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Cannabidiol promotes browning in 3T3-L1 adipocytes

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Abstract Recruitment of the brown-like phenotype in white adipocytes (browning) and activation of existing brown adipocytes are currently being investigated as a means to combat obesity. Thus, a wide variety of dietary agents that contribute to browning of white adipocytes have been identified. The present study was designed to investigate the effects of cannabidiol (CBD), a major nonpsychotropic phytocannabinoid of *Cannabis sativa*, on induction of browning in 3T3-L1 adipocytes. CBD enhanced expression of a core set of brown fat-specific marker genes (*Ucp1*, *Cited1*, *Tmem26*, *Prdm16*, *Cidea*, *Tbx1*, *Fgf21*, and *Pgc-1α*) and proteins (UCP1, PRDM16, and PGC-1α). Increased expression of UCP1 and other brown fat-specific markers contributed to the browning of 3T3-L1 adipocytes possibly via activation of PPARγ and PI3K. In addition, CBD increased protein expression levels of CPT1, ACSL, SIRT1, and PLIN while down-regulating JNK2, SREBP1, and LPL. These data suggest possible roles for CBD in browning of white adipocytes, augmentation of lipolysis, thermogenesis, and reduction of lipogenesis. In conclusion, the current data suggest that CBD plays dual modulatory roles in the form of inducing the brown-like phenotype as well as promoting lipid metabolism. Thus, CBD may be explored as a potentially promising therapeutic agent for the prevention of obesity.

Keywords Lipogenesis · Cannabidiol · Thermogenesis · Browning

Introduction

Obesity is the most common metabolic disease affecting more than 1.4 billion people worldwide [1]. It has reached global epidemic levels, leading to the development of many common medical conditions such as diabetes, cardiovascular diseases, and increased risk of cancer [2]. In obesity development, energy intake exceeds energy expenditure [3]. Any specific treatment for obesity must either reduce energy intake or increase energy expenditure, or promote both effects at the same time [4]. While decreasing caloric intake is the baseline defense against obesity, it is also critical to modify metabolic efficiency and elevate energy expenditure through key metabolic organs such as adipose tissues and skeletal muscle [5].

Recently, white and brown adipose tissues (WAT and BAT) have been proposed as two specialized types of adipose tissue in mammals with opposite functions. WAT and BAT are morphologically and functionally different tissues, with a unilocular structure in WAT and multilocular structure in BAT [6]. BAT plays a crucial role in the generation of heat by oxidizing fatty acids produced by hydrolysis of triglycerides [7]. In earlier studies, BAT was thought to function basically in newborn babies and rodents as a mechanism for easing adaptation to a cold environment [8]. However, recent studies have confirmed that adult humans also have active BAT [9, 10]. In response to various stimuli mediated by different factors, UCP1-expressing multilocular adipocytes with thermogenic capacity develop in WAT and have been named beige or brite adipocytes [7]. Recruitment of brite cells in WAT and activation of BAT are currently being investigated as being potentially beneficial strategies in the fight against obesity and related metabolic diseases [11]. As beige adipocyte induction in WAT is associated with protection against obesity in rodent models, it is

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important to understand its molecular mechanism [12]. Recent studies have demonstrated that increased conversion of white adipocytes into brown adipocytes enhances whole body energy expenditure and reduces diet-induced obesity [11, 13].

Cannabidiol (CBD) is a major nonpsychotropic phytocannabinoid (Fig. 1a) of the *Cannabis sativa* plant, which has a wide scope of medical applications [14, 15]. CBD is capable of acting as an anti-inflammatory molecule to inhibit progression of arthritis and Type 1 diabetes and has antioxidant activity [16, 17]. In a previous experiment, CBD-treated diabetic mice showed significantly reduced symptoms possibly through an immunomodulatory mechanism that altered the immune response in the periphery from Th1 to Th2 dominance [18]. CBD has also been reported to have anxiolytic, anti-psychotic, and anti-emetic effects [19, 20]. In addition, CBD has been shown to possess anti-tumor activity in human breast carcinoma and reduce tumor mass, size, and numbers in lung metastasis in animal models of breast cancer [21]. Moreover, CBD is a nonpsychoactive compound with potential in clinical research since it does not produce psychoactive effects due to low affinities for the CB1 and CB2 receptors and has no side effects when chronically administered to humans [22].

CBD is the most studied nonpsychotropic phytocannabinoid that has been already applied in clinical practice without any significant side effects [15]. Several studies have suggested that CBD is well tolerated and safe even at higher doses [23]. Although numerous ongoing clinical trials intend to explore its further therapeutic potential, the anti-obesity effect of CBD has not been explored [24].

