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4 Theophylline suppresses interleukin-6 expression by inhibiting glucocorticoid receptor
5 signaling in pre-adipocytes
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46 **Abbreviations:** 11 β -HSD, 11 β -hydroxysteroid dehydrogenases; C/EBP, CCAAT-enhancer-
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48 binding protein; DEX, dexamethasone; FFAs, free fatty acids; GC, glucocorticoid; GR,
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50 glucocorticoid receptor; GRE, glucocorticoid response element; IBMX, 3-isobutyl-1-
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52 methylxanthine; IL-6, interleukin-6; LPS, lipopolysaccharide; qPCR, quantitative real-time
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54 PCR; TNF, tumor necrosis factor
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64 **ABSTRACT**

65 Adipose tissues in obese individuals are characterized by a state of chronic low-grade
66 inflammation. Pre-adipocytes and adipocytes in this state secrete pro-inflammatory adipokines,
67 such as interleukin 6 (IL-6), which induce insulin resistance and hyperglycemia. Theophylline
68 (1,3-dimethylxanthine) exerts anti-inflammatory effects, but its effects on pro-inflammatory
69 adipokine secretion by pre-adipocytes and adipocytes have not been examined. In this study, we
70 found that theophylline decreased IL-6 secretion by 3T3-L1 pre-adipocytes and mouse-derived
71 primary pre-adipocytes. The synthetic glucocorticoid dexamethasone (DEX) induced IL-6
72 expression in 3T3-L1 pre-adipocytes, and this effect was suppressed by theophylline at the
73 mRNA level. Knockdown of CCAAT/enhancer binding protein (C/EBP) δ inhibited DEX-
74 induced IL-6 expression, and theophylline suppressed C/EBP δ expression. Furthermore,
75 theophylline suppressed transcriptional activity of the glucocorticoid receptor (GR) through
76 suppression of nuclear localization of GR. *In vivo*, glucocorticoid corticosterone treatment (100
77 $\mu\text{g}/\text{mL}$) increased fasting blood glucose and plasma IL-6 levels in C57BL/6N mice.
78 Theophylline administration (0.1% diet) reduced corticosterone-increased fasting blood glucose,
79 plasma IL-6 levels, and *Il6* gene expression in adipose tissues. These results show that
80 theophylline administration attenuated glucocorticoid-induced hyperglycemia and IL-6
81 production by inhibiting GR activity. The present findings indicate the potential of theophylline
82 as a candidate therapeutic agent to treat insulin resistance and hyperglycemia.
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104 **Keywords:** Adipocyte; CCAAT-enhancer-binding proteins; glucocorticoid receptor; interleukin-
105 6; theophylline
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112 **1. Introduction**
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124 Obesity, which is a risk factor for serious diseases such as insulin resistance, type 2 diabetes
125 mellitus, and cardiovascular disease, is associated with an increase in adipocyte number and
126 size. The accumulation of intracellular lipids during adipocyte differentiation increases
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128 adipocyte size [1]. Adipocyte differentiation is regulated by various transcription factors, such
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130 as CCAAT/enhancer binding protein (C/EBP) α , C/EBP β , C/EBP δ , and peroxisome proliferator-
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132 activated receptor γ [2]. Adipose tissue in obese individuals is characterized by chronic low-
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134 grade systemic inflammation accompanied by elevated secretion of free fatty acids (FFAs) and
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136 various pro-inflammatory cytokines, such as interleukin 6 (IL-6), tumor necrosis factor α
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138 (TNF α), and plasminogen activator inhibitor-1, which are known as adipokines [3]. These pro-
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140 inflammatory adipokines are involved in the induction of insulin resistance, which contributes
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142 to hyperglycemia and type 2 diabetes mellitus [4]. Therefore, strategies aimed at decreasing the
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144 secretion of pro-inflammatory adipokines by adipocytes are crucial to the prevention and
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146 treatment of insulin resistance.
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150 Adipose tissue contains various cells, including pre-adipocytes, mature adipocytes, and
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152 macrophages. The expression pattern of pro-inflammatory adipokines varies in these cells.
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154 TNF α is mostly produced by macrophages, whereas IL-6 is mainly produced by pre-adipocytes
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156 and mature adipocytes in adipose tissue [5]. Furthermore, IL-6 secretion is higher in pre-
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158 adipocytes than in mature adipocytes [6]. It has been reported that serum IL-6 levels and insulin
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160 resistance are strongly linked [7], and that IL-6 from adipocytes affects various tissues in
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162 autocrine and paracrine manners. In adipocyte model 3T3-L1 pre-adipocytes, IL-6 suppressed
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164 gene expression, inhibited adiponectin secretion, and acted as an insulin-sensitizing adipokine
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166 [8]. IL-6 activated SOCS3, resulting in decreased insulin sensitivity in hepatocytes [9].
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168 Furthermore, tail vein injection of IL-6-neutralizing antibodies improved insulin resistance in
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170 obese mice [10]. These findings indicated that adipose-derived IL-6 may be a target for
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172 prevention strategies against obesity-induced insulin resistance.
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174 Glucocorticoids (GCs), which are steroid hormones secreted by the adrenal cortex, have
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184 been proposed to play both adipogenic and lipolytic roles in adipose tissue [11]. GC action
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186 depends not only on blood levels, but also on tissue-specific intracellular metabolic processes
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188 catalyzed by 11 β -hydroxysteroid dehydrogenases (11 β -HSDs). 11 β -HSD converts inactive
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190 cortisone into active cortisol in humans, or into corticosterone, a major endogenous
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192 glucocorticoid, in rodents [12]. The expression of 11 β -HSD1 in adipose tissues is elevated in
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194 obese human patients [13]. GCs bind to the glucocorticoid receptor (GR) in the cytoplasm. The
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196 ligand-bound GR then translocates into the nucleus and binds to glucocorticoid-response
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198 elements (GREs) on target gene promoters, resulting in increased expression of the target genes.
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200 GCs increased plasma levels of FFAs and expression levels of pro-inflammatory adipokines
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202 such as IL-6 and TNF α in white adipose tissue of mice [14, 15]. Furthermore, chronic exposure
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204 to GCs was associated with insulin resistance, diabetes, and hepatic steatosis in rodents [16].
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206 Therefore, GC-GR signaling represents a potential therapeutic target against obesity-related
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208 insulin resistance and diabetes.
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210 Theophylline (1,3-dimethylxanthine), a methylxanthine drug, was first extracted from tea
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212 leaves. Theophylline is widely used worldwide as a therapeutic agent for respiratory diseases
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214 [17]. In addition, recent reports have shown that theophylline ameliorated acetic-acid-induced
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216 ulcerative colitis by decreasing the levels of pro-inflammatory cytokines in the mouse colon
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218 [18]. However, theophylline effects on pro-inflammatory adipokine secretion in pre-adipocytes
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220 and adipocytes have not been investigated. In this study, we assessed the molecular effects of
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222 theophylline on the secretion and expression levels of IL-6 in 3T3-L1 pre-adipocytes. We
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224 demonstrated that theophylline suppressed IL-6 gene expression by inhibiting the transcriptional
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226 activity of GR in 3T3-L1 pre-adipocytes. Furthermore, we showed that administration of
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228 theophylline decreased GC-induced hyperglycemia and plasma IL-6 levels in mice.
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232 **2. Materials and Methods**

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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 $\mu\text{g/mL}$ 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 $\mu\text{g/mL}$ 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 $\mu\text{g/mL}$) 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

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304 modifications. Briefly, primary pre-adipocytes were isolated from epididymal adipose tissue of
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306 male mice (ICR; 4-week-old). After digestion with collagenase II and centrifugation, pre-
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308 adipocytes were cultured in DMEM. When pre-adipocytes were fully confluent, they were
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310 treated with theophylline in the presence of 0.5 μ M DEX for 24 h.
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312 313 314 *2.4. siRNA*

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316 Double-stranded siRNAs for C/EBP δ were chemically synthesized (Sigma-Aldrich, Saint
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318 Louis, MO). Target sequences for siRNA duplexes were as follows: siC/EBP δ #1, 5'-
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320 CGACTTCAGCGCCTACATT-3' and siC/EBP δ #2, 5'-CGCAGACAGTGGTGAGCTT-3'. The
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322 duplexes (20 nM) were transiently transfected into 3T3-L1 pre-adipocytes using Lipofectamine
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324 RNAiMAX reagent (Invitrogen; Carlsbad, CA) and Opti-MEM (Thermo Fisher Scientific,
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326 Lafayette, CO) for 24 h, according to the manufacturer's protocol.
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330 331 *2.5 Plasmids*

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333 The consensus sequence of androgen-responsive element (ARE) is commonly recognized by
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335 GR [20]. Accordingly, we used the pARE2 [21] reporter assay vector to determine
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337 transcriptional activity of GR.
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340 341 *2.6. Measurement of secreted IL-6*

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343 The levels of IL-6 secreted by 3T3-L1 pre-adipocytes and plasma IL-6 levels in mice were
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345 determined by enzyme-linked immunosorbent assays (ELISA). A rat monoclonal anti-IL-6
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347 antibody (catalog number MAB406, 1:10,000 dilution; R&D Systems, Minneapolis, MN) was
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349 coated on 96-well plates at 4 $^{\circ}$ C overnight. The plates were washed with PBS and incubated
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351 with the sample (100 μ L) at 37 $^{\circ}$ C for 2 h. Washed plates were incubated with a biotinylated
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353 goat polyclonal anti-IL-6 antibody (Cat# BAF406, 1:1,000 dilution; R&D Systems) at 37 $^{\circ}$ C for
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355 2 h, followed by further incubation with horseradish peroxidase-conjugated streptavidin (N100;
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364 1:10,000 dilution; Thermo Fisher Scientific, Waltham, MA) at 37 °C for 1 h. The plates were
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366 washed and 3,3',5,5'-tetramethylbenzidine (100 μL; Sigma-Aldrich) was added to each well.
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368 Ten minutes later, the reaction was stopped using 2N H₂SO₄. Absorbance at 450 nm was
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370 measured in the plates by using a multi-plate reader (Bio-Rad, Richmond, CA).
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372 373 374 *2.7. Western blotting*

