D). We determined whether theophylline interacted with GR. Because likewise theophylline, theophylline-7-acetic acid suppressed DEX-induced GR transactivation and C/EBPδ protein level, we produced theophylline-affinity resin by immobilizing Sepharose on the carboxy group of theophylline-7-acetic acid. When 3T3-L1 pre-adipocytes lysates were incubated with theophylline-affinity-resin with or without theophylline, the resin pulled down GR protein in the absence of theophylline (Fig. 7E). In contrast, the interaction between the resin and GR was diminished by the incubation with theophylline. The resin did not pull down β-actin. These results suggested that theophylline suppressed GR transactivation through the interaction with GR protein.

3.8. Intake of theophylline decreases corticosterone-induced IL-6 expression in adipose tissues

To examine the effect of theophylline *in vivo*, mice were given drinking water with or without corticosterone and then fed a theophylline-containing high-fat diet for 6 weeks. Corticosterone intake led to an increase in the weight of epididymal adipose tissue, whereas theophylline decreased the corticosterone-mediated increase in adipose tissue weight (Fig. 8A). We then analyzed fasting blood glucose levels in mice. Corticosterone was found to increase blood glucose levels (188 ± 29.3 mg/dL), whereas theophylline attenuated corticosterone-mediated increase in fasting blood glucose (103 ± 16.3 mg/dL) (Fig. 8B). Furthermore, we measured IL-6 levels in plasma and adipose tissue in mice. Theophylline decreased corticosterone-induced increase in IL-6 plasma concentration (Fig. 8C). Corticosterone further enhanced *Il6* mRNA level in epididymal adipose tissues, whereas theophylline intake tended to decrease that corticosterone effect ($P = 0.0651$) (Fig. 8D). These results indicated that theophylline intake suppressed IL-6 expression in epididymal adipose tissues of mice.

4. Discussion

Obesity is a state of chronic low-grade inflammation, which promotes the secretion of certain pro-inflammatory adipokines by adipose tissues. These pro-inflammatory adipokines exert a negative influence on other tissues, such as the liver, skeletal muscle, and immune tissues, resulting in increased risk of hyperglycemia, type 2 diabetes mellitus, and cardiovascular diseases. Therefore, the suppression of pro-inflammatory adipokines represents a promising therapeutic strategy against obesity-related diseases. In this study, we focused on IL-6, one of the pro-inflammatory adipokines secreted by pre-adipocytes, and demonstrated the molecular mechanism by which theophylline suppressed the expression of IL-6 in pre-adipocytes.

IL-6 is secreted by adipose tissues, skeletal muscle, and the liver. The level of expression of IL-6 in the adipose tissue correlates with the body mass index, and plasma IL-6 is elevated in obese and diabetic subjects [27, 28]. Furthermore, the consumption of high-fat diet has been shown to induce insulin resistance and obesity through adipose tissue-derived IL-6 in mice [29]. Injection of IL-6 has been demonstrated to promote hyperglycemia in rats [30]. IL-6 levels are associated with the development of chronic hyperglycemia and insulin resistance in human patients after acute pancreatitis [31]. Several studies have described the molecular mechanism by which IL-6 induces hyperglycemia and insulin resistance in the liver and in adipocytes. Chronic exposure to IL-6 inhibited insulin-dependent tyrosine phosphorylation of the insulin receptor, as well as insulin receptor substrate-1 and -2, resulting in decreased glucose uptake and glycogenesis in the liver [32]. Furthermore, IL-6 inhibited the expression of the adiponectin gene that exerted insulin-sensitizing effects in 3T3-L1 pre-adipocytes [8]. Macrophage recruitment to adipose tissue contributes to insulin resistance; in this context, IL-6 has been found to promote the recruitment of macrophages to adipose tissue in obese mice [33]. In the present study, the administration of theophylline resulted in the attenuation of corticosterone-induced hyperglycemia and IL-6 production (Fig. 8). Therefore, our results suggest that

theophylline attenuated blood glucose levels up-regulated by GC via suppressing adipose tissue-derived IL-6 levels.

Theophylline suppressed the nuclear localization of GR, but not its protein level. GCs play a critical role in both adipogenesis and lipolysis in adipocytes. Short-term exposure to GC promoted lipolysis and release of FFAs from adipocytes [34], whereas blood FFAs induced hepatic insulin resistance [35]. In contrast, prolonged exposure to GCs induced hyperglycemia and insulin resistance, and knockdown of GR attenuated GC-mediated insulin resistance [16, 36]. Prenatal exposure to GC increased the expression of IL-6, IL-1β, and TNFα in adipose tissues [15]. Furthermore, the consumption of a high-fat diet increased the expression not only of IL-6, but also that of 11β-HSD1 in adipose tissues [37], indicating that high-fat intake increased intracellular levels of active GC in adipocytes. A selective inhibitor of 11β-HSD1 was shown to decrease serum IL-6 levels and ameliorate high-fat diet-induced insulin resistance in obese rats [37]. Therefore, IL-6 is involved in insulin resistance or hyperglycemia mediated by the dysregulation of GC-GR signaling. Previous studies have described anti-inflammatory effects of theophylline. Theophylline reduced the production of IL-6 in lipopolysaccharide (LPS)-treated primary human lung fibroblasts from patients with chronic obstructive pulmonary disease [38]. The authors suggested that theophylline suppressed the expression of IL-6 by inhibiting LPS-Toll-like receptor signaling. In addition, it has been suggested that anti-inflammatory effects of theophylline are caused by the inhibition of reactive oxygen species production [20]. Our results indicate that theophylline acted as an inhibitor of GR transactivation and thereby decreased IL-6 expression in 3T3-L1 pre-adipocytes.

Knockdown of C/EBPδ abrogated DEX-induced IL-6 expression. C/EBPδ elicited robust induction of pro-inflammatory response in macrophages. LPS-induced *IL6* mRNA expression was decreased in *Cebpd*$^{-/-}$ macrophages and glial cells [39]. GR is the main transcription factor involved in the regulation of C/EBPδ gene expression in adipocytes [40]. Several studies have reported that cytokines induced nuclear localization of GR in a GC-independent manner. TNFα

promotes insulin resistance and activates GR signaling by inducing the nuclear localization of the GR, without the requirement for GCs [36]. In addition, IL-13 promotes the nuclear translocation of the GR and enhances DEX-stimulated nuclear import of GR in human airway smooth muscle cells [41]. In this study, we showed that theophylline decreased GR nuclear localization in the presence of DEX (Fig. 6). Taken together, these results suggest that theophylline suppressed GC- and cytokine-induced GR transactivation by decreasing nulear accumulation of GR.

DEX-induced GR transactivation was suppressed by methylxanthines in which the 1-position was methylated and the 7-position was substituted by a hydrophilic group. Methylxanthine derivatives exert various pharmacological effects, such as antagonism of adenosine receptors, inhibition of phosphodiesterase, modulation of GABA receptors, and regulation of intracellular calcium levels [30]. Structure-activity relationships of methylxanthines show that their 1-methyl group is pivotal for their inhibitory effects on phosphodiesterase [42]. Furthermore, methylxanthines have been reported to exert structure-specific effects. The inhibitory effect of methylxanthines, especially of 3,7-dimethylxanthine and 1,7-dimethylxanthine at physiological concentrations, against poly(ADPribose)polymerase-1 has been shown [43], which indicated that 7-methyl group is important for this inhibitory effect. These results suggested that the suppressive effects of methylxanthines against GR transactivation depend on their molecular structure, and that the 1-methyl group and 7-hydrophilic group of theophylline are involved in the suppression of GR transactivation.

In summary, we showed that theophylline attenuated GC-induced hyperglycemia and decreased IL-6 expression by inhibiting GR signaling. Previous studies reported that GR knockout led to reduce adipogenesis during short-term differentiation [44], indicating that GR accelerates adipogenesis. Therefore, theophylline represents a potentially valuable therapeutic agent for obesity-related insulin resistance, but not for adipogenesis.