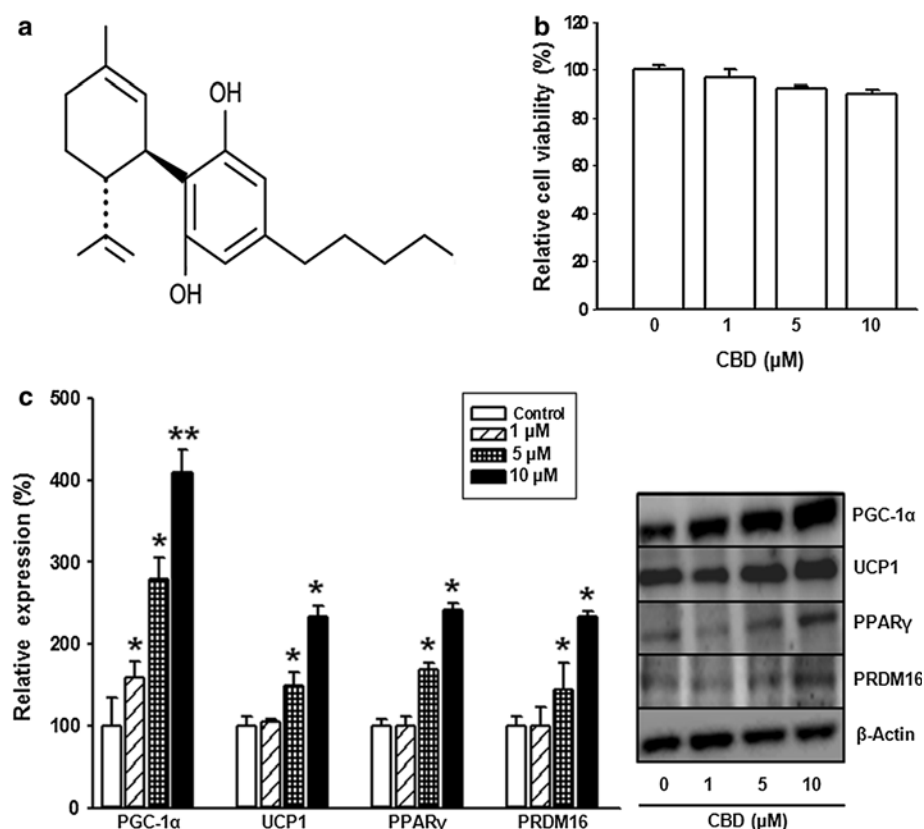
Therefore, we aimed to investigate the roles of CBD in fat browning as well as lipid metabolism in 3T3-L1 adipocytes and to elucidate the molecular mechanism underlying the fat browning effect of CBD.

Materials and methods

Chemicals

Cannabidiol (99 % purity) was purchased from Cayman Chemical and Arbor Assays (Ann Arbor, MI, USA). GW9662 and Wortmannin were purchased from Tocris Bioscience (Bristol, UK) and Sigma Chemical Co. (St. Louis, MO, USA), respectively. All other chemicals used in this study were of analytical grade.

Fig. 1 Chemical structure of cannabidiol (CBD) (a). Cytotoxicity in 3T3-L1 preadipocytes by MTT assay with CBD treatment for 72 h (b). Dose-dependent effect of CBD on expression of brown fat-specific marker proteins by Western blotting (c). 3T3-L1 cells were treated with different doses of CBD for 72 h, followed by quantification of protein expression by densitometric analysis. Data are presented as the mean \pm S.D. and differences between groups were determined by One-way analysis of variance (ANOVA) using the Statistical Package of Social Science (SPSS, version 17.0; SPSS Inc., Chicago, IL, USA) program, followed by Turkey's post hoc tests. Statistical significance between control and CBD-treated is shown as * p < 0.05 or ** p < 0.01



Cell culture and differentiation

3T3-L1 preadipocytes (ATCC, Manassas, VA, USA) were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Thermo, Waltham, MA, USA) supplemented with 10 % fetal bovine serum (FBS, PAA Laboratories, Pasching, Austria) and 1 % penicillin streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C for 48 h in a humidified atmosphere of 5 % CO₂. Differentiation was induced in confluent cells by replacing DMEM with differentiation media consisting of 10 µg/ml of insulin (Ins, Sigma, St. Louis, MO, USA), 0.25 µM dexamethasone (Dex, Sigma), and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma) in DMEM. After 2 days, cells were switched to maintenance media for another 6–8 days with media replacement every 24 h. For CBD treatment, 2-day confluent preadipocytes were incubated with different doses of CBD (1, 5, and 10 µM) during differentiation and until mature adipocyte formation. Cells treated with methanol (1:1000 dilutions) during differentiation and maintenance were used as a control. For induction of browning in 3T3-L1 adipocytes, differentiation induction media was supplemented with 50 nM triiodothyronine (T3, Sigma), and maturation medium was supplemented with 50 nM triiodothyronine and 1 µM rosiglitazone (Rosi, Abcam, Cambridge, UK) (browning cocktail). To analyze the effects of GW9662 and Wortmannin, cells were treated with GW9662 (5 µM) and Wortmannin (1 µM) during the differentiation and maturation period until harvesting of cells.