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376 3T3-L1 pre-adipocytes were incubated with theophylline in the presence of 0.5 μM DEX for
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378 2 h or 24 h. SDS-PAGE and western blotting were performed as described previously [22]. Cell
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380 lysates were analyzed by western blotting using the following rabbit polyclonal antibodies: anti-
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382 C/EBPδ (Cat# sc-9315) and anti-C/EBPβ (Cat# sc-150; Santa Cruz Biotechnology, Santa Cruz,
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384 CA), anti-GR (Cat# 12041S; Cell Signaling, Danvers, MA), anti-IL6 (Cat# ab6672), and anti-
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386 Histone H3 (Cat# ab1791; Abcam, Cambridge, UK). In addition, a mouse monoclonal anti-β-
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388 actin antibody (Cat# sc-47778; Santa Cruz Biotechnology) was used. The immunoreactive
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390 proteins were reacted with Immunostar LD (Wako, Osaka, Japan) and visualized using Ez-
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392 Capture MG (ATTO Co., Tokyo, Japan).
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396 *2.8. Quantitative real-time PCR (qPCR)*

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398 Total RNA was extracted from 3T3-L1 pre-adipocytes using TRIzol (Invitrogen). cDNAs
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400 were synthesized using RevaTra Ace and subjected to qPCR using sets of specific primers (see
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402 Table S1 for the sequences). qPCR was performed with SYBR PremixEx Taq II (Takara Bio,
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404 Shiga, Japan) using a two-step PCR method on a Thermal Cycler Dice real-time system (Takara
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406 Bio.). The relative expression levels of each gene were calculated using the 2^{-ΔΔCt} (CT, cycle
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408 threshold) method, and data were normalized to the expression level of *Gapdh*, which was used
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410 as endogenous control.
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414 *2.9. Luciferase reporter assay*

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424 The luciferase reporter assay was performed as described previously [23]. 3T3-L1 pre-
425 adipocytes were transiently transfected with the reporter vectors pARE2-TATA-Luc and pRL-
426 SV40 (control reporter vector; Promega) using Lipofectamine 2000 for 24 h. After the medium
427 was replaced, cells were incubated with 0.5 μ M DEX and theophylline. Transfection efficiency
428 was normalized by luminescence levels in pRL-SV40-transfected cells. Firefly and Renilla
429 luciferase activities were measured using a Dual Luciferase reporter assay kit and GloMax
430 20/20 Luminometer (Promega). Data were expressed as relative light units (RLU; firefly levels
431 divided by Renilla levels).

442 *2.10. Immunostaining*

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

460 *2.11. Subcellular fractionation*

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462 Confluent 3T3-L1 pre-adipocytes were incubated with theophylline, RU486 (1 μ M), or
463 xanthine (100 nM) in the presence of DEX for 30 min. The cells were lysed in hypotonic buffer
464 containing 10 mM KCl, 1.5 mM MgCl₂, 10 mM Hepes-NaOH (pH 7.5) and protease cocktail
465 (Nacalai Tesque, Kyoto, Japan). Subcellular fractionation was performed as described
466 previously [21]. Proteins in each fraction were analyzed by western blotting.

474 *2.12. Chromatin immunoprecipitation (ChIP)*

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

500 *2.13. Pull-down assay*

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

524 *2.14. Statistical analysis*

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526 Data were analyzed using the Student's *t* test or one-way analysis of variance (ANOVA),
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528 with Turkey's post hoc test, if appropriate. Statistical analysis was performed using JMP
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530 statistical software version 11.2.0 (SAS Institute. Cary, NC). Data are expressed as the mean \pm
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532 standard deviation (S.D.). All statistical analyses were conducted with a significance level of α
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534 = 0.05 ($P < 0.05$).

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546 **3. Results**
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550 *3.1. Theophylline decreases IL-6 secretion by 3T3-L1 pre-adipocytes*
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552 We examined the effect of theophylline on IL-6 secretion in 3T3-L1 pre-adipocytes. IL-6
553 secretion levels by 3T3-L1 pre-adipocytes were increased by the treatment with a
554 differentiation-inducing reagent that contained DEX, IBMX, and insulin. At concentrations
555 above 100 nM, theophylline decreased the reagent-induced secretion of IL-6 (Fig. 1A). In
556 addition, theophylline concentration-dependently suppressed the reagent-induced increase in IL-
557 6 protein (Fig. 1B). To identify the active component of the differentiation-inducing reagent that
558 was responsible for inducing IL-6 expression, 3T3-L1 pre-adipocytes were treated with DEX,
559 IBMX, or insulin. IL-6 protein level was induced by DEX, but not by IBMX or insulin (Fig.
560 1C). Furthermore, theophylline suppressed DEX-induced increase in IL-6 expression at protein
561 and mRNA levels (Fig. 1D and 1E). These results indicated that theophylline suppressed DEX-
562 induced IL-6 expression at the transcriptional level in 3T3-L1 pre-adipocytes.
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576 *3.2. Theophylline decreases IL-6 secretion by primary pre-adipocytes*
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578 We examined the effect of theophylline on the levels of secreted and intracellular IL-6 in
579 primary pre-adipocytes from male and female mice. DEX increased the level of secreted IL-6 in
580 male and female pre-adipocytes fourfold and twofold, respectively, whereas theophylline
581 counteracted this effect of DEX (Fig. 2A). In addition, theophylline suppressed protein level of
582 IL-6 in both male and female primary pre-adipocytes (Fig. 2B). These results indicated that
583 theophylline suppressed DEX-induced IL-6 expression not only in mouse 3T3-L1 pre-
584 adipocytes, but also in primary pre-adipocytes.
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594 *3.3. C/EBP δ is involved in the expression of IL-6*
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604 The promoter region of the *Il6* gene contains a CCAAT box motif, which is the binding site
605 of C/EBPs [24]. We found that DEX increased C/EBP δ protein level in 3T3-L1 pre-adipocytes
606 (Fig. 3A). Furthermore, to determine whether DEX-induced C/EBP δ involved in IL-6
607 expression, 3T3-L1 pre-adipocytes were transiently transfected with two siRNAs with different
608 sequences to avoid off-target effects. C/EBP δ siRNA reduced DEX-induced protein expression
609 of C/EBP δ (Fig. 3A), and knockdown of C/EBP δ attenuated DEX-induced IL-6 secretion and
610 protein expression (Fig. 3B and 3C). **These results suggested that DEX up-regulated *Il6***
611 **expression through C/EBP δ in 3T3-L1 pre-adipocytes.**
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622 3.4. Theophylline decreases C/EBP δ expression at the mRNA level

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624 We examined the effect of theophylline on C/EBP δ expression in 3T3-L1 pre-adipocytes.
625 Theophylline decreased DEX-induced increase in C/EBP δ protein level in a concentration-
626 dependent manner (Fig. 4A). However, DEX and theophylline did not affect C/EBP β protein
627 level. Although the proteasome inhibitor MG132 increased C/EBP δ protein levels in the
628 absence of theophylline, this compound did not restore C/EBP δ protein level, reduced by
629 theophylline, to normal levels (Fig. 4B). Furthermore, we observed that theophylline suppressed
630 DEX-induced gene expression of *Cebpd* (Fig. 4C). These results indicated that theophylline
631 suppressed DEX-induced IL-6 expression by decreasing C/EBP δ expression in 3T3-L1 pre-
632 adipocytes.
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644 3.5. Theophylline suppresses the transcriptional activity of GR

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646 DEX is a GR agonist, therefore it was expected that the GR antagonist RU486 inhibited
647 DEX-induced increase in the levels of secreted and intracellular IL-6 (Fig. 5A). We then
648 examined whether theophylline suppressed the transcriptional activity of GR using a luciferase
649 reporter assay for GR transactivation. Theophylline suppressed DEX-enhanced GR
650 transactivation in a concentration-dependent manner (Fig. 5B). However, theophylline did not
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664 decrease GR protein levels (Fig. 5C). We assumed that theophylline regulated the binding of
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666 GR to glucocorticoid-responsive genes. Multi-genome analysis using the MAPPER database
667 [25, 26] showed that the promoter region of *Cebpd* contained two GRE candidates: one located
668 at -2110 to -2096, and the other at -1301 to -1289 of *Cebpd*. We performed the ChIP assay
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670 using 3T-L1 cell lysates and three sets of PCR primers, as shown in Fig. 5D (left panel). When
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672 the ChIP assay was performed using the primer sets P2 and P3, but not set P1, DEX increased
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674 the relative level of interaction between GR and DNA (Fig. 5D, right panel). Theophylline
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676 decreased DEX-induced interaction between GR and DNA (Fig. 5D, right panel). Theophylline
677
678 decreased DEX-induced interaction between GR and DNA. These results indicated that
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680 theophylline suppressed DEX-induced binding of GR to DNA.
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684 *3.6. Theophylline decreases the nuclear localization of GR*

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686 We investigated the effect of theophylline on the intracellular localization of GR in 3T3-L1
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688 pre-adipocytes using immunofluorescence microscopy. GR was distributed throughout the cell
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690 in the absence of DEX, and DEX increased the nuclear localization of GR. In contrast,
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692 treatment with theophylline resulted in decreased nuclear localization of GR in the presence of
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694 DEX (Fig. 6A). In addition, cellular fractionation showed that RU486 decreased DEX-induced
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696 nuclear accumulation of GR (Fig. 6B). Likewise, theophylline decreased its nuclear
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698 accumulation. However, xanthine did not suppress nuclear import of GR in the presence of
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700 DEX. These results indicated that theophylline suppressed GR transactivation by decreasing
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702 nuclear accumulation of GR.
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706 *3.7. Theophylline interacts with GR in 3T3-L1 pre-adipocytes*