Conflicts of interest

The authors declare that there are no conflicts of interest

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Hightlight

- Theophylline decreased glucocorticoid-induced IL6 production.
- Theophylline decreased IL6 expression by decreasing C/EBPδ expression.
- Theophylline suppressed the nuclear translocation of glucocorticoid receptor.
- Intake of theophylline ameliorated glucocorticoid-induced hyperglycemia in mice.

Theophylline suppresses interleukin-6 expression by inhibiting glucocorticoid receptor signaling in pre-adipocytes

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Abbreviations: 11β-HSD, 11β-hydroxysteroid dehydrogenases; C/EBP, CCAAT-enhancer-binding protein; DEX, dexamethasone; FFAs, free fatty acids; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid response element; IBMX, 3-isobutyl-1-methylxanthine; IL-6, interleukin-6; LPS, lipopolysaccharide; qPCR, quantitative real-time PCR; TNF, tumor necrosis factor

ABSTRACT

Adipose tissues in obese individuals are characterized by a state of chronic low-grade inflammation. Pre-adipocytes and adipocytes in this state secrete pro-inflammatory adipokines, such as interleukin 6 (IL-6), which induce insulin resistance and hyperglycemia. Theophylline (1,3-dimethylxanthine) exerts anti-inflammatory effects, but its effects on pro-inflammatory adipokine secretion by pre-adipocytes and adipocytes have not been examined. In this study, we found that theophylline decreased IL-6 secretion by 3T3-L1 pre-adipocytes and mouse-derived primary pre-adipocytes. The synthetic glucocorticoid dexamethasone (DEX) induced IL-6 expression in 3T3-L1 pre-adipocytes, and this effect was suppressed by theophylline at the mRNA level. Knockdown of CCAAT/enhancer binding protein (C/EBP) δ inhibited DEX-induced IL-6 expression, and theophylline suppressed C/EBPδ expression. Furthermore, theophylline suppressed transcriptional activity of the glucocorticoid receptor (GR) through suppression of nuclear localization of GR. *In vivo*, glucocorticoid corticosterone treatment (100 μg/mL) increased fasting blood glucose and plasma IL-6 levels in C57BL/6N mice. Theophylline administration (0.1% diet) reduced corticosterone-increased fasting blood glucose, plasma IL-6 levels, and *Il6* gene expression in adipose tissues. These results show that theophylline administration attenuated glucocorticoid-induced hyperglycemia and IL-6 production by inhibiting GR activity. The present findings indicate the potential of theophylline as a candidate therapeutic agent to treat insulin resistance and hyperglycemia.

Keywords: Adipocyte; CCAAT-enhancer-binding proteins; glucocorticoid receptor; interleukin-6; theophylline

1. Introduction

Obesity, which is a risk factor for serious diseases such as insulin resistance, type 2 diabetes mellitus, and cardiovascular disease, is associated with an increase in adipocyte number and size. The accumulation of intracellular lipids during adipocyte differentiation increases adipocyte size [1]. Adipocyte differentiation is regulated by various transcription factors, such as CCAAT/enhancer binding protein (C/EBP)α, C/EBPβ, C/EBPδ, and peroxisome proliferator-activated receptor γ [2]. Adipose tissue in obese individuals is characterized by chronic low-grade systemic inflammation accompanied by elevated secretion of free fatty acids (FFAs) and various pro-inflammatory cytokines, such as interleukin 6 (IL-6), tumor necrosis factor α (TNFα), and plasminogen activator inhibitor-1, which are known as adipokines [3]. These pro-inflammatory adipokines are involved in the induction of insulin resistance, which contributes to hyperglycemia and type 2 diabetes mellitus [4]. Therefore, strategies aimed at decreasing the secretion of pro-inflammatory adipokines by adipocytes are crucial to the prevention and treatment of insulin resistance.

Adipose tissue contains various cells, including pre-adipocytes, mature adipocytes, and macrophages. The expression pattern of pro-inflammatory adipokines varies in these cells. TNFα is mostly produced by macrophages, whereas IL-6 is mainly produced by pre-adipocytes and mature adipocytes in adipose tissue [5]. Furthermore, IL-6 secretion is higher in pre-adipocytes than in mature adipocytes [6]. It has been reported that serum IL-6 levels and insulin resistance are strongly linked [7], and that IL-6 from adipocytes affects various tissues in autocrine and paracrine manners. In adipocyte model 3T3-L1 pre-adipocytes, IL-6 suppressed gene expression, inhibited adiponectin secretion, and acted as an insulin-sensitizing adipokine [8]. IL-6 activated SOC3, resulting in decreased insulin sensitivity in hepatocytes [9]. Furthermore, tail vein injection of IL-6-neutralizing antibodies improved insulin resistance in obese mice [10]. These findings indicated that adipose-derived IL-6 may be a target for prevention strategies against obesity-induced insulin resistance.

Glucocorticoids (GCs), which are steroid hormones secreted by the adrenal cortex, have

been proposed to play both adipogenic and lipolytic roles in adipose tissue [11]. GC action depends not only on blood levels, but also on tissue-specific intracellular metabolic processes catalyzed by 11β-hydroxysteroid dehydrogenases (11β-HSDs). 11β-HSD converts inactive cortisone into active cortisol in humans, or into corticosterone, a major endogenous glucocorticoid, in rodents [12]. The expression of 11β-HSD1 in adipose tissues is elevated in obese human patients [13]. GCs bind to the glucocorticoid receptor (GR) in the cytoplasm. The ligand-bound GR then translocates into the nucleus and binds to glucocorticoid-response elements (GREs) on target gene promoters, resulting in increased expression of the target genes. GCs increased plasma levels of FFAs and expression levels of pro-inflammatory adipokines such as IL-6 and TNFα in white adipose tissue of mice [14, 15]. Furthermore, chronic exposure to GCs was associated with insulin resistance, diabetes, and hepatic steatosis in rodents [16]. Therefore, GC-GR signaling represents a potential therapeutic target against obesity-related insulin resistance and diabetes.

Theophylline (1,3-dimethylxanthine), a methylxanthine drug, was first extracted from tea leaves. Theophylline is widely used worldwide as a therapeutic agent for respiratory diseases [17]. In addition, recent reports have shown that theophylline ameliorated acetic-acid-induced ulcerative colitis by decreasing the levels of pro-inflammatory cytokines in the mouse colon [18]. However, theophylline effects on pro-inflammatory adipokine secretion in pre-adipocytes and adipocytes have not been investigated. In this study, we assessed the molecular effects of theophylline on the secretion and expression levels of IL-6 in 3T3-L1 pre-adipocytes. We demonstrated that theophylline suppressed IL-6 gene expression by inhibiting the transcriptional activity of GR in 3T3-L1 pre-adipocytes. Furthermore, we showed that administration of theophylline decreased GC-induced hyperglycemia and plasma IL-6 levels in mice.

2. Materials and Methods

2.1. Animal experiments

All animal experiments conformed to the protocols approved by the Institutional Animal Care and Use Committee of Shinshu University Animal Experimentation Regulations (Permission Number 280042) and the Guide for Care and Use of Laboratory animals (NIH Publications No. 8023, revised 1978). Five-week-old male C57BL/6N mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and housed under controlled temperature (20 ± 3 °C) with a 12 h light-dark cycle. Mice had free access to food and water. Six-week-old mice were randomly divided into three groups: vehicle group (Veh), corticosterone group (CORT), and theophylline group (TP) (n = 6 in each group). Veh group was fed High-Fat Diet 32 (CLEA Japan, Inc., Tokyo, Japan), and given free access to drinking water. CORT and TP groups were given drinking water containing 100 μg/mL corticosterone. CORT group was fed High-Fat Diet 32, and TP group was fed High-Fat Diet 32 containing 0.1% (w/w) theophylline. Six weeks after the start of the study, the mice were sacrificed under anesthesia, and their plasma and epididymal adipose tissues were harvested.