Cell viability assay

Preadipocytes were seeded in a 96-well plate at a density of 1×10^4 cells/well and incubated until more than 70 % confluency. Cells were treated with different concentrations of CBD (1, 5, and 10 µM) for 72 h. Preadipocytes treated with methanol (1:1000) were used as a control. After 72 h, media was removed and treated with MTT according to the manufacturer's instructions. Absorbance was measured at 570 nm with background subtraction at 690 nm using a microplate reader, Tecan Infinite M200 Pro (Mannedorf, Switzerland). Six replicate wells were used for each data point throughout the experiments.

Oil Red O staining

Preadipocytes were seeded in a 6-well plate and allowed to reach 70 % confluency. Cells were treated with cocktail and CBD in the differentiation and maturation medium. After 72 h of treatment, cells were washed with phosphate-buffered saline (PBS), fixation with 10 % formalin for 1 h at room temperature, and washed again three times with

deionized water. A mixture of Oil Red O solution (0.6 % Oil Red O dye in isopropanol) and water at a ratio of 6:4 was layered onto cells for 20 min, followed by washing four times with deionized water. Intracellular lipid accumulation was quantified using ORO staining. The stained lipid droplets were visualized using an inverted microscope. Intracellular lipid content was quantified after extracting ORO bound to cells with 100 % isopropanol, and absorbance at 500 nm was determined in triplicate wells using a microplate reader.

Immunofluorescence

Cells grown on poly-L-lysine-pretreated coverslips were fixed with 4 % *p*-formaldehyde, followed by washing with 1× PBS two times for 5 min. Un-reacted groups were then blocked by incubation with 1 % BSA in PBS-T for 1 h at room temperature. After washing with 1× PBS twice for 5 min, permeabilization was carried out with 0.1 % triton X-100 (Sigma). Next, cells were washed with PBS three times and incubated with polyclonal anti-UCPI and PRDM16 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in antibody buffer diluted 1:200 overnight at 4 °C, followed by three washes with PBS. Cells were then incubated with FITC-conjugated anti-goat secondary antibody (1:500 dilutions). DAPI (Invitrogen) was used to stain nuclei of cells. Fluorescence images were captured using a confocal laser scanning microscope LSM700 (Carl Zeiss, Oberkochen Germany). Analysis of images was performed by software Zen 2009 (Carl Zeiss). For staining of mitochondria, MitoTracker Red® (1 mM, Cell Signaling Technology, Beverly, MA, USA) was directly added to the growing media at a concentration of 20–25 nM, and cells were kept for 30–40 min at 37 °C. After incubation, cells were washed with 1× PBS and fixed with 4 % *p*-formaldehyde, followed by a single wash with PBS and immunostaining.

Total RNA extraction

Preadipocytes were cultured in 6-well cell culture plates and incubated until more than 70 % confluency. Differentiation was initiated by addition of differentiation media, followed by replacement replaced with maintenance media after 2 days (changed every 24 h). Cells were also treated with either CBD alone or combined with browning cocktail. On day 10 after maturation, cells were harvested and then used to extract total RNA using a total RNA isolation kit (RNA-spin, iNtRON Biotechnology, Seongnam, Korea). Quantitative and qualitative ratio metric analysis of RNA was carried out using a microplate reader, Tecan Infinite M200 Pro (Mannedorf, Switzerland). RNA integrity was confirmed using 1.5 % agarose gel.

Real-time RT-PCR

cDNA was synthesized from RNA (1 µg) using Maxime RT premix (iNtRON Biotechnology). Power SYBR green (Roche Diagnostics GmbH, Mannheim, Germany) was employed to quantitatively determine transcription levels of genes with RT-PCR (Stratagene 246 mix 3000p QPCR System, Agilent Technologies, Santa Clara, CA, USA). PCR reactions were run in duplicate for each sample, and transcription levels of every gene were normalized to the level of *β-actin*. Sequences of primer sets used in this study are listed in Table 1.

