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Activation of type 2 cannabinoid receptors (CB2R) promotes fatty acid oxidation through the SIRT1/PGC-1α pathway

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ABSTRACT

Abnormal fatty acid oxidation has been associated with obesity and type 2 diabetes. At the transcriptional level, peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α) has been reported to strongly increase the ability of hormone nuclear receptors PPARα and ERRα to drive transcription of fatty acid oxidation enzymes. In this study, we report that a specific agonist of the type 2 cannabinoid receptor (CB2R) can lead to fatty acid oxidation through the PGC-1α pathway. We have found that CB2R is expressed in differentiated C2C12 myotubes, and that use of the specific agonist trans-caryophyllene (TC) stimulates sirtuin 1 (SIRT1) deacetylase activity by increasing the phosphorylation of cAMP response element-binding protein (CREB), thus leading to increased levels of PGC-1α deacetylation. This use of TC treatment increases the expression of genes linked to the fatty acid oxidation pathway in a SIRT1/PGC-1α-dependent mechanism and also drastically accelerates the rate of complete fatty acid oxidation in C2C12 myotubes, neither of which occur when CB2R mRNA is knocked down using siRNA. These results reveal that activation of CB2R by a selective agonist promotes lipid oxidation through a signaling/transcriptional pathway. Our findings imply that pharmacological manipulation of CB2R may provide therapeutic possibilities to treat metabolic diseases associated with lipid dysregulation.

1. Introduction

The increasing occurrence of obesity presents a global health problem which correlates with the growing incidence of conditions such as type 2 diabetes [1]. Fatty acids are central structural and functional components of lipid metabolism and the abnormal oxidation of fatty acids has been associated with obesity and type 2 diabetes [2]. Fatty acid oxidation rates are regulated at both transcriptional and non-transcriptional levels. At the transcriptional level, peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α) was reported to strongly increase the ability of the hormone nuclear receptors PPARα and ERRα to promote transcription of fatty acid oxidation enzymes [3]. The activity of PGC-1α is regulated by deacetylase Sirtin1 (SIRT1) [4]. Previous studies have shown that SIRT1 deacetylase activity is increased through different nutrient and signaling pathways, including glucose restriction, AMP-activated protein kinase (AMPK) activation and cAMP response element-binding protein (CREB) activation [5]. SIRT1/PGC-1α pathway has been considered to be an important mediator of fatty acid oxidation.

During the past decade, the biological effects of cannabinoids have been attracting more and more interest. Two main subtypes of cannabinoid receptors, type 1 cannabinoid receptors (CB1R) and type 2 cannabinoid receptors (CB2R), have been identified in mammalian tissues. Selective agonists and antagonists for these receptors have been identified [6]. CB1R is reported to be highly expressed in the brain [6], while CB2R was initially considered to be primarily expressed in immune cells [7]. However, recent studies have intriguingly demonstrated that this receptor can also be found in the brain [8], in endothelial cells of various origins [9], and in vascular smooth muscle cells [10]. A bicyclic sesquiterpene, trans-caryophyllene (TC), has been reported to be a CB2R selective agonist, which binds to CB2R but not CB1R and results in activation of the Gi/Go subtype of G proteins [11]. A previous study has demonstrated that TC could produce neuroprotective effects in ischemic models both *in vitro* and *in vivo* by activating CB2Rs. Further study has verified that increased levels of AMPK and phosphorylation of CREB are both involved [12]. However, the effects of CB2R agonists on SIRT1 deacetylase, PGC-1α activity, and fatty acid oxidation rates are as yet unknown. In this study, we identify CB2R expression at the mRNA and protein level in myotubes, however we do not see CB1R expression at the protein level. Addition of a known CB2R agonist, TC, stimulates SIRT1 deacetylase activity by increasing the phosphorylation of CREB. As a result, PGC-1α is deacetylated and activated, resulting in an increase in the expression of genes linked to complete oxidation of fatty acids. Confirmation that this agonist works through CB2R was

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demonstrated by selective knockdown of CB2R which resulted in the loss of TC activity on the activation of SIRT1/PGC-1α and oxidation of fatty acids.