2.2. Cells and cell culture

Murine 3T3-L1 pre-adipocytes were purchased from JCRB Cell Bank (Osaka, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine serum, 100 μg/mL streptomycin, and 100 units/mL penicillin. The cells were maintained at 37 °C in the atmosphere of 95% air and 5% CO_2 and at 98% humidity. The cells were incubated with the synthetic glucocorticoid dexamethasone (DEX; 0.5 μM), 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM), and insulin (10 μg/mL) in DMEM containing high levels of glucose (4.5 g/L glucose) and 10% fetal bovine serum for 24 h.

2.3 Primary pre-adipocyte cultures

Primary pre-adipocyte cultures were generated as previously described [19], with minor

modifications. Briefly, primary pre-adipocytes were isolated from epididymal adipose tissue of male mice (ICR; 4-week-old). After digestion with collagenase II and centrifugation, pre-adipocytes were cultured in DMEM. When pre-adipocytes were fully confluent, they were treated with theophylline in the presence of 0.5 μM DEX for 24 h.

2.4. siRNA

Double-stranded siRNAs for C/EBPδ were chemically synthesized (Sigma-Aldrich, Saint Louis, MO). Target sequences for siRNA duplexes were as follows: siC/EBPδ#1, 5′-CGACTTCAGCGCCTACATT-3′ and siC/EBPδ#2, 5′-CGCAGACAGTGGTGAGCTT-3′. The duplexes (20 nM) were transiently transfected into 3T3-L1 pre-adipocytes using Lipofectamine RNAiMAX reagent (Invitrogen; Carlsbad, CA) and Opti-MEM (Thermo Fisher Scientific, Lafayette, CO) for 24 h, according to the manufacturer's protocol.

2.5 Plasmids

The consensus sequence of androgen-responsive element (ARE) is commonly recognized by GR [20]. Accordingly, we used the pARE2 [21] reporter assay vector to determine transcriptional activity of GR.

2.6. Measurement of secreted IL-6

The levels of IL-6 secreted by 3T3-L1 pre-adipocytes and plasma IL-6 levels in mice were determined by enzyme-linked immunosorbent assays (ELISA). A rat monoclonal anti-IL-6 antibody (catalog number MAB406, 1:10,000 dilution; R&D Systems, Minneapolis, MN) was coated on 96-well plates at 4 °C overnight. The plates were washed with PBS and incubated with the sample (100 μL) at 37 °C for 2 h. Washed plates were incubated with a biotinylated goat polyclonal anti-IL-6 antibody (Cat# BAF406, 1:1,000 dilution; R&D Systems) at 37 °C for 2 h, followed by further incubation with horseradish peroxidase-conjugated streptavidin (N100;

1:10,000 dilution; Thermo Fisher Scientific, Waltham, MA) at 37 °C for 1 h. The plates were washed and 3,3′,5,5′-tetramethylbenzidine (100 μL; Sigma-Aldrich) was added to each well. Ten minutes later, the reaction was stopped using 2N H_2SO_4. Absorbance at 450 nm was measured in the plates by using a multi-plate reader (Bio-Rad, Richmond, CA).

2.7. Western blotting

3T3-L1 pre-adipocytes were incubated with theophylline in the presence of 0.5 μM DEX for 2 h or 24 h. SDS-PAGE and western blotting were performed as described previously [22]. Cell lysates were analyzed by western blotting using the following rabbit polyclonal antibodies: anti-C/EBPδ (Cat# sc-9315) and anti-C/EBPβ (Cat# sc-150; Santa Cruz Biotechnology, Santa Cruz, CA), anti-GR (Cat# 12041S; Cell Signaling, Danvers, MA), anti-IL6 (Cat# ab6672), and anti-Histone H3 (Cat# ab1791; Abcam, Cambridge, UK). In addition, a mouse monoclonal anti-β-actin antibody (Cat# sc-47778; Santa Cruz Biotechnology) was used. The immunoreactive proteins were reacted with Immunostar LD (Wako, Osaka, Japan) and visualized using Ez-Capture MG (ATTO Co., Tokyo, Japan).

2.8. Quantitative real-time PCR (qPCR)

Total RNA was extracted from 3T3-L1 pre-adipocytes using TRIzol (Invitrogen). cDNAs were synthesized using RevaTra Ace and subjected to qPCR using sets of specific primers (see Table S1 for the sequences). qPCR was performed with SYBR PremixEx Taq II (Takara Bio, Shiga, Japan) using a two-step PCR method on a Thermal Cycler Dice real-time system (Takara Bio.). The relative expression levels of each gene were calculated using the $2^{-\Delta\Delta Ct}$ (CT, cycle threshold) method, and data were normalized to the expression level of *Gapdh*, which was used as endogenous control.

2.9. Luciferase reporter assay

The luciferase reporter assay was performed as described previously [23]. 3T3-L1 pre-adipocytes were transiently transfected with the reporter vectors pARE2-TATA-Luc and pRL-SV40 (control reporter vector; Promega) using Lipofectamine 2000 for 24 h. After the medium was replaced, cells were incubated with 0.5 μM DEX and theophylline. Transfection efficiency was normalized by luminescence levels in pRL-SV40-transfected cells. Firefly and Renilla luciferase activities were measured using a Dual Luciferase reporter assay kit and GloMax 20/20 Luminometer (Promega). Data were expressed as relative light units (RLU; firefly levels divided by Renilla levels).

2.10. Immunostaining

Immunostaining was performed as described previously [22]. In brief, fixed cell samples were incubated with a rabbit polyclonal anti-GR antibody in phosphate buffered saline containing 3% bovine serum albumin at 4 °C overnight, followed by the incubation with Alexa Fluor 488-conjugated secondary anti-rabbit IgG at room temperature for 1 h. The nuclei were stained with Hoechst 33258 (1 μg/mL; Dojindo Lab, Kumamoto, Japan) at room temperature for 10 min, followed by inspection using an EVOS FL Auto microscope (Thermo Fisher Scientific).

2.11. Subcellular fractionation

Confluent 3T3-L1 pre-adipocytes were incubated with theophylline, RU486 (1 μM), or xanthine (100 nM) in the presence of DEX for 30 min. The cells were lysed in hypotonic buffer containing 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Hepes-NaOH (pH 7.5) and protease cocktail (Nacalai Tesque, Kyoto, Japan). Subcellular fractionation was performed as described previously [21]. Proteins in each fraction were analyzed by western blotting.

2.12. Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed as described previously [23]. In brief, 3T3-L1 pre-adipocytes were incubated with 100 nM theophylline and 0.5 μM DEX for 30 min. The promoter region of the *Cebpd* gene was amplified by qPCR using a set of specific primers (see supplemental Table S1 for sequences). qPCR profiles were obtained after running the following program: 94 °C for 30 s, 65 °C for 15 s, 72 °C for 20 s. The relative amounts of each promoter region were calculated using the $2^{-\Delta\Delta Ct}$ method, and the data were normalized to that of the input sample.

2.13. Statistical analysis

Data were analyzed using the Student's *t* test or one-way analysis of variance (ANOVA), with Turkey's post hoc test, if appropriate. Statistical analysis was performed using JMP statistical software version 11.2.0 (SAS Institute. Cary, NC). Data are expressed as the mean ± standard deviation (S.D.). All statistical analyses were conducted with a significance level of $\alpha = 0.05$ ($P < 0.05$).