Immunoblot analysis

Cell lysates were prepared using RIPA buffer (Sigma) by homogenization and centrifugation at 14,000×g for 20 min. Cell extract was diluted in 5× sample buffer (50 mM Tris at pH 6.8, 2 % SDS, 10 % glycerol, 5 % β-mercaptoethanol, and 0.1 % bromophenol blue) and heated for 5 min at 95 °C before 8, 10, or 12 % SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, samples were transferred to a polyvinylidene difluoride membrane (PVDF, Santa Cruz Biotechnology) and then blocked for 1 h with TBS-T (10 mM Tris-HCl, 150 mM NaCl, and 0.1 % Tween 20) containing 5 % skim milk or bovine albumin serum (BSA). The membrane was rinsed three times consecutively with TBS-T buffer, followed by overnight incubation with 1:1000 dilutions of primary polyclonal antibodies anti-β-actin, anti-PPARγ, anti-PRDM16, anti-UCP1, anti-PGC-1α, anti-CPT1, anti-SIRT1, anti-LPL, anti-JNK2, anti-ACSL, anti-SREBP1, (Santa Cruz Biotechnology), anti-PI3K (Cell Signaling Technology), and anti-PLIN, (Abcam) in TBS-T buffer containing 1 % skim milk or BSA. After three washes, the membrane was incubated for 1 h with horseradish peroxidase-conjugated anti-goat IgG or anti-mouse IgG or anti-rabbit IgG secondary antibody (1:1000, Santa Cruz Biotechnology) in TBS-T buffer containing either 1 % skim milk or BSA. Development was carried out using enhanced chemiluminescence (West Zol, iNtRON

Biotechnology). Quantification of band intensities was performed using ImageJ software (NIH).

Statistical analysis

All data are presented as the mean ± S.D. of at least three independent experiments. Statistical significance among multiple groups was analyzed using One-way ANOVA using the Statistical Package of Social Science (SPSS, version 17.0; SPSS Inc., Chicago, IL, USA) program, followed by Turkey's post hoc test. Statistical significances were indicated as either $p < 0.05$ or $p < 0.01$.

Results

CBD induces brown-like phenotype in a dose-dependent manner in 3T3-L1 adipocytes

To determine the effective dose of CBD for browning of 3T3-L1 adipocytes, cytotoxicity of CBD was first evaluated by MTT assay. CBD showed no significant cytotoxicity within the range of 10 µM (Fig. 1b). Hence, we used 10 µM CBD for the further experiments. 3T3-L1 pre-adipocytes were differentiated in the presence of methanol (control group), cocktail (positive control group), and CBD (treatment group), followed by maintenance media. We sought to investigate the effects of different concentrations of CBD (1–10 µM) on the brown fat-like phenotype in 3T3-L1 cells. After browning cocktail (50 nM triiodothyronine and 1 µM rosiglitazone) treatment, CBD synergistically up-regulates brown fat markers (PGC-1α, UCP1, PPARγ, and PRDM16) in a dose-dependent manner (Fig. 1c). As shown in Fig. 2a, b, CBD treatment also resulted in the up-regulation of brown fat-specific genes and proteins compared to vehicle and positive control (cocktail group). The increased expression of brown fat markers, including PGC-1α, UCP1, PPARγ, and PRDM16, as well as *Ucp1*, *Cited1*, *Tmem26*, *Prdm16*, *Cidea*, *Tbx1*, *Fgf21*, and *Pgc-1α*, provides evidence for the conversion of 3T3-L1 cells into beige cells. In addition, enhanced

Table 1 Primer sequences used for real-time quantitative PCR

Gene	Forward	Reverse
<i>Ucp1</i>	CCTGCCTCTCTCGGAAACAA	GTAGCGGGGTTTGATCCCAT
<i>Cited</i>	GCGGTAAAAGATCGCAAGGC	TTGTAGAAGGGGTGGCAGTA
<i>Tmem26</i>	GAAACAGTATTGCAGCACCC	CCAGACCGGTTACATACCA
<i>Prdm16</i>	GATGGGAGATGCTGACGGAT	TGATCTGACACATGGCGAGG
<i>Cidea</i>	CGGGAATAGCCAGAGTCACC	TGTGCATCGGATGTCGTAGG
<i>Tbx1</i>	CGAATGTTCCCCACGTTCCA	GTCTACTCGGCCAGGTGTAG
<i>Fgf21</i>	CGTCTGCCTCAGAAGGACTC	TCTACCATGCTCAGGGGGTC
<i>Pgc-1α</i>	ATGTGCAGCCAAGACTCTGTA	CGCTACACCACTTCAATCCAC