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708 3T3-L1 pre-adipocytes were treated with six xanthine derivatives, including theophylline, to
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710 determine their effect on GR transactivation (Fig. 7A). Xanthine has three methylation sites at
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712 positions 1, 3, and 7. Theophylline-7-acetic acid and 1-methylxanthine, as well as theophylline,
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714 suppressed DEX-enhanced GR transactivation in 3T3-L1 pre-adipocytes (Fig. 7B). In contrast,
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723 xanthine, 3,7-dimethylxanthine and 1,3,7-trimethylxanthine did not affect DEX-enhanced GR
724 transactivation. When the effects of xanthine derivatives on protein levels of C/EBP δ and levels
725 of secreted IL-6 were examined, similar results were obtained (Fig. 7C and 7D). We determined
726 whether theophylline interacted with GR. **Because likewise theophylline, theophylline-7-acetic**
727 **acid suppressed DEX-induced GR transactivation and C/EBP δ protein level, we produced**
728 **theophylline-affinity resin by immobilizing Sepharose on the carboxy group of theophylline-7-**
729 **acetic acid.** When 3T3-L1 pre-adipocytes lysates were incubated with theophylline-affinity-
730 resin with or without theophylline, the resin pulled down GR protein in the absence of
731 theophylline (Fig. 7E). In contrast, the interaction between the resin and GR was diminished by
732 the incubation with theophylline. The resin did not pull down β -actin. These results suggested
733 that theophylline suppressed GR transactivation through the interaction with GR protein.
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748 *3.8. Intake of theophylline decreases corticosterone-induced IL-6 expression in adipose tissues*

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750 To examine the effect of theophylline *in vivo*, mice were given drinking water with or
751 without corticosterone and then fed a theophylline-containing high-fat diet for 6 weeks.
752 Corticosterone intake led to an increase in the weight of epididymal adipose tissue, whereas
753 theophylline decreased the corticosterone-mediated increase in adipose tissue weight (Fig. 8A).
754 We then analyzed fasting blood glucose levels in mice. Corticosterone was found to increase
755 blood glucose levels (188 ± 29.3 mg/dL), whereas theophylline attenuated corticosterone-
756 mediated increase in fasting blood glucose (103 ± 16.3 mg/dL) (Fig. 8B). Furthermore, we
757 measured IL-6 levels in plasma and adipose tissue in mice. Theophylline decreased
758 corticosterone-induced increase in IL-6 plasma concentration (Fig. 8C). Corticosterone further
759 enhanced *Il6* mRNA level in epididymal adipose tissues, whereas theophylline intake tended to
760 decrease that corticosterone effect ($P = 0.0651$) (Fig. 8D). These results indicated that
761 theophylline intake suppressed IL-6 expression in epididymal adipose tissues of mice.
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784 **4. Discussion**
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788 Obesity is a state of chronic low-grade inflammation, which promotes the secretion of certain
789 pro-inflammatory adipokines by adipose tissues. These pro-inflammatory adipokines exert a
790 negative influence on other tissues, such as the liver, skeletal muscle, and immune tissues,
791 resulting in increased risk of hyperglycemia, type 2 diabetes mellitus, and cardiovascular
792 diseases. Therefore, the suppression of pro-inflammatory adipokines represents a promising
793 therapeutic strategy against obesity-related diseases. In this study, we focused on IL-6, one of
794 the pro-inflammatory adipokines secreted by pre-adipocytes, and demonstrated the molecular
795 mechanism by which theophylline suppressed the expression of IL-6 in pre-adipocytes.
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800 IL-6 is secreted by adipose tissues, skeletal muscle, and the liver. The level of expression of
801 IL-6 in the adipose tissue correlates with the body mass index, and plasma IL-6 is elevated in
802 obese and diabetic subjects [27, 28]. Furthermore, the consumption of high-fat diet has been
803 shown to induce insulin resistance and obesity through adipose tissue-derived IL-6 in mice [29].
804 Injection of IL-6 has been demonstrated to promote hyperglycemia in rats [30]. IL-6 levels are
805 associated with the development of chronic hyperglycemia and insulin resistance in human
806 patients after acute pancreatitis [31]. Several studies have described the molecular mechanism
807 by which IL-6 induces hyperglycemia and insulin resistance in the liver and in adipocytes.
808 Chronic exposure to IL-6 inhibited insulin-dependent tyrosine phosphorylation of the insulin
809 receptor, as well as insulin receptor substrate-1 and -2, resulting in decreased glucose uptake
810 and glycogenesis in the liver [32]. Furthermore, IL-6 inhibited the expression of the adiponectin
811 gene that exerted insulin-sensitizing effects in 3T3-L1 pre-adipocytes [8]. Macrophage
812 recruitment to adipose tissue contributes to insulin resistance; in this context, IL-6 has been
813 found to promote the recruitment of macrophages to adipose tissue in obese mice [33]. In the
814 present study, the administration of theophylline resulted in the attenuation of corticosterone-
815 induced hyperglycemia and IL-6 production (Fig. 8). Therefore, our results suggest that
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843 theophylline attenuated blood glucose levels up-regulated by GC via suppressing adipose tissue-
844 derived IL-6 levels.
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847 Theophylline suppressed the nuclear localization of GR, but not its protein level. GCs play a
848 critical role in both adipogenesis and lipolysis in adipocytes. Short-term exposure to GC
849 promoted lipolysis and release of FFAs from adipocytes [34], whereas blood FFAs induced
850 hepatic insulin resistance [35]. In contrast, prolonged exposure to GCs induced hyperglycemia
851 and insulin resistance, and knockdown of GR attenuated GC-mediated insulin resistance [16,
852 36]. Prenatal exposure to GC increased the expression of IL-6, IL-1 β , and TNF α in adipose
853 tissues [15]. Furthermore, the consumption of a high-fat diet increased the expression not only
854 of IL-6, but also that of 11 β -HSD1 in adipose tissues [37], indicating that high-fat intake
855 increased intracellular levels of active GC in adipocytes. A selective inhibitor of 11 β -HSD1 was
856 shown to decrease serum IL-6 levels and ameliorate high-fat diet-induced insulin resistance in
857 obese rats [37]. Therefore, IL-6 is involved in insulin resistance or hyperglycemia mediated by
858 the dysregulation of GC-GR signaling. Previous studies have described anti-inflammatory
859 effects of theophylline. Theophylline reduced the production of IL-6 in lipopolysaccharide
860 (LPS)-treated primary human lung fibroblasts from patients with chronic obstructive pulmonary
861 disease [38]. The authors suggested that theophylline suppressed the expression of IL-6 by
862 inhibiting LPS-Toll-like receptor signaling. In addition, it has been suggested that anti-
863 inflammatory effects of theophylline are caused by the inhibition of reactive oxygen species
864 production [20]. Our results indicate that theophylline acted as an inhibitor of GR
865 transactivation and thereby decreased IL-6 expression in 3T3-L1 pre-adipocytes.
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867 Knockdown of C/EBP δ abrogated DEX-induced IL-6 expression. C/EBP δ elicited robust
868 induction of pro-inflammatory response in macrophages. LPS-induced *IL6* mRNA expression
869 was decreased in *Cebpd*^{-/-} macrophages and glial cells [39]. GR is the main transcription factor
870 involved in the regulation of C/EBP δ gene expression in adipocytes [40]. Several studies have
871 reported that cytokines induced nuclear localization of GR in a GC-independent manner. TNF α
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904 promotes insulin resistance and activates GR signaling by inducing the nuclear localization of
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906 the GR, without the requirement for GCs [36]. In addition, IL-13 promotes the nuclear
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908 translocation of the GR and enhances DEX-stimulated nuclear import of GR in human airway
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910 smooth muscle cells [41]. In this study, we showed that theophylline decreased GR nuclear
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912 localization in the presence of DEX (Fig. 6). Taken together, these results suggest that
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914 theophylline suppressed GC- and cytokine-induced GR transactivation by decreasing nuclear
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916 accumulation of GR.
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918 DEX-induced GR transactivation was suppressed by methylxanthines in which the 1-position
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920 was methylated and the 7-position was substituted by a hydrophilic group. Methylxanthine
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922 derivatives exert various pharmacological effects, such as antagonism of adenosine receptors,
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924 inhibition of phosphodiesterase, modulation of GABA receptors, and regulation of intracellular
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926 calcium levels [30]. Structure-activity relationships of methylxanthines show that their 1-methyl
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928 group is pivotal for their inhibitory effects on phosphodiesterase [42]. Furthermore,
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930 methylxanthines have been reported to exert structure-specific effects. The inhibitory effect of
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932 methylxanthines, especially of 3,7-dimethylxanthine and 1,7-dimethylxanthine at physiological
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934 concentrations, against poly(ADPribose)polymerase-1 has been shown [43], which indicated
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936 that 7-methyl group is important for this inhibitory effect. These results suggested that the
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938 suppressive effects of methylxanthines against GR transactivation depend on their molecular
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940 structure, and that the 1-methyl group and 7-hydrophilic group of theophylline are involved in
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942 the suppression of GR transactivation.
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944 In summary, we showed that theophylline attenuated GC-induced hyperglycemia and
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946 decreased IL-6 expression by inhibiting GR signaling. Previous studies reported that GR
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948 knockout led to reduce adipogenesis during short-term differentiation [44], indicating that GR
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950 accelerates adipogenesis. Therefore, theophylline represents a potentially valuable therapeutic
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952 agent for obesity-related insulin resistance, but not for adipogenesis.
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Conflicts of interest