2. Materials and methods

2.1. Cell culture

C2C12 cells of mice were grown in Dulbecco's modified low-glucose Eagle (DMEM) medium containing 10% FBS (v/v), 4.0 mM glutamine, and 1% penicillin and streptomycin. Cells were cultured in a humidified incubator with 5% CO_2 at 37 °C. When cells were 70–80% confluent, the differentiation of myoblasts into myotubes was induced by switching the medium to DMEM containing 2% horse serum (GIBCO, Grand Island, NY, USA) for 72 h. Cells were treated with 1 μM trans-caryophyllene (TC) (Sigma–Aldrich, St. Louis, MO, USA) for 48 h. Cellular transfection to knockdown CB2R protein expression was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Briefly, 24 h before transfection, cells (5×10^5) were plated in 0.5 ml cultural medium and allowed to grow overnight at 37 °C and 5% CO_2. The small interfering RNA (siRNA) transfection complex, formed by combining transfection reagent and 50 nM siRNA (QIAGEN, USA; Ctrl_Allstars_1 Negative Control (Neg siRNA) or Mm_CNR2_9 target sequence: AAGGCCCAAGGTCCTCGGTTA) in serum-free Opti-MEM Medium, was added dropwise to cells. The successful knockdown of CB2R was verified by western blot analysis.

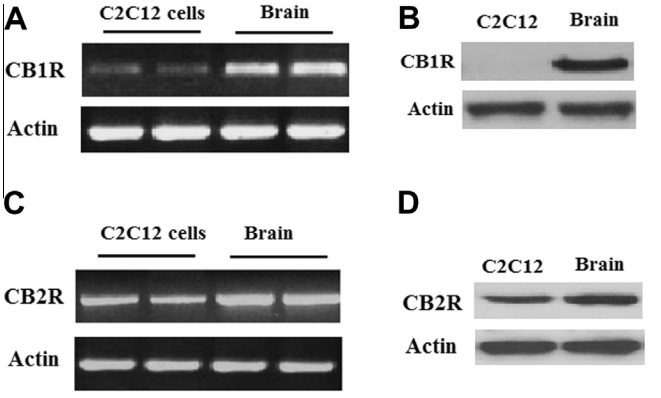

Fig. 1. Differentiated C2C12 myotubes express CB2R. (A) RT-PCR for expression of CB1R, with brain samples used as a positive control and actin serving as the housekeeping gene. (B) CB1R was not observed by western blot analysis. (C) RT-PCR for expression of CB2R, with brain samples used as a positive control and actin serving as the housekeeping gene. (D) Western blot analysis revealed that CB2R was expressed in C2C12 myotubes.

2.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA from cultured cells was isolated using Trizol reagent (Invitrogen) in accordance with the manual instructions. Two micrograms of total RNA was used to synthesize cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster, CA, USA). Synthesized cDNA by reverse transcriptase polymerase chain reaction (RT-PCR) was used to study the expression of CB1R and CB2R in C2C12 cells. The primers used for CB1R are: forward (5′-CCAAGAAAAGATGACGGCAG-3′) and reverse (5′-AGGATGACACATAGCACCAG-3′). The primers used for CB2R are: forward (5′-TCGCTTACATCCTTCAGACAG-3′) and reverse (5′-TCTT CCCTCCCAACTCCTTC-3′). The primers used for β-actin are: forward (5′-CTGTCGAGTCGCGTCCACCC-3′), and reverse (5′-GCTTTGCACA TGCCGGAGCC-3′).