3. Results

3.1. Theophylline decreases IL-6 secretion by 3T3-L1 pre-adipocytes

We examined the effect of theophylline on IL-6 secretion in 3T3-L1 pre-adipocytes. IL-6 secretion levels by 3T3-L1 pre-adipocytes were increased by the treatment with a differentiation-inducing reagent that contained DEX, IBMX, and insulin. At concentrations above 100 nM, theophylline decreased the reagent-induced secretion of IL-6 (Fig. 1A). In addition, theophylline concentration-dependently suppressed the reagent-induced increase in IL-6 protein (Fig. 1B). To identify the active component of the differentiation-inducing reagent that was responsible for inducing IL-6 expression, 3T3-L1 pre-adipocytes were treated with DEX, IBMX, or insulin. IL-6 protein level was induced by DEX, but not by IBMX or insulin (Fig.

1C). Furthermore, theophylline suppressed DEX-induced increase in IL-6 expression at protein and mRNA levels (Fig. 1D and 1E). These results indicated that theophylline suppressed DEX-induced IL-6 expression at the transcriptional level in 3T3-L1 pre-adipocytes.

3.2. Theophylline decreases IL-6 secretion by primary pre-adipocytes

We examined the effect of theophylline on the levels of secreted and intracellular IL-6 in primary pre-adipocytes from male and female mice. DEX increased the level of secreted IL-6 in male and female pre-adipocytes fourfold and twofold, respectively, whereas theophylline counteracted this effect of DEX (Fig. 2A). In addition, theophylline suppressed protein level of IL-6 in both male and female primary pre-adipocytes (Fig. 2B). These results indicated that theophylline suppressed DEX-induced IL-6 expression not only in mouse 3T3-L1 pre-adipocytes, but also in primary pre-adipocytes.

3.3.Theophylline suppresses C/EBPδ-regulated IL-6 promoter activity

The promoter region of the *Il6* gene contains a CCAAT box motif, which is the binding site of C/EBPs [24]. We found that DEX increased C/EBPδ protein level in 3T3-L1 pre-adipocytes (Fig. 3A). Furthermore, to determine whether DEX-induced C/EBPδ involved in IL-6 expression, 3T3-L1 pre-adipocytes were transiently transfected with two siRNAs with different sequences to avoid off-target effects. C/EBPδ siRNA reduced DEX-induced protein expression of C/EBPδ (Fig. 3A), and knockdown of C/EBPδ attenuated DEX-induced IL-6 secretion and protein expression (Fig. 3B and 3C). These results suggested that DEX up-regulated *Il6* promoter activity through C/EBPδ, and that theophylline suppressed DEX-induced *Il6* promoter activity in 3T3-L1 pre-adipocytes.

3.4. Theophylline decreases C/EBPδ expression at the mRNA level

We examined the effect of theophylline on C/EBPδ expression in 3T3-L1 pre-adipocytes.

Theophylline decreased DEX-induced increase in C/EBPδ protein level in a concentration-dependent manner (Fig. 4A). However, DEX and theophylline did not affect C/EBPβ protein level. Although the proteasome inhibitor MG132 increased C/EBPδ protein levels in the absence of theophylline, this compound did not restore C/EBPδ protein level, reduced by theophylline, to normal levels (Fig. 4B). Furthermore, we observed that theophylline suppressed DEX-induced gene expression of *Cebpd* (Fig. 4C). These results indicated that theophylline suppressed DEX-induced IL-6 expression by decreasing C/EBPδ expression in 3T3-L1 pre-adipocytes.

3.5. Theophylline suppresses the transcriptional activity of GR

DEX is a GR agonist, therefore it was expected that the GR antagonist RU486 inhibited DEX-induced increase in the levels of secreted and intracellular IL-6 (Fig. 5A). We then examined whether theophylline suppressed the transcriptional activity of GR using a luciferase reporter assay for GR transactivation. Theophylline suppressed DEX-enhanced GR transactivation in a concentration-dependent manner (Fig. 5B). However, theophylline did not decrease GR protein levels (Fig. 5C). We assumed that theophylline regulated the binding of GR to glucocorticoid-responsive genes. Multi-genome analysis using the MAPPER database [25, 26] showed that the promoter region of *Cebpd* contained two GRE candidates: one located at −2110 to −2096, and the other at −1301 to −1289 of *Cebpd*. We performed the ChIP assay using 3T-L1 cell lysates and three sets of PCR primers, as shown in Fig. 5D (left panel). When the ChIP assay was performed using the primer sets P2 and P3, but not set P1, DEX increased the relative level of interaction between GR and DNA (Fig. 5D, right panel). Theophylline decreased DEX-induced interaction between GR and DNA. These results indicated that theophylline suppressed DEX-induced binding of GR to DNA.

3.6. Theophylline decreases the nuclear localization of GR

We investigated the effect of theophylline on the intracellular localization of GR in 3T3-L1 pre-adipocytes using immunofluorescence microscopy. GR was distributed throughout the cell in the absence of DEX, and DEX increased the nuclear localization of GR. In contrast, treatment with theophylline resulted in decreased nuclear localization of GR in the presence of DEX (Fig. 6A). In addition, cellular fractionation showed that RU486 decreased DEX-induced nuclear accumulation of GR (Fig. 6B). Likewise, theophylline decreased its nuclear accumulation. However, xanthine did not suppress nuclear import of GR in the presence of DEX. These results indicated that theophylline suppressed GR transactivation by decreasing nuclear accumulation of GR.

3.7. Theophylline interacts with GR in 3T3-L1 pre-adipocytes

3T3-L1 pre-adipocytes were treated with six xanthine derivatives, including theophylline, to determine their effect on GR transactivation (Fig. 7A). Xanthine has three methylation sites at positions 1, 3, and 7. Theophylline-7-acetic acid and 1-methylxanthine, as well as theophylline, suppressed DEX-enhanced GR transactivation in 3T3-L1 pre-adipocytes (Fig. 7B). In contrast, xanthine, 3,7-dimethylxanthine and 1,3,7-trimethylxanthine did not affect DEX-enhanced GR transactivation. When the effects of xanthine derivatives on protein levels of C/EBPδ and levels of secreted IL-6 were examined, similar results were obtained (Fig. 7C and 7D). To determine whether theophylline interacted with GR, theophylline-7-acetic acid was immobilized on Sepharose through its carboxy group. When 3T3-L1 pre-adipocytes lysates were incubated with theophylline-affinity-resin with or without theophylline, the resin pulled down GR protein in the absence of theophylline (Fig. 7E). In contrast, the interaction between the resin and GR was diminished by the incubation with theophylline. The resin did not pull down β-actin. These results suggested that theophylline suppressed GR transactivation through the interaction with GR protein.

3.8. Intake of theophylline decreases corticosterone-induced IL-6 expression in adipose tissues

To examine the effect of theophylline *in vivo*, mice were given drinking water with or without corticosterone and then fed a theophylline-containing high-fat diet for 6 weeks. Corticosterone intake led to an increase in the weight of epididymal adipose tissue, whereas theophylline decreased the corticosterone-mediated increase in adipose tissue weight (Fig. 8A). We then analyzed fasting blood glucose levels in mice. Corticosterone was found to increase blood glucose levels (188 ± 29.3 mg/dL), whereas theophylline attenuated corticosterone-mediated increase in fasting blood glucose (103 ± 16.3 mg/dL) (Fig. 8B). Furthermore, we measured IL-6 levels in plasma and adipose tissue in mice. Theophylline decreased corticosterone-induced increase in IL-6 plasma concentration (Fig. 8C). Corticosterone further enhanced *Il6* mRNA level in epididymal adipose tissues, whereas theophylline intake tended to decrease that corticosterone effect (Fig. 8D). These results indicated that theophylline intake suppressed IL-6 expression in epididymal adipose tissues of mice.