The authors declare that there are no conflicts of interest

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6 **Hightlight**
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- 9 • Theophylline decreased glucocorticoid-induced IL6 production.
- 10 • Theophylline decreased IL6 expression by decreasing C/EBP δ expression.
- 11 • Theophylline suppressed the nuclear translocation of glucocorticoid receptor.
- 12 • Intake of theophylline ameliorated glucocorticoid-induced hyperglycemia in mice.
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4 Theophylline suppresses interleukin-6 expression by inhibiting glucocorticoid receptor
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46 **Abbreviations:** 11 β -HSD, 11 β -hydroxysteroid dehydrogenases; C/EBP, CCAAT-enhancer-
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48 binding protein; DEX, dexamethasone; FFAs, free fatty acids; GC, glucocorticoid; GR,
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50 glucocorticoid receptor; GRE, glucocorticoid response element; IBMX, 3-isobutyl-1-
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52 methylxanthine; IL-6, interleukin-6; LPS, lipopolysaccharide; qPCR, quantitative real-time
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54 PCR; TNF, tumor necrosis factor
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63
64 **ABSTRACT**

65 Adipose tissues in obese individuals are characterized by a state of chronic low-grade
66 inflammation. Pre-adipocytes and adipocytes in this state secrete pro-inflammatory adipokines,
67 such as interleukin 6 (IL-6), which induce insulin resistance and hyperglycemia. Theophylline
68 (1,3-dimethylxanthine) exerts anti-inflammatory effects, but its effects on pro-inflammatory
69 adipokine secretion by pre-adipocytes and adipocytes have not been examined. In this study, we
70 found that theophylline decreased IL-6 secretion by 3T3-L1 pre-adipocytes and mouse-derived
71 primary pre-adipocytes. The synthetic glucocorticoid dexamethasone (DEX) induced IL-6
72 expression in 3T3-L1 pre-adipocytes, and this effect was suppressed by theophylline at the
73 mRNA level. Knockdown of CCAAT/enhancer binding protein (C/EBP) δ inhibited DEX-
74 induced IL-6 expression, and theophylline suppressed C/EBP δ expression. Furthermore,
75 theophylline suppressed transcriptional activity of the glucocorticoid receptor (GR) through
76 suppression of nuclear localization of GR. *In vivo*, glucocorticoid corticosterone treatment (100
77 $\mu\text{g}/\text{mL}$) increased fasting blood glucose and plasma IL-6 levels in C57BL/6N mice.
78 Theophylline administration (0.1% diet) reduced corticosterone-increased fasting blood glucose,
79 plasma IL-6 levels, and *Il6* gene expression in adipose tissues. These results show that
80 theophylline administration attenuated glucocorticoid-induced hyperglycemia and IL-6
81 production by inhibiting GR activity. The present findings indicate the potential of theophylline
82 as a candidate therapeutic agent to treat insulin resistance and hyperglycemia.
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104 **Keywords:** Adipocyte; CCAAT-enhancer-binding proteins; glucocorticoid receptor; interleukin-
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112 **1. Introduction**
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124 Obesity, which is a risk factor for serious diseases such as insulin resistance, type 2 diabetes
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126 mellitus, and cardiovascular disease, is associated with an increase in adipocyte number and
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128 size. The accumulation of intracellular lipids during adipocyte differentiation increases
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130 adipocyte size [1]. Adipocyte differentiation is regulated by various transcription factors, such
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132 as CCAAT/enhancer binding protein (C/EBP) α , C/EBP β , C/EBP δ , and peroxisome proliferator-
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134 activated receptor γ [2]. Adipose tissue in obese individuals is characterized by chronic low-
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136 grade systemic inflammation accompanied by elevated secretion of free fatty acids (FFAs) and
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138 various pro-inflammatory cytokines, such as interleukin 6 (IL-6), tumor necrosis factor α
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140 (TNF α), and plasminogen activator inhibitor-1, which are known as adipokines [3]. These pro-
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142 inflammatory adipokines are involved in the induction of insulin resistance, which contributes
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144 to hyperglycemia and type 2 diabetes mellitus [4]. Therefore, strategies aimed at decreasing the
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146 secretion of pro-inflammatory adipokines by adipocytes are crucial to the prevention and
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148 treatment of insulin resistance.
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150 Adipose tissue contains various cells, including pre-adipocytes, mature adipocytes, and
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152 macrophages. The expression pattern of pro-inflammatory adipokines varies in these cells.
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154 TNF α is mostly produced by macrophages, whereas IL-6 is mainly produced by pre-adipocytes
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156 and mature adipocytes in adipose tissue [5]. Furthermore, IL-6 secretion is higher in pre-
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158 adipocytes than in mature adipocytes [6]. It has been reported that serum IL-6 levels and insulin
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160 resistance are strongly linked [7], and that IL-6 from adipocytes affects various tissues in
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162 autocrine and paracrine manners. In adipocyte model 3T3-L1 pre-adipocytes, IL-6 suppressed
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164 gene expression, inhibited adiponectin secretion, and acted as an insulin-sensitizing adipokine
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166 [8]. IL-6 activated SOCS3, resulting in decreased insulin sensitivity in hepatocytes [9].
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168 Furthermore, tail vein injection of IL-6-neutralizing antibodies improved insulin resistance in
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170 obese mice [10]. These findings indicated that adipose-derived IL-6 may be a target for
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172 prevention strategies against obesity-induced insulin resistance.
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174 Glucocorticoids (GCs), which are steroid hormones secreted by the adrenal cortex, have
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184 been proposed to play both adipogenic and lipolytic roles in adipose tissue [11]. GC action
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186 depends not only on blood levels, but also on tissue-specific intracellular metabolic processes
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188 catalyzed by 11 β -hydroxysteroid dehydrogenases (11 β -HSDs). 11 β -HSD converts inactive
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190 cortisone into active cortisol in humans, or into corticosterone, a major endogenous
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192 glucocorticoid, in rodents [12]. The expression of 11 β -HSD1 in adipose tissues is elevated in
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194 obese human patients [13]. GCs bind to the glucocorticoid receptor (GR) in the cytoplasm. The
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196 ligand-bound GR then translocates into the nucleus and binds to glucocorticoid-response
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198 elements (GREs) on target gene promoters, resulting in increased expression of the target genes.
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200 GCs increased plasma levels of FFAs and expression levels of pro-inflammatory adipokines
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202 such as IL-6 and TNF α in white adipose tissue of mice [14, 15]. Furthermore, chronic exposure
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204 to GCs was associated with insulin resistance, diabetes, and hepatic steatosis in rodents [16].
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206 Therefore, GC-GR signaling represents a potential therapeutic target against obesity-related
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208 insulin resistance and diabetes.
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210 Theophylline (1,3-dimethylxanthine), a methylxanthine drug, was first extracted from tea
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212 leaves. Theophylline is widely used worldwide as a therapeutic agent for respiratory diseases
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214 [17]. In addition, recent reports have shown that theophylline ameliorated acetic-acid-induced
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216 ulcerative colitis by decreasing the levels of pro-inflammatory cytokines in the mouse colon
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218 [18]. However, theophylline effects on pro-inflammatory adipokine secretion in pre-adipocytes
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220 and adipocytes have not been investigated. In this study, we assessed the molecular effects of
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222 theophylline on the secretion and expression levels of IL-6 in 3T3-L1 pre-adipocytes. We
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224 demonstrated that theophylline suppressed IL-6 gene expression by inhibiting the transcriptional
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226 activity of GR in 3T3-L1 pre-adipocytes. Furthermore, we showed that administration of
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228 theophylline decreased GC-induced hyperglycemia and plasma IL-6 levels in mice.
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232 **2. Materials and Methods**

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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% CO₂ 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

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304 modifications. Briefly, primary pre-adipocytes were isolated from epididymal adipose tissue of
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306 male mice (ICR; 4-week-old). After digestion with collagenase II and centrifugation, pre-
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308 adipocytes were cultured in DMEM. When pre-adipocytes were fully confluent, they were
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310 treated with theophylline in the presence of 0.5 μ M DEX for 24 h.
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312 313 314 *2.4. siRNA*

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316 Double-stranded siRNAs for C/EBP δ were chemically synthesized (Sigma-Aldrich, Saint
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318 Louis, MO). Target sequences for siRNA duplexes were as follows: siC/EBP δ #1, 5'-
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320 CGACTTCAGCGCCTACATT-3' and siC/EBP δ #2, 5'-CGCAGACAGTGGTGAGCTT-3'. The
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322 duplexes (20 nM) were transiently transfected into 3T3-L1 pre-adipocytes using Lipofectamine
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324 RNAiMAX reagent (Invitrogen; Carlsbad, CA) and Opti-MEM (Thermo Fisher Scientific,
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326 Lafayette, CO) for 24 h, according to the manufacturer's protocol.
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330 331 *2.5 Plasmids*

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333 The consensus sequence of androgen-responsive element (ARE) is commonly recognized by
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335 GR [20]. Accordingly, we used the pARE2 [21] reporter assay vector to determine
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337 transcriptional activity of GR.
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340 341 *2.6. Measurement of secreted IL-6*

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343 The levels of IL-6 secreted by 3T3-L1 pre-adipocytes and plasma IL-6 levels in mice were
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345 determined by enzyme-linked immunosorbent assays (ELISA). A rat monoclonal anti-IL-6
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347 antibody (catalog number MAB406, 1:10,000 dilution; R&D Systems, Minneapolis, MN) was
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349 coated on 96-well plates at 4 $^{\circ}$ C overnight. The plates were washed with PBS and incubated
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351 with the sample (100 μ L) at 37 $^{\circ}$ C for 2 h. Washed plates were incubated with a biotinylated
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353 goat polyclonal anti-IL-6 antibody (Cat# BAF406, 1:1,000 dilution; R&D Systems) at 37 $^{\circ}$ C for
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355 2 h, followed by further incubation with horseradish peroxidase-conjugated streptavidin (N100;
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364 1:10,000 dilution; Thermo Fisher Scientific, Waltham, MA) at 37 °C for 1 h. The plates were
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366 washed and 3,3',5,5'-tetramethylbenzidine (100 μL; Sigma-Aldrich) was added to each well.
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368 Ten minutes later, the reaction was stopped using 2N H₂SO₄. Absorbance at 450 nm was
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370 measured in the plates by using a multi-plate reader (Bio-Rad, Richmond, CA).
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372 373 374 *2.7. Western blotting*