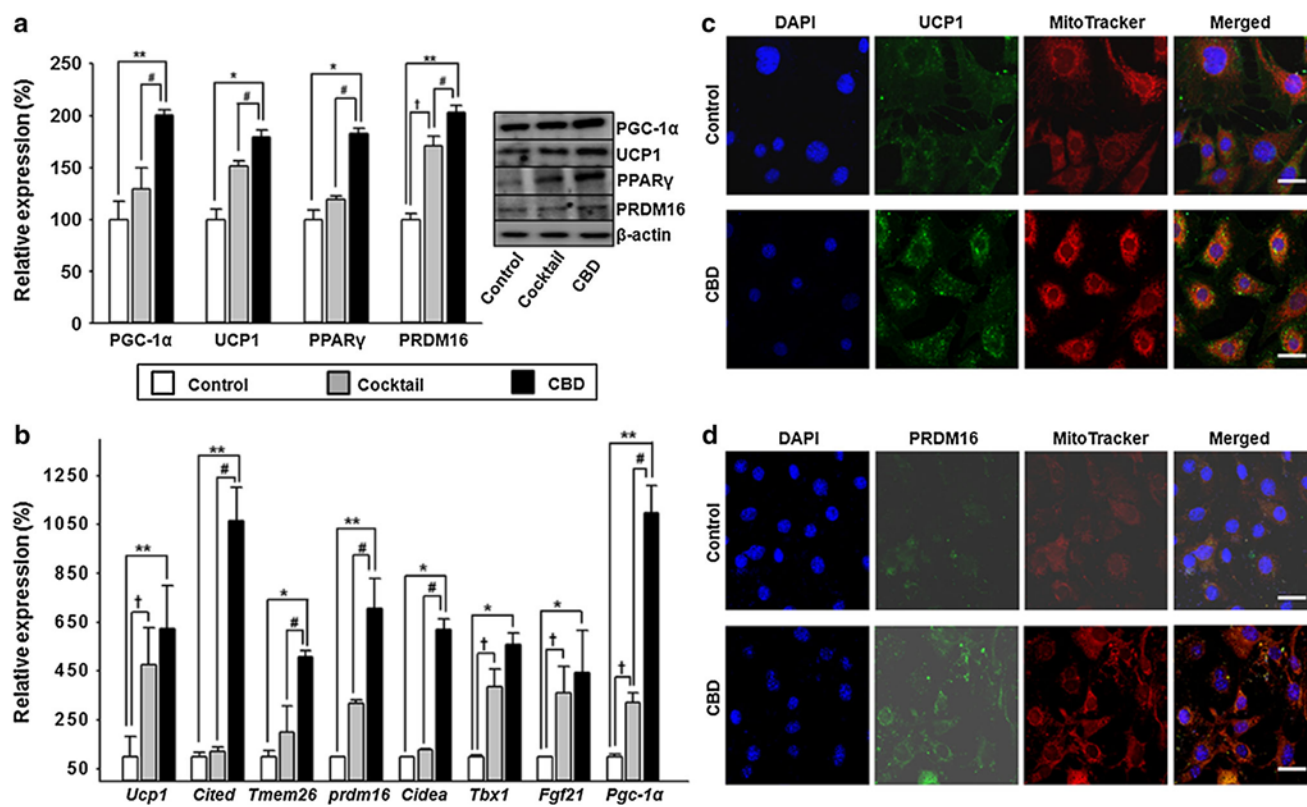


Fig. 2 CBD increases the expression of brown fat-specific proteins (a) and genes (b) in 3T3-L1 adipocytes. After 4–6 days of CBD treatment, cells were fixed with *p*-formaldehyde, subjected to staining with MitoTracker Red®, and then ICC staining for UCP1 (c) and PRDM16 (d). The representative confocal microscopic images show chances of mitochondrial membrane potential and statistics of fluorescence intensity. Immunofluorescent images were captured at 400X magnification and scale bars = 50 μ m. Expression patterns of brown fat cell-specific genes were measured by real-time RT-PCR,

whereas expression levels of proteins were measured by immunoblotting and quantified by densitometric analysis. Relative intensities (%) of proteins and mRNA were normalized with β -actin. All data are presented as the mean \pm S.D., and differences between groups were determined by One-way Analysis of Variance (ANOVA). Statistical significance between control and CBD-treated group is shown as * $p < 0.05$ and ** $p < 0.01$; control and cocktail-treated group depicted as † $p < 0.05$; cocktail and CBD-treated group is noted as # $p < 0.05$

expressions of UCP1 and PRDM16 upon CBD treatment was reconfirmed at the cellular level based on higher intensities of these two markers in immunofluorescent images (Fig. 2c, d).