4. Discussion

Obesity is a state of chronic low-grade inflammation, which promotes the secretion of certain pro-inflammatory adipokines by adipose tissues. These pro-inflammatory adipokines exert a negative influence on other tissues, such as the liver, skeletal muscle, and immune tissues, resulting in increased risk of hyperglycemia, type 2 diabetes mellitus, and cardiovascular diseases. Therefore, the suppression of pro-inflammatory adipokines represents a promising therapeutic strategy against obesity-related diseases. In this study, we focused on IL-6, one of the pro-inflammatory adipokines secreted by pre-adipocytes, and demonstrated the molecular mechanism by which theophylline suppressed the expression of IL-6 in pre-adipocytes.

IL-6 is secreted by adipose tissues, skeletal muscle, and the liver. The level of expression of IL-6 in the adipose tissue correlates with the body mass index, and plasma IL-6 is elevated in

obese and diabetic subjects [27, 28]. Furthermore, the consumption of high-fat diet has been shown to induce insulin resistance and obesity through adipose tissue-derived IL-6 in mice [29]. Injection of IL-6 has been demonstrated to promote hyperglycemia in rats [30]. IL-6 levels are associated with the development of chronic hyperglycemia and insulin resistance in human patients after acute pancreatitis [31]. Several studies have described the molecular mechanism by which IL-6 induces hyperglycemia and insulin resistance in the liver and in adipocytes. Chronic exposure to IL-6 inhibited insulin-dependent tyrosine phosphorylation of the insulin receptor, as well as insulin receptor substrate-1 and -2, resulting in decreased glucose uptake and glycogenesis in the liver [32]. Furthermore, IL-6 inhibited the expression of the adiponectin gene that exerted insulin-sensitizing effects in 3T3-L1 pre-adipocytes [8]. Macrophage recruitment to adipose tissue contributes to insulin resistance; in this context, IL-6 has been found to promote the recruitment of macrophages to adipose tissue in obese mice [33]. In the present study, the administration of theophylline resulted in the attenuation of corticosterone-induced hyperglycemia and IL-6 production (Fig. 8). Therefore, our results suggest that theophylline attenuated blood glucose levels up-regulated by GC via suppressing adipose tissue-derived IL-6 levels.

Theophylline suppressed the nuclear localization of GR, but not its protein level. GCs play a critical role in both adipogenesis and lipolysis in adipocytes. Short-term exposure to GC promoted lipolysis and release of FFAs from adipocytes [34], whereas blood FFAs induced hepatic insulin resistance [35]. In contrast, prolonged exposure to GCs induced hyperglycemia and insulin resistance, and knockdown of GR attenuated GC-mediated insulin resistance [16, 36]. Prenatal exposure to GC increased the expression of IL-6, IL-1β, and TNFα in adipose tissues [15]. Furthermore, the consumption of a high-fat diet increased the expression not only of IL-6, but also that of 11β-HSD1 in adipose tissues [37], indicating that high-fat intake increased intracellular levels of active GC in adipocytes. A selective inhibitor of 11β-HSD1 was shown to decrease serum IL-6 levels and ameliorate high-fat diet-induced insulin resistance in

obese rats [37]. Therefore, IL-6 is involved in insulin resistance or hyperglycemia mediated by the dysregulation of GC-GR signaling. Previous studies have described anti-inflammatory effects of theophylline. Theophylline reduced the production of IL-6 in lipopolysaccharide (LPS)-treated primary human lung fibroblasts from patients with chronic obstructive pulmonary disease [38]. The authors suggested that theophylline suppressed the expression of IL-6 by inhibiting LPS-Toll-like receptor signaling. In addition, it has been suggested that anti-inflammatory effects of theophylline are caused by the inhibition of reactive oxygen species production [20]. Our results indicate that theophylline acted as an inhibitor of GR transactivation and thereby decreased IL-6 expression in 3T3-L1 pre-adipocytes.

Knockdown of C/EBPδ abrogated DEX-induced IL-6 expression. C/EBPδ elicited robust induction of pro-inflammatory response in macrophages. LPS-induced *IL6* mRNA expression was decreased in *Cebpd*$^{-/-}$ macrophages and glial cells [39]. GR is the main transcription factor involved in the regulation of C/EBPδ gene expression in adipocytes [40]. Several studies have reported that cytokines induced nuclear localization of GR in a GC-independent manner. TNF promotes insulin resistance and activates GR signaling by inducing the nuclear localization of the GR, without the requirement for GCs [36]. In addition, IL-13 promotes the nuclear translocation of the GR and enhances DEX-stimulated nuclear import of GR in human airway smooth muscle cells [41]. In this study, we showed that theophylline decreased not only GR nuclear localization, but also GR binding to DNA in the presence of DEX (Fig. 5D). Taken together, these results suggest that theophylline suppressed GC- and cytokine-induced GR transactivation by decreasing the interaction between GR and DNA.

DEX-induced GR transactivation was suppressed by methylxanthines in which the 1-position was methylated and the 7-position was substituted by a hydrophilic group. Methylxanthine derivatives exert various pharmacological effects, such as antagonism of adenosine receptors, inhibition of phosphodiesterase, modulation of GABA receptors, and regulation of intracellular calcium levels [30]. Structure-activity relationships of methylxanthines show that their 1-methyl

group is pivotal for their inhibitory effects on phosphodiesterase [42]. Furthermore, methylxanthines have been reported to exert structure-specific effects. The inhibitory effect of methylxanthines, especially of 3,7-dimethylxanthine and 1,7-dimethylxanthine at physiological concentrations, against poly(ADPribose)polymerase-1 has been shown [43], which indicated that 7-methyl group is important for this inhibitory effect. These results suggested that the suppressive effects of methylxanthines against GR transactivation depend on their molecular structure, and that the 1-methyl group and 7-hydrophilic group of theophylline are involved in the suppression of GR transactivation.

In summary, we showed that theophylline attenuated GC-induced hyperglycemia and decreased IL-6 expression by inhibiting GR signaling. Previous studies reported that GR knockout led to reduce adipogenesis during short-term differentiation [44], indicating that GR accelerates adipogenesis. Therefore, theophylline represents a potentially valuable therapeutic agent for obesity-related insulin resistance, but not for adipogenesis.

Conflicts of interest

The authors declare that there are no conflicts of interest

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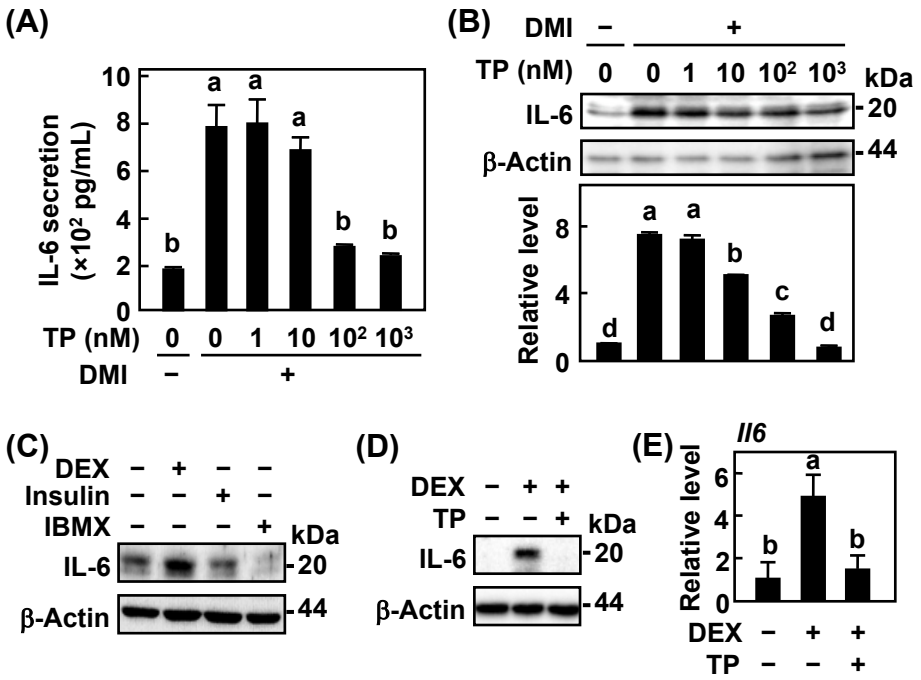