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376 3T3-L1 pre-adipocytes were incubated with theophylline in the presence of 0.5 μM DEX for
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378 2 h or 24 h. SDS-PAGE and western blotting were performed as described previously [22]. Cell
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380 lysates were analyzed by western blotting using the following rabbit polyclonal antibodies: anti-
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382 C/EBPδ (Cat# sc-9315) and anti-C/EBPβ (Cat# sc-150; Santa Cruz Biotechnology, Santa Cruz,
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384 CA), anti-GR (Cat# 12041S; Cell Signaling, Danvers, MA), anti-IL6 (Cat# ab6672), and anti-
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386 Histone H3 (Cat# ab1791; Abcam, Cambridge, UK). In addition, a mouse monoclonal anti-β-
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388 actin antibody (Cat# sc-47778; Santa Cruz Biotechnology) was used. The immunoreactive
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390 proteins were reacted with Immunostar LD (Wako, Osaka, Japan) and visualized using Ez-
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392 Capture MG (ATTO Co., Tokyo, Japan).
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396 *2.8. Quantitative real-time PCR (qPCR)*

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398 Total RNA was extracted from 3T3-L1 pre-adipocytes using TRIzol (Invitrogen). cDNAs
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400 were synthesized using RevaTra Ace and subjected to qPCR using sets of specific primers (see
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402 Table S1 for the sequences). qPCR was performed with SYBR PremixEx Taq II (Takara Bio,
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404 Shiga, Japan) using a two-step PCR method on a Thermal Cycler Dice real-time system (Takara
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406 Bio.). The relative expression levels of each gene were calculated using the 2^{-ΔΔCt} (CT, cycle
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408 threshold) method, and data were normalized to the expression level of *Gapdh*, which was used
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410 as endogenous control.
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414 *2.9. Luciferase reporter assay*

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424 The luciferase reporter assay was performed as described previously [23]. 3T3-L1 pre-
425 adipocytes were transiently transfected with the reporter vectors pARE2-TATA-Luc and pRL-
426 SV40 (control reporter vector; Promega) using Lipofectamine 2000 for 24 h. After the medium
427 was replaced, cells were incubated with 0.5 μ M DEX and theophylline. Transfection efficiency
428 was normalized by luminescence levels in pRL-SV40-transfected cells. Firefly and Renilla
429 luciferase activities were measured using a Dual Luciferase reporter assay kit and GloMax
430 20/20 Luminometer (Promega). Data were expressed as relative light units (RLU; firefly levels
431 divided by Renilla levels).

442 *2.10. Immunostaining*

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

460 *2.11. Subcellular fractionation*

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462 Confluent 3T3-L1 pre-adipocytes were incubated with theophylline, RU486 (1 μ M), or
463 xanthine (100 nM) in the presence of DEX for 30 min. The cells were lysed in hypotonic buffer
464 containing 10 mM KCl, 1.5 mM MgCl₂, 10 mM Hepes-NaOH (pH 7.5) and protease cocktail
465 (Nacalai Tesque, Kyoto, Japan). Subcellular fractionation was performed as described
466 previously [21]. Proteins in each fraction were analyzed by western blotting.

474 *2.12. Chromatin immunoprecipitation (ChIP)*

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484 Chromatin immunoprecipitation was performed as described previously [23]. In brief, 3T3-
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486 L1 pre-adipocytes were incubated with 100 nM theophylline and 0.5 μ M DEX for 30 min. The
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488 promoter region of the *Cebpd* gene was amplified by qPCR using a set of specific primers (see
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490 supplemental Table S1 for sequences). qPCR profiles were obtained after running the following
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492 program: 94 °C for 30 s, 65 °C for 15 s, 72 °C for 20 s. The relative amounts of each promoter
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494 region were calculated using the $2^{-\Delta\Delta C_t}$ method, and the data were normalized to that of the input
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500 2.13. Statistical analysis

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502 Data were analyzed using the Student's *t* test or one-way analysis of variance (ANOVA),
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504 with Turkey's post hoc test, if appropriate. Statistical analysis was performed using JMP
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506 statistical software version 11.2.0 (SAS Institute. Cary, NC). Data are expressed as the mean \pm
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508 standard deviation (S.D.). All statistical analyses were conducted with a significance level of α
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510 = 0.05 ($P < 0.05$).
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514 3. Results

518 3.1. Theophylline decreases IL-6 secretion by 3T3-L1 pre-adipocytes

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520 We examined the effect of theophylline on IL-6 secretion in 3T3-L1 pre-adipocytes. IL-6
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522 secretion levels by 3T3-L1 pre-adipocytes were increased by the treatment with a
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524 differentiation-inducing reagent that contained DEX, IBMX, and insulin. At concentrations
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526 above 100 nM, theophylline decreased the reagent-induced secretion of IL-6 (Fig. 1A). In
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528 addition, theophylline concentration-dependently suppressed the reagent-induced increase in IL-
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530 6 protein (Fig. 1B). To identify the active component of the differentiation-inducing reagent that
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532 was responsible for inducing IL-6 expression, 3T3-L1 pre-adipocytes were treated with DEX,
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534 IBMX, or insulin. IL-6 protein level was induced by DEX, but not by IBMX or insulin (Fig.
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544 1C). Furthermore, theophylline suppressed DEX-induced increase in IL-6 expression at protein
545 and mRNA levels (Fig. 1D and 1E). These results indicated that theophylline suppressed DEX-
546 induced IL-6 expression at the transcriptional level in 3T3-L1 pre-adipocytes.
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551 552 *3.2. Theophylline decreases IL-6 secretion by primary pre-adipocytes*

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554 We examined the effect of theophylline on the levels of secreted and intracellular IL-6 in
555 primary pre-adipocytes from male and female mice. DEX increased the level of secreted IL-6 in
556 male and female pre-adipocytes fourfold and twofold, respectively, whereas theophylline
557 counteracted this effect of DEX (Fig. 2A). In addition, theophylline suppressed protein level of
558 IL-6 in both male and female primary pre-adipocytes (Fig. 2B). These results indicated that
559 theophylline suppressed DEX-induced IL-6 expression not only in mouse 3T3-L1 pre-
560 adipocytes, but also in primary pre-adipocytes.
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570 571 *3.3. Theophylline suppresses C/EBP δ -regulated IL-6 promoter activity*

572 The promoter region of the *Il6* gene contains a CCAAT box motif, which is the binding site
573 of C/EBPs [24]. We found that DEX increased C/EBP δ protein level in 3T3-L1 pre-adipocytes
574 (Fig. 3A). Furthermore, to determine whether DEX-induced C/EBP δ involved in IL-6
575 expression, 3T3-L1 pre-adipocytes were transiently transfected with two siRNAs with different
576 sequences to avoid off-target effects. C/EBP δ siRNA reduced DEX-induced protein expression
577 of C/EBP δ (Fig. 3A), and knockdown of C/EBP δ attenuated DEX-induced IL-6 secretion and
578 protein expression (Fig. 3B and 3C). These results suggested that DEX up-regulated *Il6*
579 promoter activity through C/EBP δ , and that theophylline suppressed DEX-induced *Il6* promoter
580 activity in 3T3-L1 pre-adipocytes.
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591 592 *3.4. Theophylline decreases C/EBP δ expression at the mRNA level*

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594 We examined the effect of theophylline on C/EBP δ expression in 3T3-L1 pre-adipocytes.
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604 Theophylline decreased DEX-induced increase in C/EBP δ protein level in a concentration-
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606 dependent manner (Fig. 4A). However, DEX and theophylline did not affect C/EBP β protein
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608 level. Although the proteasome inhibitor MG132 increased C/EBP δ protein levels in the
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610 absence of theophylline, this compound did not restore C/EBP δ protein level, reduced by
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612 theophylline, to normal levels (Fig. 4B). Furthermore, we observed that theophylline suppressed
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614 DEX-induced gene expression of *Cebpd* (Fig. 4C). These results indicated that theophylline
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616 suppressed DEX-induced IL-6 expression by decreasing C/EBP δ expression in 3T3-L1 pre-
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618 adipocytes.
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622 3.5. Theophylline suppresses the transcriptional activity of GR

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624 DEX is a GR agonist, therefore it was expected that the GR antagonist RU486 inhibited
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626 DEX-induced increase in the levels of secreted and intracellular IL-6 (Fig. 5A). We then
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628 examined whether theophylline suppressed the transcriptional activity of GR using a luciferase
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630 reporter assay for GR transactivation. Theophylline suppressed DEX-enhanced GR
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632 transactivation in a concentration-dependent manner (Fig. 5B). However, theophylline did not
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634 decrease GR protein levels (Fig. 5C). We assumed that theophylline regulated the binding of
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636 GR to glucocorticoid-responsive genes. Multi-genome analysis using the MAPPER database
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638 [25, 26] showed that the promoter region of *Cebpd* contained two GRE candidates: one located
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640 at -2110 to -2096, and the other at -1301 to -1289 of *Cebpd*. We performed the ChIP assay
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642 using 3T-L1 cell lysates and three sets of PCR primers, as shown in Fig. 5D (left panel). When
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644 the ChIP assay was performed using the primer sets P2 and P3, but not set P1, DEX increased
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646 the relative level of interaction between GR and DNA (Fig. 5D, right panel). Theophylline
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648 decreased DEX-induced interaction between GR and DNA. These results indicated that
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650 theophylline suppressed DEX-induced binding of GR to DNA.
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654 3.6. Theophylline decreases the nuclear localization of GR