CBD regulates lipid metabolism in 3T3-L1 adipocytes

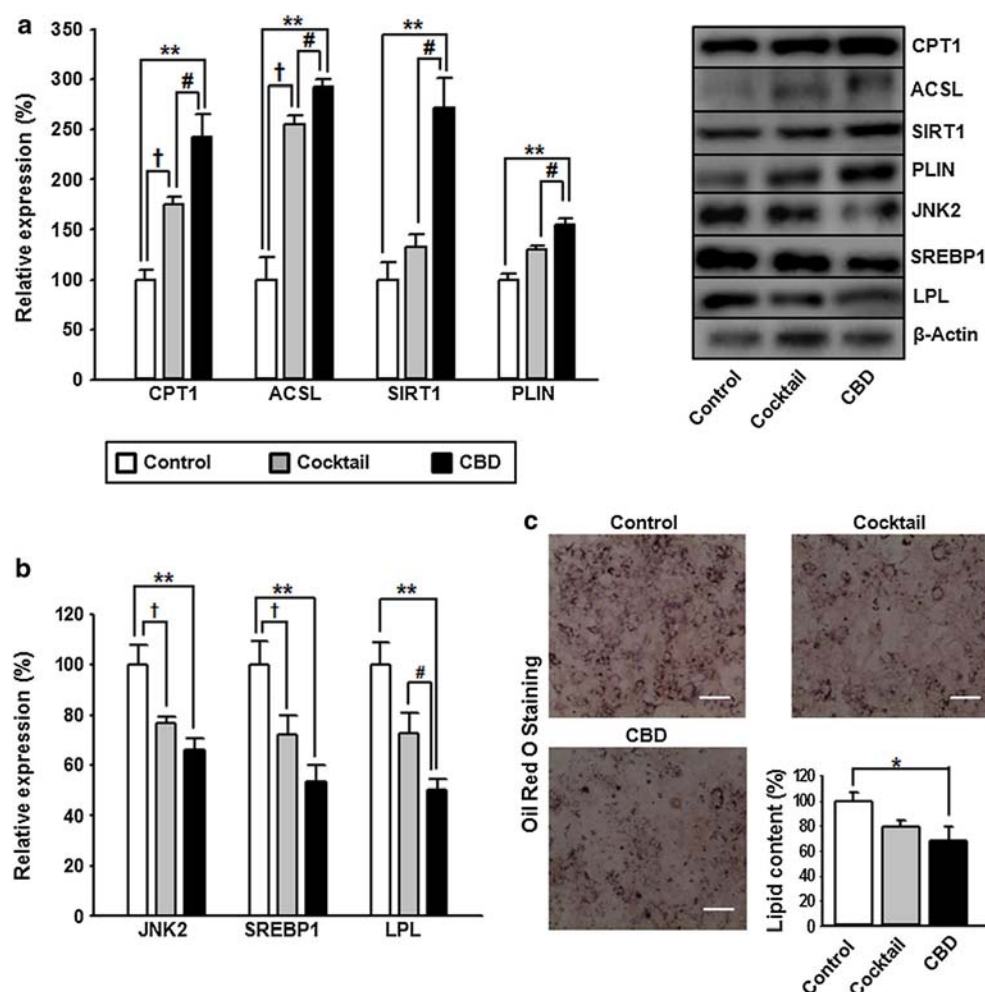
In addition to induction of beige adipocytes, we examined whether CBD stimulates metabolic activity of 3T3-L1 adipocytes. Our results demonstrate that CBD treatment led to significant elevation of CPT1, ACSL, SIRT1, and PLIN protein levels (Fig. 3a), suggesting enhanced lipolysis, fatty acid oxidation, and mitochondrial biogenesis. In contrast, expression levels of JNK2, SREBP1, and LPL were down-regulated upon CBD treatment (Fig. 3b), suggesting reduced lipogenesis. CBD (10 μ M) significantly inhibited lipid accumulation in the treatment group as compared to the control group (Fig. 3c). Further, lipid content was quantified after extraction of ORO bound to

cells with 100 % isopropanol and was found to be reduced upon CBD treatment, confirming inhibition of lipid droplets by CBD.

Stimulatory effect of CBD on browning of 3T3-L1 adipocytes is dependent on PPAR γ and PI3K activation

To understand the possible mechanism underlying the browning effect of CBD, we first determined the expression levels of PPAR γ and PI3K, which are master metabolic switches of adipocyte differentiation, adipogenesis, and metabolism. Expression levels of PPAR γ and PI3K were significantly elevated in the presence of CBD. These results led us to hypothesize that CBD induced the brown fat phenotype via PPAR γ and PI3K activation. To test this hypothesis, we treated cells with PPAR γ inhibitor (GW9662) and PI3K inhibitor (Wortmannin) in two separate experiments. As mentioned in Fig. 4a, b, inhibition of PPAR γ and PI3K expression in 3T3-L1 adipocytes

Fig. 3 Effects of CBD on expression of marker proteins in 3T3-L1 adipocytes responsible for adipogenesis and lipolysis (a), and lipogenesis (b). Representative images of Oil Red O staining were taken at 200X and scale bars = 50 μ m (c). Protein expression was measured by immunoblotting and quantified by densitometric analysis



precluded brown adipogenesis, as evidenced by suppressed protein levels of PGC-1 α , UCP1, and PRDM16. Therefore, it may be concluded that CBD promotes brown adipogenesis, at least partly by activating the PPAR γ and PI3K signaling pathways.