Fig. 1. Expression pattern of IL-6 following the treatment with theophylline.
(A) IL-6 secretion by 3T3-L1 pre-adipocytes treated with DMI and theophylline (TP; 100 nM) for 24 h. (B) Western blotting of IL-6 in 3T3-L1 pre-adipocytes treated with DMI and TP (100 nM). (C) IL-6 protein level in 3T3-L1 pre-adipocytes treated with dexamethasone (DEX; 0.5 μM), 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM), or insulin (10 μg/mL insulin) for 24 h. (D) Western blotting of IL-6 in 3T3-L1 pre-adipocytes treated with 0.5 μM DEX and 100 nM TP for 24 h. (E) *Il6* gene expression in 3T3-L1 pre-adipocytes treated with 0.5 μM DEX and 100 nM TP for 24 h. For western blotting, the intensity of each band was quantified using ImageJ 1.44, and the ratio of the level of each protein was normalized to that of β-actin (loading control) level. Data are presented as the mean ± S.D. ($n = 3$). Values in groups indicated by different letters are significantly different ($P < 0.05$). The results are representative of data from three independent experiments. DMI means 0.5 μM DEX, 0.5 mM IBMX, and 10 μg/mL insulin.

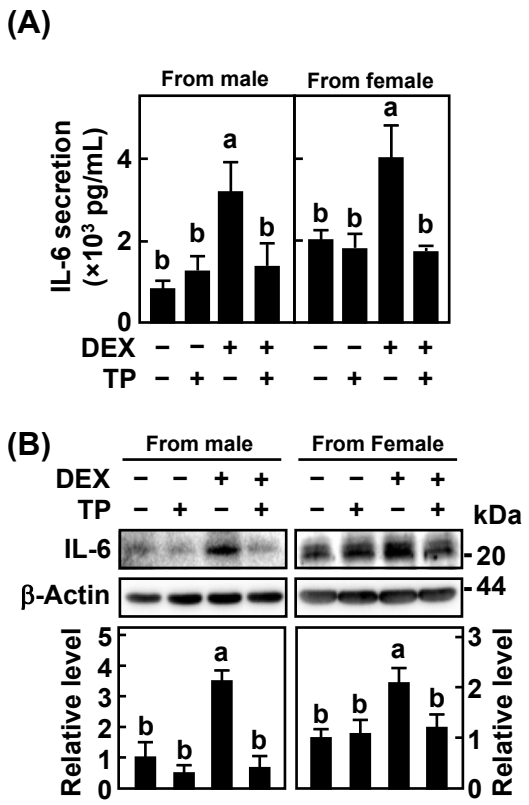

Fig. 2. Suppressive effect of theophylline on primary pre-adipocytes from male and female mice. (A) IL-6 secretion by mouse primary pre-adipocytes treated with theophylline (TP; 100 nM) in the presence of dexamethasone (DEX; 0.5 μM) for 24 h. (B) Western blotting of IL-6 in mouse primary pre-adipocytes treated with 0.5 μM DEX and 100 nM TP for 24 h. For western blotting, the intensity of each band was quantified using ImageJ 1.44, and the ratio of the level of each protein was normalized to that of β-actin (loading control) level. Data are presented as the mean ± S.D. ($n = 3$). Values in groups indicated by different letters are significantly different ($P < 0.05$). The results are representative of data from three independent experiments.

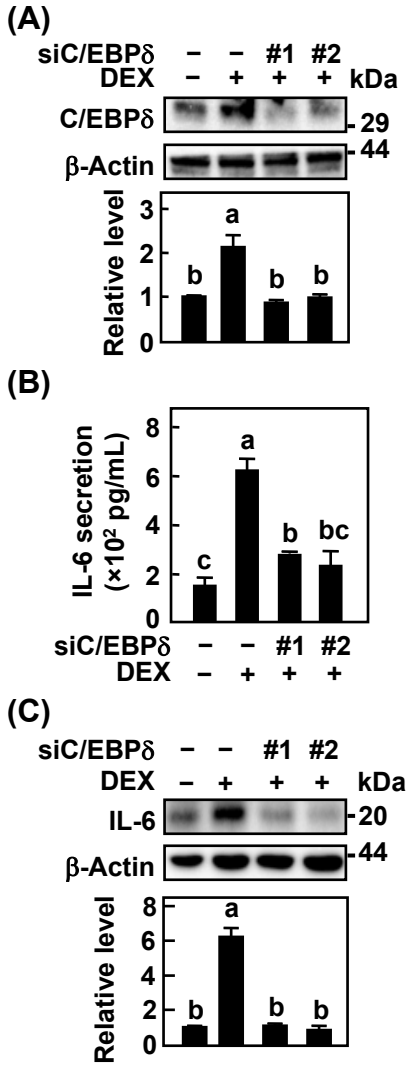

Fig. 3. Involvement of C/EBPδ in dexamethasone-induced Il6 expression.
(A) Western blotting of C/EBPδ in 3T3-L1 pre-adipocytes transiently transfected with control siRNA or siRNA against C/EBPδ (siC/EBPδ). (B) IL-6 secretion by 3T3-L1 pre-adipocytes after treatment with siRNA and dexamethasone (DEX; 0.5 μM) for 24 h. (C) Western blot analysis of IL-6 in 3T3-L1 pre-adipocytes treated with siRNA and 0.5 μM DEX for 24 h. For western blotting, the intensity of each band was quantified using ImageJ 1.44, and the ratio of each band was normalized to that of β-actin (loading control) level. Data are presented as the mean ± S.D. ($n = 3$). Values in groups indicated by different letters are significantly different ($P < 0.05$). The results are representative of data from three independent experiments.

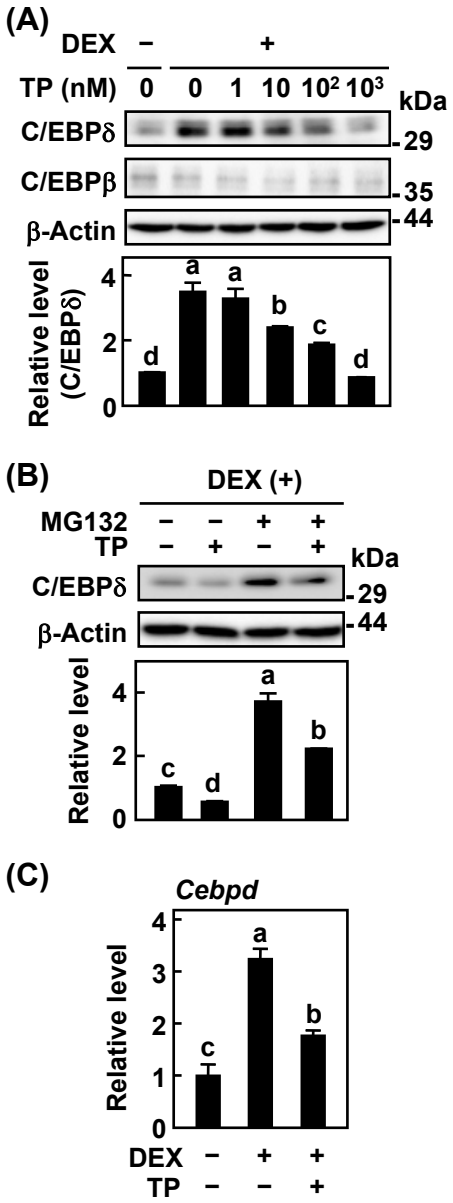

Fig. 4. Effect of theophylline on the expression of C/EBPδ.
(A) Western blotting of C/EBPδ in 3T3-L1 pre-adipocytes treated with theophylline (TP; 100 nM) for 2 h. (B) Western blotting of C/EBPδ in 3T3-L1 pre-adipocytes after TP treatment with or without MG132 (10 μM) for 2 h; **P* < 0.05 vs. TP(−). (C) *Cebpd* expression in 3T3-L1 pre-adipocytes treated with dexamethasone (DEX; 0.5 μM) and TP (100 nM) for 2 h. For western blotting, the intensity of each band was quantified using ImageJ 1.44, and the ratio of each protein level was normalized to that of β-actin (loading control) level. Data are presented as the mean ± S.D. ($n = 3$). Values in groups indicated by different letters are significantly different ($P < 0.05$). The results are representative of data from three independent experiments.