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663 We investigated the effect of theophylline on the intracellular localization of GR in 3T3-L1
664 pre-adipocytes using immunofluorescence microscopy. GR was distributed throughout the cell
665 in the absence of DEX, and DEX increased the nuclear localization of GR. In contrast,
666 treatment with theophylline resulted in decreased nuclear localization of GR in the presence of
667 DEX (Fig. 6A). In addition, cellular fractionation showed that RU486 decreased DEX-induced
668 nuclear accumulation of GR (Fig. 6B). Likewise, theophylline decreased its nuclear
669 accumulation. However, xanthine did not suppress nuclear import of GR in the presence of
670 DEX. These results indicated that theophylline suppressed GR transactivation by decreasing
671 nuclear accumulation of GR.
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684 *3.7. Theophylline interacts with GR in 3T3-L1 pre-adipocytes*

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686 3T3-L1 pre-adipocytes were treated with six xanthine derivatives, including theophylline, to
687 determine their effect on GR transactivation (Fig. 7A). Xanthine has three methylation sites at
688 positions 1, 3, and 7. Theophylline-7-acetic acid and 1-methylxanthine, as well as theophylline,
689 suppressed DEX-enhanced GR transactivation in 3T3-L1 pre-adipocytes (Fig. 7B). In contrast,
690 xanthine, 3,7-dimethylxanthine and 1,3,7-trimethylxanthine did not affect DEX-enhanced GR
691 transactivation. When the effects of xanthine derivatives on protein levels of C/EBP δ and levels
692 of secreted IL-6 were examined, similar results were obtained (Fig. 7C and 7D). To determine
693 whether theophylline interacted with GR, theophylline-7-acetic acid was immobilized on
694 Sepharose through its carboxy group. When 3T3-L1 pre-adipocytes lysates were incubated with
695 theophylline-affinity-resin with or without theophylline, the resin pulled down GR protein in the
696 absence of theophylline (Fig. 7E). In contrast, the interaction between the resin and GR was
697 diminished by the incubation with theophylline. The resin did not pull down β -actin. These
698 results suggested that theophylline suppressed GR transactivation through the interaction with
699 GR protein.
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724 *3.8. Intake of theophylline decreases corticosterone-induced IL-6 expression in adipose tissues*
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726 To examine the effect of theophylline *in vivo*, mice were given drinking water with or
727 without corticosterone and then fed a theophylline-containing high-fat diet for 6 weeks.
728 Corticosterone intake led to an increase in the weight of epididymal adipose tissue, whereas
729 theophylline decreased the corticosterone-mediated increase in adipose tissue weight (Fig. 8A).
730 We then analyzed fasting blood glucose levels in mice. Corticosterone was found to increase
731 blood glucose levels (188 ± 29.3 mg/dL), whereas theophylline attenuated corticosterone-
732 mediated increase in fasting blood glucose (103 ± 16.3 mg/dL) (Fig. 8B). Furthermore, we
733 measured IL-6 levels in plasma and adipose tissue in mice. Theophylline decreased
734 corticosterone-induced increase in IL-6 plasma concentration (Fig. 8C). Corticosterone further
735 enhanced *Il6* mRNA level in epididymal adipose tissues, whereas theophylline intake tended to
736 decrease that corticosterone effect (Fig. 8D). These results indicated that theophylline intake
737 suppressed IL-6 expression in epididymal adipose tissues of mice.
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752 **4. Discussion**
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756 Obesity is a state of chronic low-grade inflammation, which promotes the secretion of certain
757 pro-inflammatory adipokines by adipose tissues. These pro-inflammatory adipokines exert a
758 negative influence on other tissues, such as the liver, skeletal muscle, and immune tissues,
759 resulting in increased risk of hyperglycemia, type 2 diabetes mellitus, and cardiovascular
760 diseases. Therefore, the suppression of pro-inflammatory adipokines represents a promising
761 therapeutic strategy against obesity-related diseases. In this study, we focused on IL-6, one of
762 the pro-inflammatory adipokines secreted by pre-adipocytes, and demonstrated the molecular
763 mechanism by which theophylline suppressed the expression of IL-6 in pre-adipocytes.
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772 IL-6 is secreted by adipose tissues, skeletal muscle, and the liver. The level of expression of
773 IL-6 in the adipose tissue correlates with the body mass index, and plasma IL-6 is elevated in
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784 obese and diabetic subjects [27, 28]. Furthermore, the consumption of high-fat diet has been
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786 shown to induce insulin resistance and obesity through adipose tissue-derived IL-6 in mice [29].
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788 Injection of IL-6 has been demonstrated to promote hyperglycemia in rats [30]. IL-6 levels are
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790 associated with the development of chronic hyperglycemia and insulin resistance in human
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792 patients after acute pancreatitis [31]. Several studies have described the molecular mechanism
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794 by which IL-6 induces hyperglycemia and insulin resistance in the liver and in adipocytes.
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796 Chronic exposure to IL-6 inhibited insulin-dependent tyrosine phosphorylation of the insulin
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798 receptor, as well as insulin receptor substrate-1 and -2, resulting in decreased glucose uptake
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800 and glycogenesis in the liver [32]. Furthermore, IL-6 inhibited the expression of the adiponectin
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802 gene that exerted insulin-sensitizing effects in 3T3-L1 pre-adipocytes [8]. Macrophage
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804 recruitment to adipose tissue contributes to insulin resistance; in this context, IL-6 has been
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806 found to promote the recruitment of macrophages to adipose tissue in obese mice [33]. In the
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808 present study, the administration of theophylline resulted in the attenuation of corticosterone-
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810 induced hyperglycemia and IL-6 production (Fig. 8). Therefore, our results suggest that
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812 theophylline attenuated blood glucose levels up-regulated by GC via suppressing adipose tissue-
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814 derived IL-6 levels.

816 Theophylline suppressed the nuclear localization of GR, but not its protein level. GCs play a
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818 critical role in both adipogenesis and lipolysis in adipocytes. Short-term exposure to GC
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820 promoted lipolysis and release of FFAs from adipocytes [34], whereas blood FFAs induced
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822 hepatic insulin resistance [35]. In contrast, prolonged exposure to GCs induced hyperglycemia
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824 and insulin resistance, and knockdown of GR attenuated GC-mediated insulin resistance [16,
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826 36]. Prenatal exposure to GC increased the expression of IL-6, IL-1 β , and TNF α in adipose
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828 tissues [15]. Furthermore, the consumption of a high-fat diet increased the expression not only
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830 of IL-6, but also that of 11 β -HSD1 in adipose tissues [37], indicating that high-fat intake
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832 increased intracellular levels of active GC in adipocytes. A selective inhibitor of 11 β -HSD1 was
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834 shown to decrease serum IL-6 levels and ameliorate high-fat diet-induced insulin resistance in
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844 obese rats [37]. Therefore, IL-6 is involved in insulin resistance or hyperglycemia mediated by
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846 the dysregulation of GC-GR signaling. Previous studies have described anti-inflammatory
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848 effects of theophylline. Theophylline reduced the production of IL-6 in lipopolysaccharide
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850 (LPS)-treated primary human lung fibroblasts from patients with chronic obstructive pulmonary
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852 disease [38]. The authors suggested that theophylline suppressed the expression of IL-6 by
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854 inhibiting LPS-Toll-like receptor signaling. In addition, it has been suggested that anti-
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856 inflammatory effects of theophylline are caused by the inhibition of reactive oxygen species
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858 production [20]. Our results indicate that theophylline acted as an inhibitor of GR
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860 transactivation and thereby decreased IL-6 expression in 3T3-L1 pre-adipocytes.

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

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886 DEX-induced GR transactivation was suppressed by methylxanthines in which the 1-position
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888 was methylated and the 7-position was substituted by a hydrophilic group. Methylxanthine
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890 derivatives exert various pharmacological effects, such as antagonism of adenosine receptors,
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892 inhibition of phosphodiesterase, modulation of GABA receptors, and regulation of intracellular
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894 calcium levels [30]. Structure-activity relationships of methylxanthines show that their 1-methyl
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904 group is pivotal for their inhibitory effects on phosphodiesterase [42]. Furthermore,
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906 methylxanthines have been reported to exert structure-specific effects. The inhibitory effect of
907
908 methylxanthines, especially of 3,7-dimethylxanthine and 1,7-dimethylxanthine at physiological
909
910 concentrations, against poly(ADPribose)polymerase-1 has been shown [43], which indicated
911
912 that 7-methyl group is important for this inhibitory effect. These results suggested that the
913
914 suppressive effects of methylxanthines against GR transactivation depend on their molecular
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916 structure, and that the 1-methyl group and 7-hydrophilic group of theophylline are involved in
917
918 the suppression of GR transactivation.

919
920 In summary, we showed that theophylline attenuated GC-induced hyperglycemia and
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922 decreased IL-6 expression by inhibiting GR signaling. Previous studies reported that GR
923
924 knockout led to reduce adipogenesis during short-term differentiation [44], indicating that GR
925
926 accelerates adipogenesis. Therefore, theophylline represents a potentially valuable therapeutic
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928 agent for obesity-related insulin resistance, but not for adipogenesis.