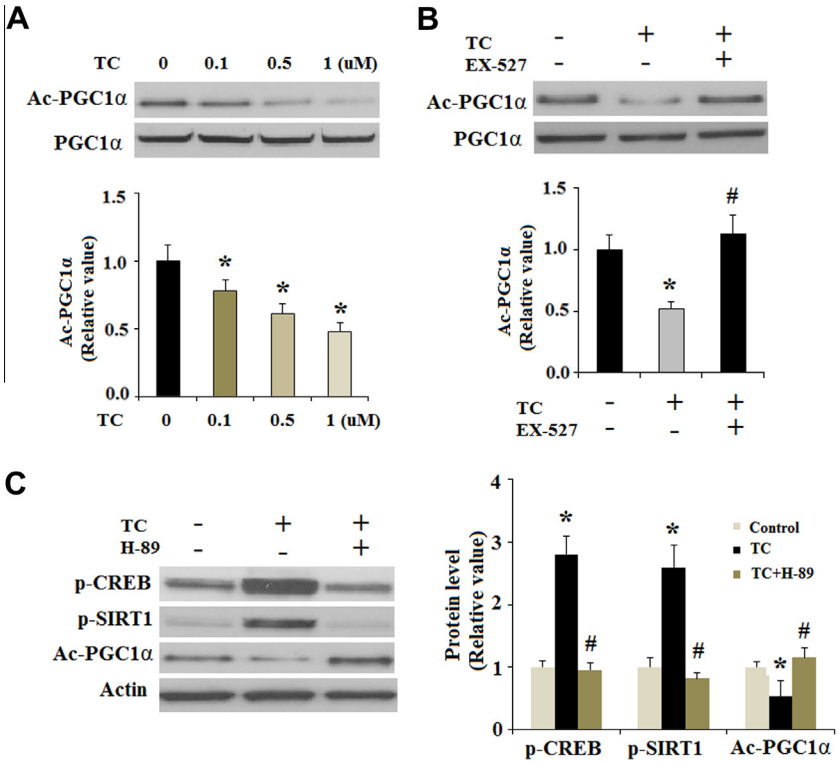

Fig. 2. TC, as a specific agonist of CB2R, induces PGC1α deacetylation through SIRT1 in C2C12 myotubes. (A) TC deacetylates PGC-1α in a concentration-dependent manner. Acetylated PGC-1α was detected using the acetyl-lysine-specific antibody following PGC-1α immunoprecipation ($^*p < 0.01$ vs. non-treatment group); (B) TC deacetylates PGC-1α in a SIRT1-dependent manner. C2C12 myotubes were treated with 0.5 μM TC, with control cells pretreated with EX-527 (2 μM) for 4 h before the addition of TC ($^*p < 0.01$ vs. non-treatment group; $^\#p < 0.01$ vs. TC treatment group); (C) TC induces phosphorylation of the PKA substrate p-CREB. The effects of TC on phosphorylation of SIRT1 and deacetylation of PGC-1α were abolished by the PKA inhibitor H-89 ($^*p < 0.01$ vs. control group; $^\#p < 0.01$ vs. TC treatment group).