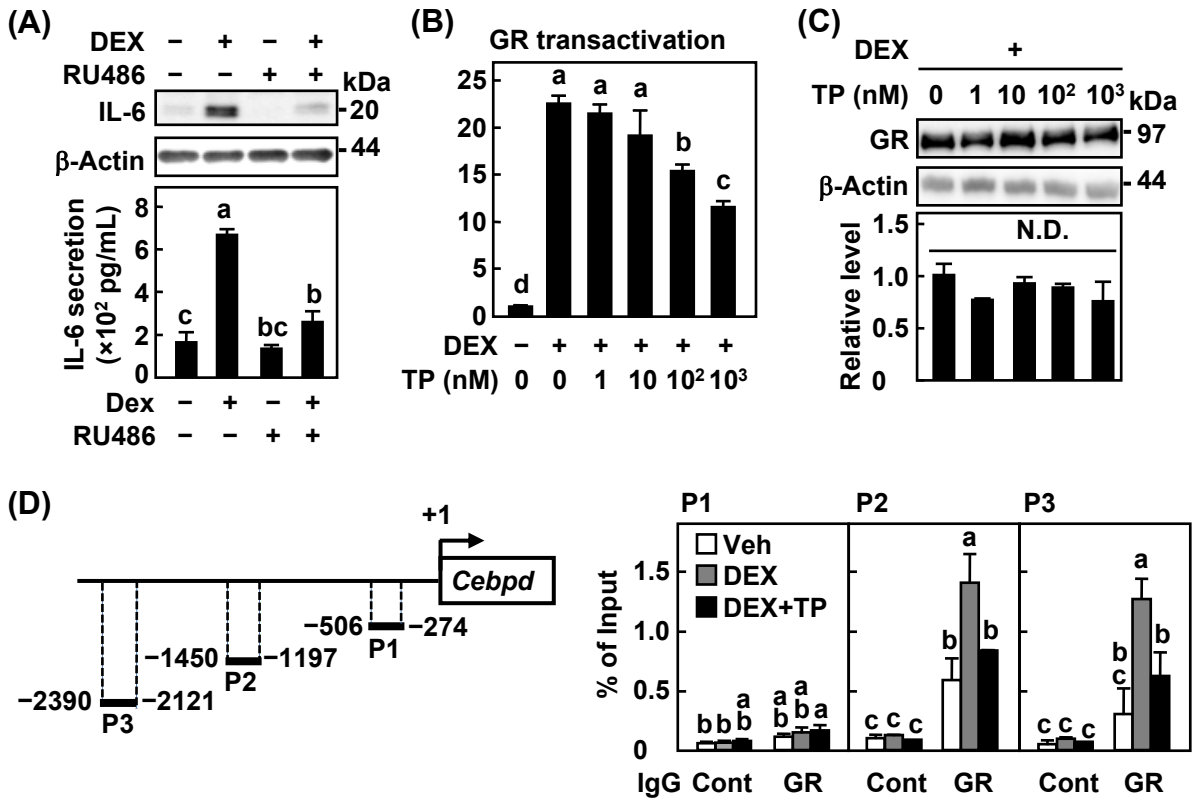

Fig. 5. Effect of theophylline on the transcriptional activity of GR.
(A) Western blotting of IL-6 in 3T3-L1 pre-adipocytes after treatment with dexamethasone (DEX; 0.5 μM) and 1 μM RU486, an antagonist of the glucocorticoid receptor (GR). (B) Transcriptional activity of GR in 3T3-L1 pre-adipocytes. pARE2-TATA-Luc reporter vectors were transfected into 3T3-L1 pre-adipocytes. Then, cells were incubated with 0.5 μM DEX and theophylline (TP) for 3 h. (C) Western blotting of GR in 3T3-L1 pre-adipocytes treated with 0.5 μM DEX and TP for 3 h. (D) Schematic representation of the promoter regions of the *Cebpd* gene (left panel). Protein-DNA complexes from 3T3-L1 pre-adipocytes were immunoprecipitated with control (Cont) IgG or anti-GR (GR) IgG. Immunoprecipitated DNA was analyzed by qPCR (right panel). For western blotting, the intensity of each band was quantified using ImageJ 1.44, and the ratio of each protein level was normalized to that of β-actin (loading control) level. Data are presented as the mean ± S.D. ($n = 3$). Values in groups indicated by different letters are significantly different ($P < 0.05$). The results are representative of data from three independent experiments.

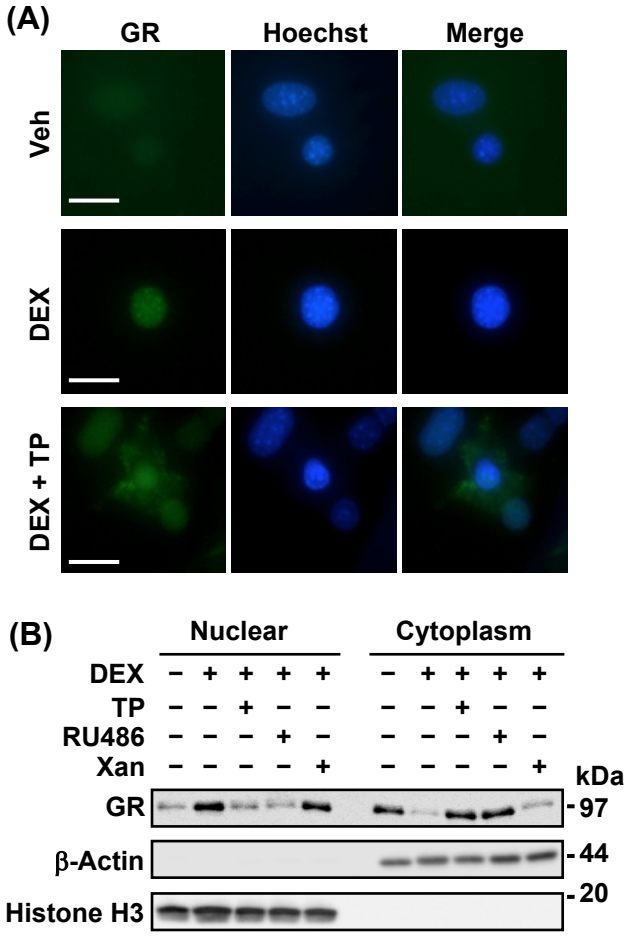

Fig. 6. Intracellular localization of GR in the presence of theophylline.
(A) Immunofluorescence analysis of the glucocorticoid receptor (GR, green) in 3T3-L1 pre-adipocytes incubated with or without dexamethasone (DEX; 0.5 μM) and theophylline (TP; 100 nM) for 30 min. Nuclei were stained with Hoechst33258 (blue). Scale bar = 20 μm. (B) Subcellular distribution of GR in 3T3-L1 pre-adipocytes treated with TP (100 nM), xanthine (100 nM) or RU486 (1 μM) in the presence of 0.5 μM DEX for 30 min. Nuclear and cytoplasmic proteins were analyzed by western blotting with antibodies against GR, histone H3 (a nuclear marker), and β-actin (a cytoplasm marker). Images are representative of three independent experiments.