Discussion

In this study, we present evidence for the first time that CBD treatment has effective potential to induce the brown fat phenotype in cultured white adipocytes by significant elevation of brown fat-specific genes and proteins. Although an earlier study demonstrated that CBD is able to reduce obesity and other metabolic disorders by inhibiting lipogenesis [25], it is still unknown how CBD decreases body weight. Here, we tried to examine whether CBD could increase thermogenesis. We successfully demonstrated that CBD along with a browning cocktail synergistically promotes the beige phenotype and stimulates the thermogenic program through significant up-regulation of brown fat-specific genes and proteins. It has already been

reported that *Tmem26*, *Tbx1*, and *Cited* are brown fat-specific markers that are enriched in beige cells [26]. Our results provide evidence that CBD significantly up-regulated expression of these three browning-specific genes, suggesting a possible role for CBD in inducing the beige phenotype. We also demonstrated that CBD significantly enhanced UCP1 and PGC-1 α at both the mRNA and protein levels, reflecting the obvious acquisition of BAT features in 3T3-L1 adipocytes [27, 28].

Enhanced mitochondrial numbers and activity are characteristic features of browning of adipose tissues [12]. The current results demonstrate that CBD increases biogenesis of mitochondria, as supported by confocal microscopic detection of PRDM16 as well as elevated expression of marker proteins involved in mitochondrial function such as CPT1, ACSL, and SIRT1 [29, 30]. Interaction between CPT1 and ACSL has been found to play an important role in the transfer of activated fatty acids through the mitochondrial outer membrane [29]. It is also well known that CPT1 and SIRT1 play significant roles in mitochondrial activity and fatty acid oxidation in brown adipocytes [31].