931 932 **Conflicts of interest**

933
934 The authors declare that there are no conflicts of interest
935
936

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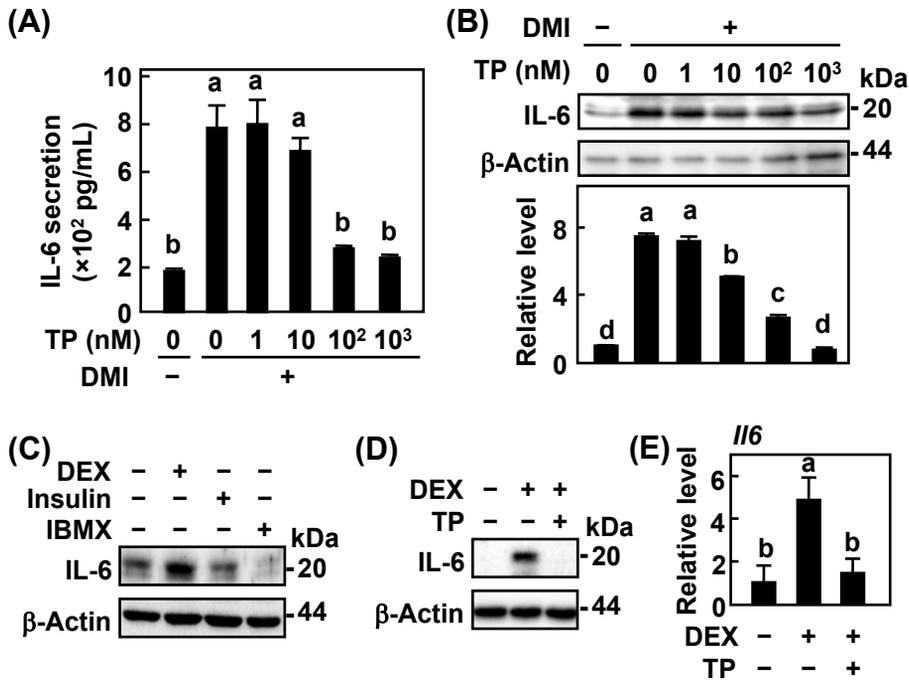
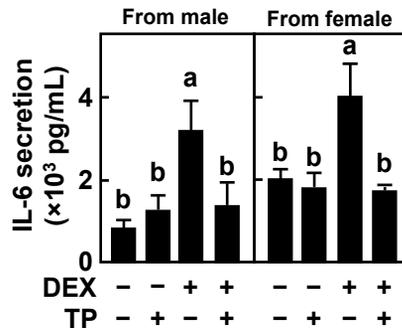


Fig. 1. Expression pattern of IL-6 following the treatment with theophylline.

(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 μ M), 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM), or insulin (10 μ g/mL insulin) for 24 h. (D) Western blotting of IL-6 in 3T3-L1 pre-adipocytes treated with 0.5 μ M DEX and 100 nM TP for 24 h. (E) *I/6* gene expression in 3T3-L1 pre-adipocytes treated with 0.5 μ M 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 \pm 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 μ M DEX, 0.5 mM IBMX, and 10 μ g/mL insulin.

(A)



(B)

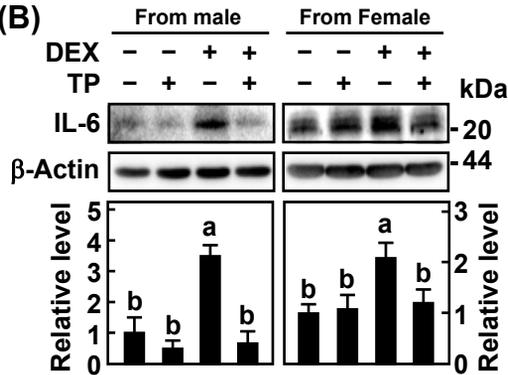


Fig. 2. Suppressive effect of theophylline on primary pre-adipocytes from male and female mice.

(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.

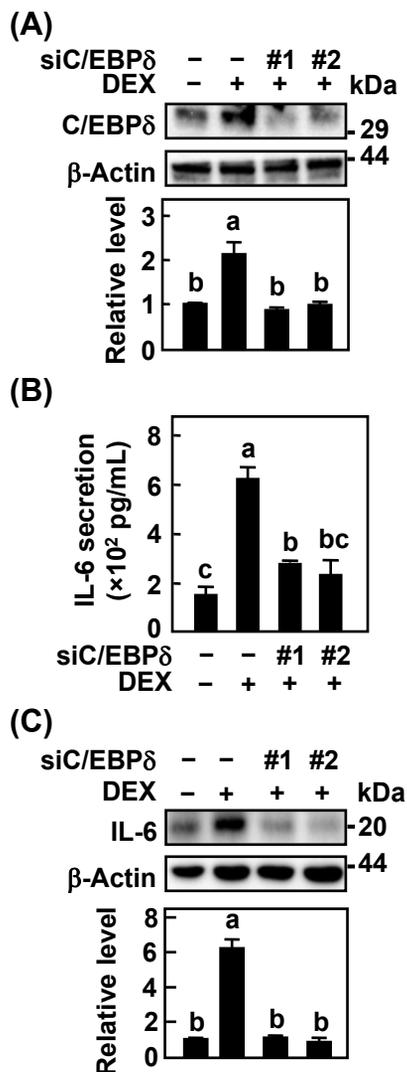


Fig. 3. Involvement of C/EBP δ in dexamethasone-induced IL6 expression.

(A) Western blotting of C/EBP δ in 3T3-L1 pre-adipocytes transiently transfected with control siRNA or siRNA against C/EBP δ (siC/EBP δ). (B) IL-6 secretion by 3T3-L1 pre-adipocytes after treatment with siRNA and dexamethasone (DEX; 0.5 μ M) for 24 h. (C) Western blot analysis of IL-6 in 3T3-L1 pre-adipocytes treated with siRNA and 0.5 μ M 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 \pm 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.

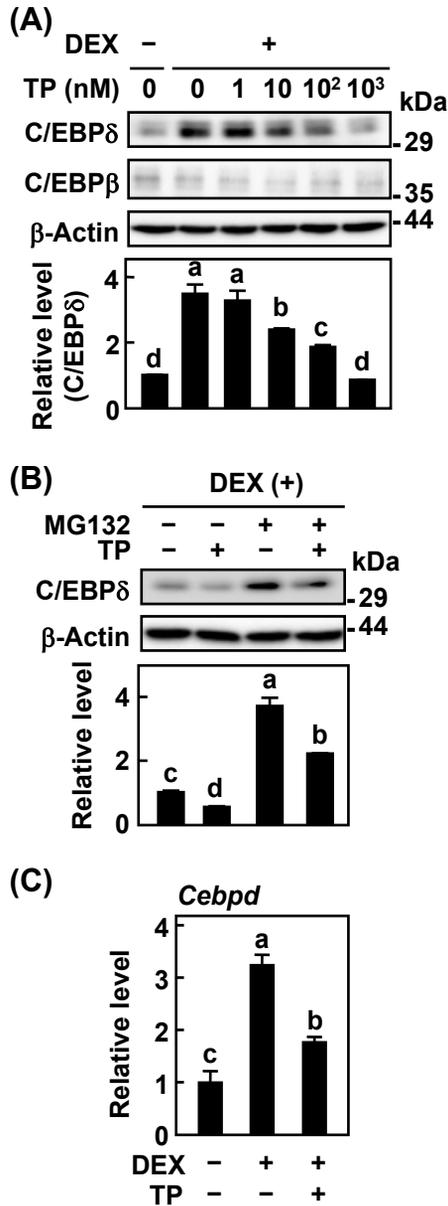


Fig. 4. Effect of theophylline on the expression of C/EBP δ .

(A) Western blotting of C/EBP δ in 3T3-L1 pre-adipocytes treated with theophylline (TP; 100 nM) for 2 h. (B) Western blotting of C/EBP δ in 3T3-L1 pre-adipocytes after TP treatment with or without MG132 (10 μ M) for 2 h; * P < 0.05 vs. TP(-). (C) *Cebpd* expression in 3T3-L1 pre-adipocytes treated with dexamethasone (DEX; 0.5 μ M) 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 \pm 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.

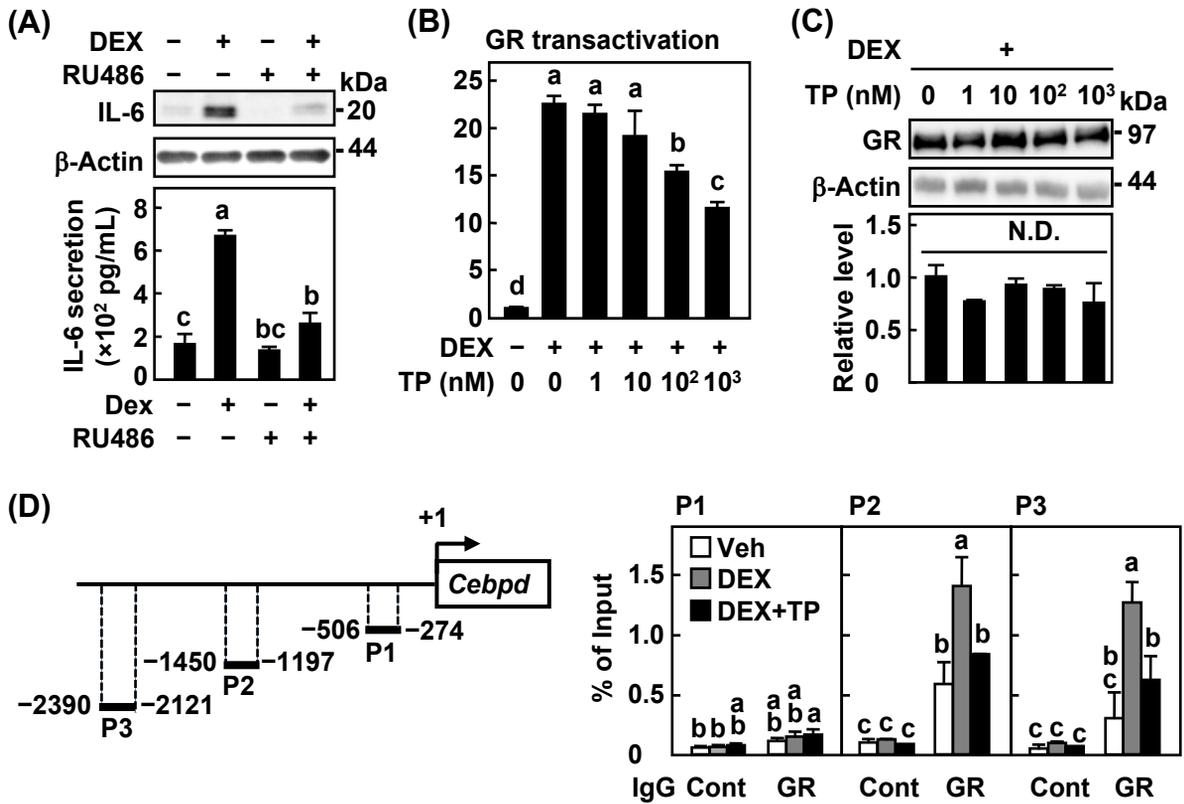


Fig. 5. Effect of theophylline on the transcriptional activity of GR.