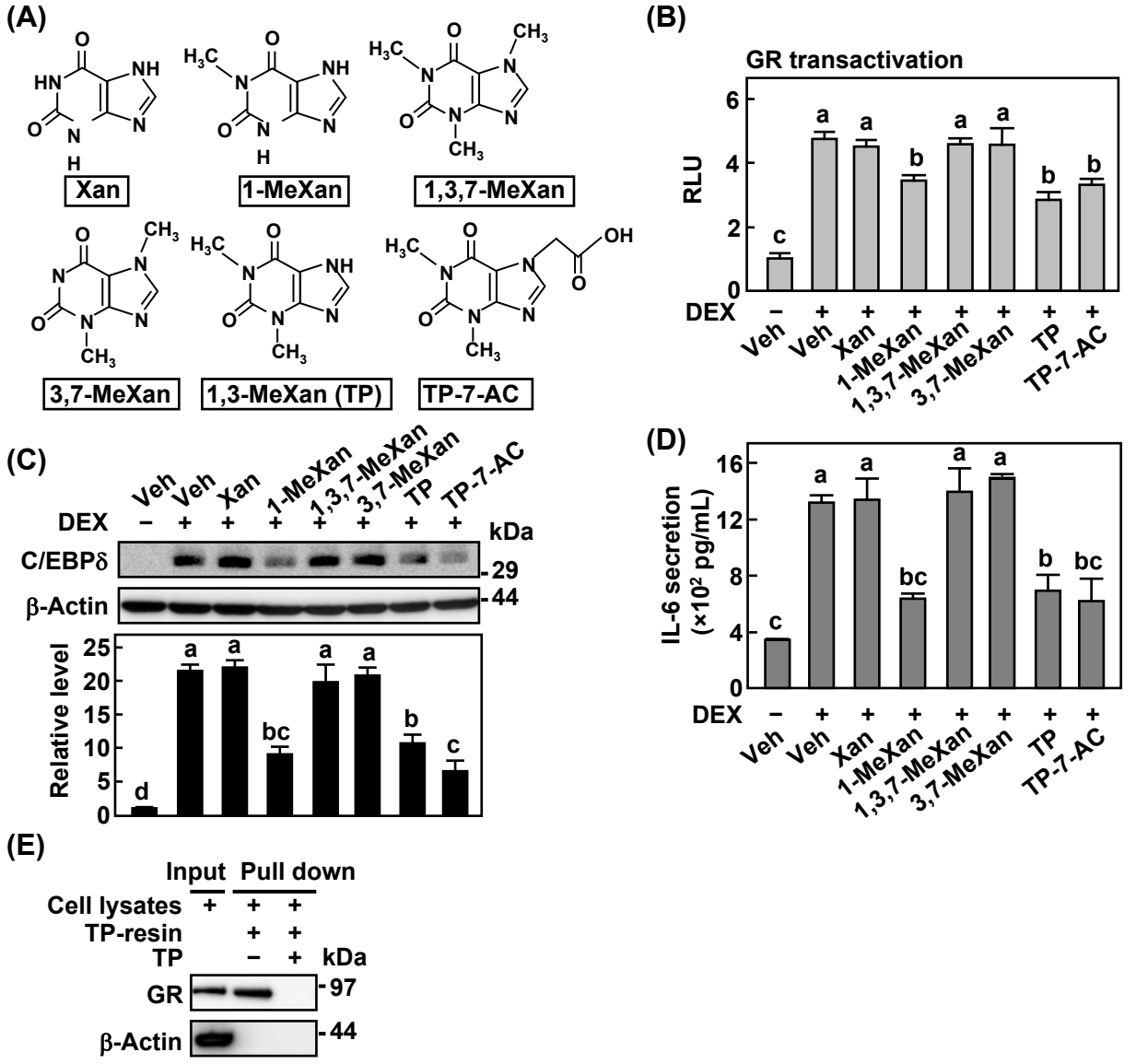

Fig. 7. Suppressive effects of theophylline and xanthine derivatives on GR transactivation. (A) Chemical structures. (B) Transcriptional activity of the glucocorticoid receptor (GR) in 3T3-L1 pre-adipocytes; luciferase reporter vectors were transfected into 3T3-L1 pre-adipocytes, which were then incubated with xanthine derivatives (100 nM) in the presence of dexamethasone (DEX; 0.5 μM) for 3 h. (C) Western blotting analysis of C/EBPδ in 3T3-L1 pre-adipocytes treated with xanthine derivatives (100 nM) in the presence of 0.5 μM DEX for 3 h. The intensity of each band was quantified using ImageJ 1.44, and the ratio of each protein level was normalized to that of β-actin (loading control) level. (D) IL-6 secretion by 3T3-L1 pre-adipocytes treated with xanthine derivatives (100 nM) in the presence of 0.5 μM DEX for 24 h. (E) Proteins interacting with theophylline-affinity resin (TP-resin) were pulled down and analyzed by western blotting. Data are presented as the mean ± S.D. (n = 3). Values in groups indicated by different letters are significantly different ($P < 0.05$). The results are representative of data from three independent experiments.

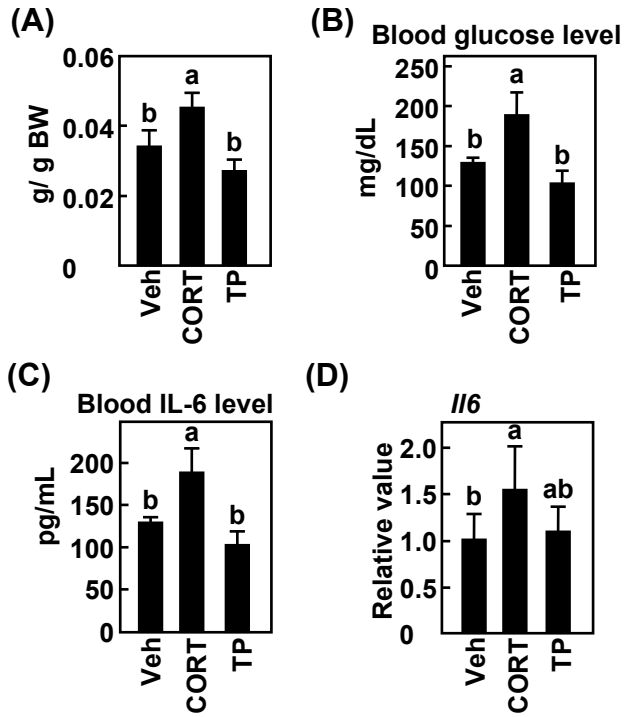

Fig. 8. In vivo effect of theophylline on blood glucose level and plasma IL-6 level.
(A) The weights of epididymis adipose tissue were normalized to body weight (BW). Veh group received high-fat diet and water. CORT group received high-fat diet and corticosterone-containing water. TP group received theophylline-containing high-fat diet and corticosterone-containing water for 6 weeks (n = 6 per group). (B) Blood glucose levels in mice fasted for 12 h. (C) Plasma IL-6 levels in mice of different experimental groups. (D) *Il6* expression in epididymis adipose tissues of mice of different experimental groups. Data are presented as the mean ± S.D. (n = 6). Values in groups indicated by different letters are significantly different ($P < 0.05$).

Table S1: Primer Sequences

	Forward; 5´ to 3´	Reverse; 5´ to 3´
Cebpd	GATCTGCACGGCCTGTTGTA	CTCCACTGCCCACCTGTCA
Gapdh	ACAACTTTGGCATTGTGGAA	GATGCAGGGATGATGTTCTG
Il6	AGTCCGGAGAGGAGACTTCA	ATTTCCACGATTTCCCAGAG
P1	TGCTGAACCTAACCTCGACG	CAGGACGCCTTCAGACATAG
P2	CACGGTAGCCTGTCCTTCTG	GTTAAAGCCAGCGCACATGT
P3	CCATTCTCATTCGAACCGCC	ATCTCCCTGAGGTCCTGCTT