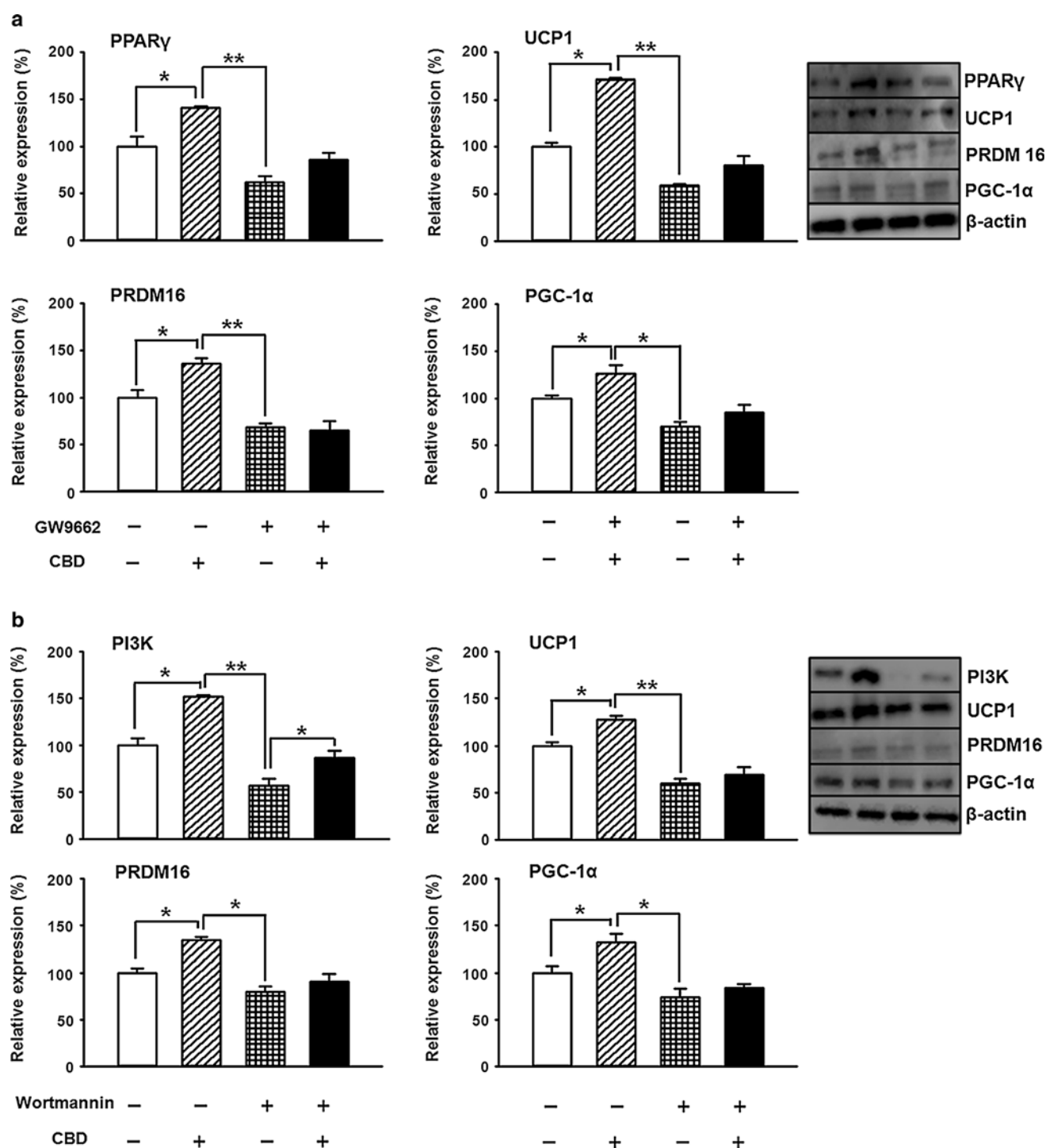


Fig. 4 Effects of **a** GW9662 (PPAR γ inhibitor) and **b** Wortmannin (PI3K inhibitor) on expression levels of browning marker proteins. GW9662 (5 μ M) and Wortmannin (1 μ M) were treated to 3T3-L1 adipocytes, and expression levels of proteins were determined by immunoblotting. All data are presented as the mean \pm S.D., and

differences among groups were determined by One-way Analysis of Variance (ANOVA) using the Statistical Package of Social Science (SPSS, version 17.0; SPSS Inc., Chicago, IL, USA) Program, followed by Turkey's post hoc tests. Statistical significance among different groups are shown as * p < 0.05 and ** p < 0.01

In addition, CBD treatment led to reduced expression of important proteins involved in lipogenesis such as JNK2, SREBP1, and LPL. It has already been reported that

SREBP1, a key transcription factor of lipogenesis, regulates lipid metabolism via the JNK2 pathway [32]. Although the role of SREBP1 in liver lipogenesis has been

extensively explored, its physiological role in adipocytes is still ambiguous. Targeted disruption of the *Srebp1* gene markedly suppresses expression of an entire class of lipogenic enzymes during fully induced lipogenesis in a refed state [33]. Therefore, alleviated expression of SREBP1 and JNK2 is likely to reflect the inhibitory action of CBD in regard to lipogenesis. CBD also appears to play a significant role in the down-regulation of LPL, which assists fatty acid uptake and de novo lipogenesis in adipocytes [34]. Differential expression of tissue-specific LPL has been implicated in the pathogenesis of lipid disorders, obesity, and atherosclerosis [35]. Previously, it has been observed that deficiency of LPL in adipocytes could lead to the reduction of fat volume but with a brownish appearance [36]. Thus, reduced LPL expression upon CBD treatment may alleviate lipogenesis and promote browning of adipocytes. Moreover, the current data show that the treatment of 3T3-L1 adipocytes with CBD reduced the number of lipid droplets, which provides less space to store triacylglycerol during lipogenesis [37].

During energy homeostasis, activation of PPAR γ -mediated pathways plays a crucial role [38]. Therefore, it is important to identify molecular pathways mediating the browning action of white adipocytes by CBD. Different pathways are known to be associated with thermogenesis. For example, PGC-1 α , a master regulator of mitochondrial biogenesis, drives thermogenesis in BAT, and its expression is regulated by diverse signaling pathways involving PPAR γ and cAMP/PKA/p38 MAPK [39, 40]. Numerous natural compounds such as ginsenoside Rb1 and apelin are able to induce browning through regulation of the PPAR γ and PI3K pathways, respectively [41]. However, recent studies also showed that PI3K signaling activation plays an important role in brown adipogenesis [42]. Our data show that CBD significantly increased the expression levels of PPAR γ and PI3K in 3T3-L1 adipocytes. This effect was abrogated by their inhibitors GW9662 and Wortmannin, respectively, which abolished CBD-induced expression of browning-specific markers. This indicates that the PPAR γ and PI3K signaling pathways, at least in part, play significant roles in the CBD-induced browning of adipocytes.

In conclusion, CBD modulates inhibition of lipogenesis and induction of brown-like phenotype in 3T3-L1 adipocytes. CBD stimulates expression of brown cell-specific genes in adipocytes via PPAR γ and PI3K activation, thereby displaying potential therapeutic implications for the treatment of obesity. Our findings provide initial in vitro experimental evidence in support of the possible therapeutic use of CBD for the induction of fat browning, although an in vivo study is needed.

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Compliance with ethical standards

Conflicts of interest The authors declared no conflicts of interest.

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