(A) Western blotting of IL-6 in 3T3-L1 pre-adipocytes after treatment with dexamethasone (DEX; 0.5 μ M) and 1 μ M 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 μ M DEX and theophylline (TP) for 3 h. (C) Western blotting of GR in 3T3-L1 pre-adipocytes treated with 0.5 μ M 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 \pm 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.

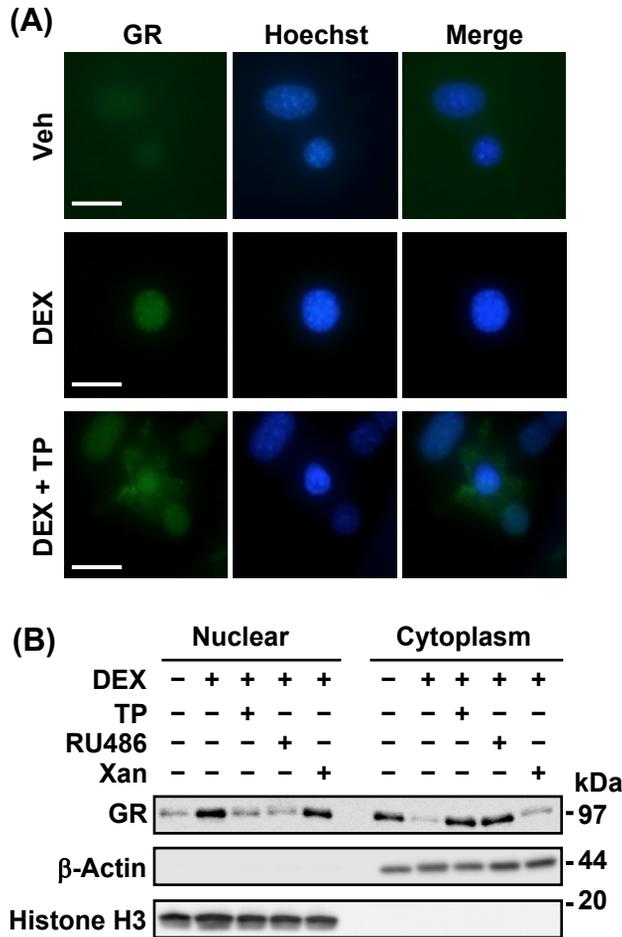


Fig. 6. Intracellular localization of GR in the presence of theophylline.

(A) Immunofluorescence analysis of the glucocorticoid receptor (GR, green) in 3T3-L1 pre-adipocytes incubated with or without dexamethasone (DEX; 0.5 μ M) and theophylline (TP; 100 nM) for 30 min. Nuclei were stained with Hoechst33258 (blue). Scale bar = 20 μ m. (B) Subcellular distribution of GR in 3T3-L1 pre-adipocytes treated with TP (100 nM), xanthine (100 nM) or RU486 (1 μ M) in the presence of 0.5 μ M 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.

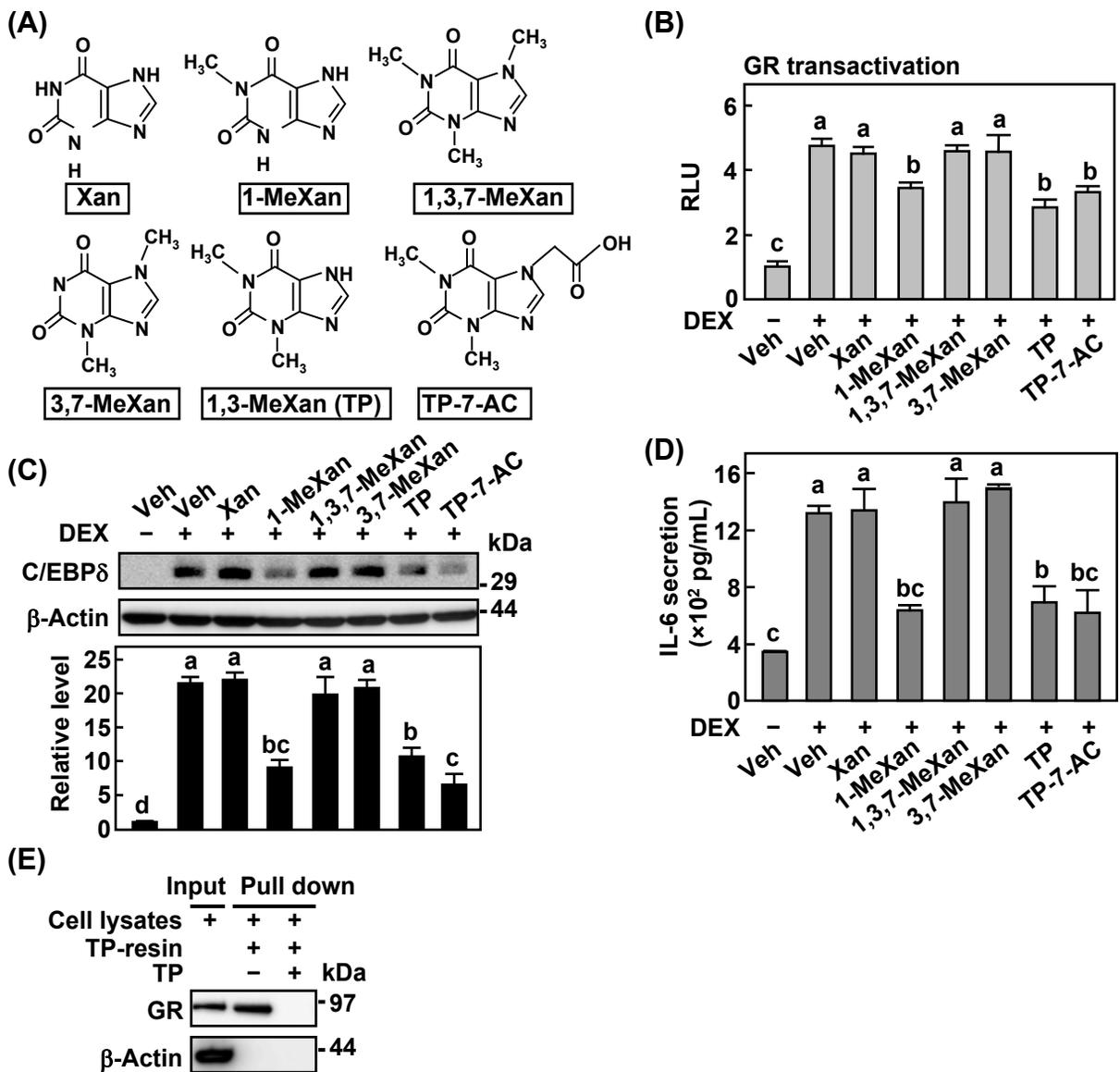


Fig. 7. Suppressive effects of theophylline and xanthine derivatives on GR transactivation.

(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 μ M) for 3 h. (C) Western blotting analysis of C/EBP δ in 3T3-L1 pre-adipocytes treated with xanthine derivatives (100 nM) in the presence of 0.5 μ M 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 μ M 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 \pm 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.

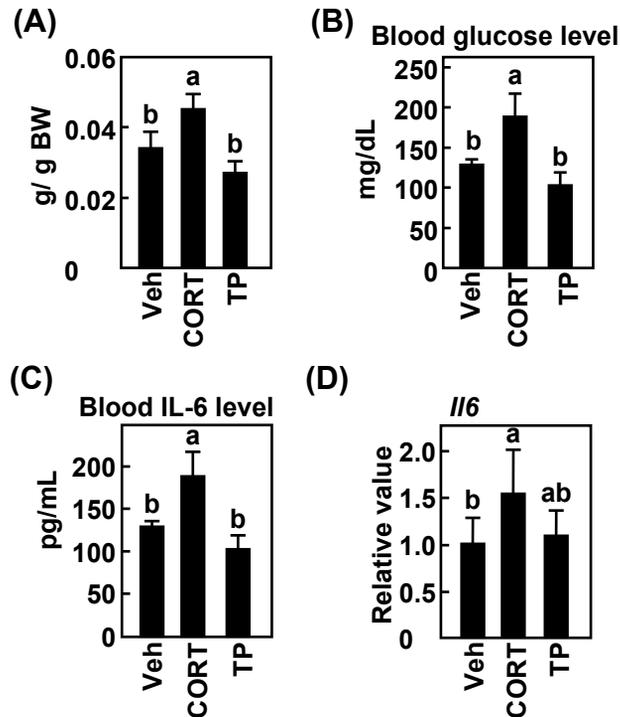


Fig. 8. In vivo effect of theophylline on blood glucose level and plasma IL-6 level.

(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 \pm S.D. ($n = 6$). Values in groups indicated by different letters are significantly different ($P < 0.05$).

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Table S1: Primer Sequences

	Forward; 5' to 3'	Reverse; 5' to 3'
<i>Cebpd</i>	GATCTGCACGGCCTGTTGTA	CTCCACTGCCACCTGTCA
<i>Gapdh</i>	ACAACCTTGGCATTGTGGAA	GATGCAGGGATGATGTTCTG
<i>Ilf6</i>	AGTCCGGAGAGGAGACTTCA	ATTCCACGATTTCCAGAG
P1	TGCTGAACCTAACCTCGACG	CAGGACGCCTTCAGACATAG
P2	CACGGTAGCCTGTCTTCTG	GTAAAGCCAGCGCACATGT
P3	CCATTCTCATTGAACCGCC	ATCTCCCTGAGGTCCTGCTT