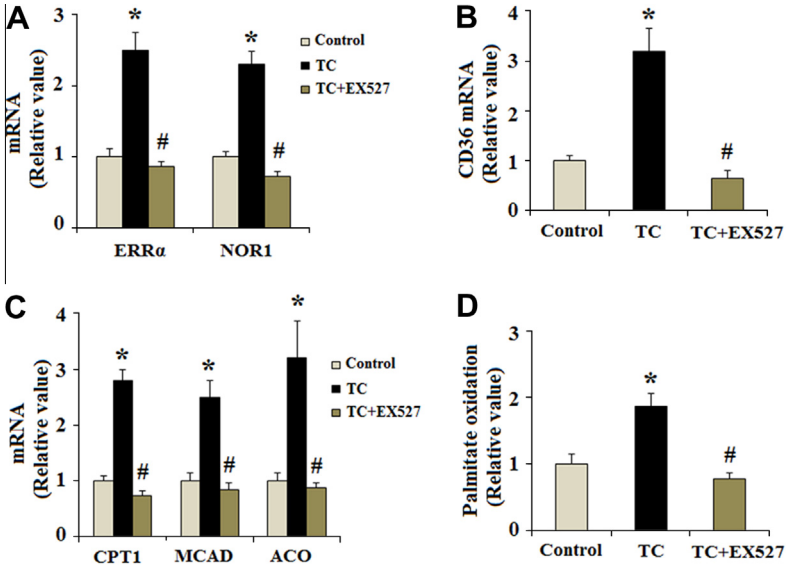

Fig. 3. TC, as a specific agonist of CB2R, increases expression of genes linked to fatty acid oxidation in cultured muscle cells. (A) Total RNA extracted from myotubes was used to measure the indicated genes using qRT-PCR. TC treatment increases fatty acid oxidation transcriptional regulatory genes ERRα and NOR1, which is abolished by EX-527; (B) TC treatment increases fatty acid transport genes CD36, which is abolished by EX-527; (C) TC increases expression of all three mitochondrial β-oxidation genes, CPT1, MCAD, ACO, an effect that is inhibited by EX-527; (D) TC increases the rate of fatty acid oxidation, in C2C12 myotubes, which is inhibited by EX-527. (*$p < 0.01$ vs. control group; #$p < 0.01$ vs. TC treatment group).

2.3. Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA in C2C12 cells was isolated using Trizol (Invitrogen) in accordance with the manual instructions. Two micrograms of total RNA was used to synthesize cDNA using high capacity cDNA reverse transcription kit (Applied Biosystems). Synthesized cDNA was used to perform quantitative real time polymerase chain reaction (qRT-PCR) with SYBR GREEN PCR Master Mix (Applied Biosystems). The following primers were used: MCAD: forward (5′-GAA GGTTGAACTCGCTAGGC-3′) and reverse (5′-GCTAGCTGATTGGCA ATGTC-3′); PDK4: forward (5′- CCGCTGTCCATGAAGCA-3′) and reverse (5′-GCAGAAAAGCAAAGGACGTT-3′); ERRα: forward (5′-CAAG AGCATCCCAGGCTT-3′) and reverse (5′-GCACTTCCATCCACACACTC-3′); ACO: forward (5′-AGAACCCATTTGCACACCTTG-3′) and reverse (5′- AGCGTCCGTATCTTGAGTCCT-3′); ACAD9: forward (5′-TTTCCAG AGGTCAGTCAACATGA-3′) and reverse (5′-TGG TCAATTTTT CGAGAGTCCAC-3′); ACADSB forward (5′-CCCAACCTGCTTGTCTCC TTG-3′) and reverse (5′-ATCCCTGGATCACCGATTTCT-3′).

2.4. Rates of fatty acid oxidation

Fatty acid oxidation assays were performed following the method described previously [13]. Differentiated C2C12 myotubes were incubated in the presence of ^{14}C-palmitate. As an indication of fatty acid oxidation, ^{14}C-palmitate oxidation was measured as ^{14}C-labeled CO_2 production over a 3 h-period. Whatman paper was wetted with 100 μl of phenylethylamine-methanol (1:1) to trap the ^{14}C-labeled CO_2 produced during the incubation period. Finally, radioactivity was measured on a scintillation counter.

2.5. Western blot analysis

Cell lysates were prepared using lysis buffer (Cell signaling technology, Danvers, MA, USA) which was supplemented with a protease and phosphatase inhibitor cocktail (Sigma–Aldrich). The protein lysates were subject to 10% SDS–PAGE and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). After blocking with 5% nonfat skimmed milk for 2 h at RT, the membranes were probed with primary antibodies overnight at 4 °C. After subsequent washing with TBST, the secondary antibody was added to horse radish peroxidase (HRP) (Pierce Biotechnology, Rockford, IL, USA) and was incubated at RT for 1 h. Blots were developed with enhanced chemiluminescence (ECL) kit (Pierce Biotechnology). The following antibodies were used in this study: rabbit anti-CB1R and anti-CB2R antibodies (Abcam, USA); p-CREB (Abcam, USA); p-SIRT1 (Abcam, USA), and anti-β-actin (Cell signaling technology, USA).

2.6. PGC-1α acetylation assays

PGC-1α lysine acetylation was analyzed by immunoprecipitation of PGC-1α followed by western blot using acetyl-lysine antibodies (Cell signaling technology, USA) as previously described [4]. After the indicated treatment, C2C12 cells were lysed and PGC-1α was immunoprecipitated using anti-PGC-1α (Abcam, USA) and examined for acetylation.

2.7. Statistical analysis

Data are the means ± standard error of the mean (SEM). Statistical analysis was performed by a two-tailed Student's *t*-test. $p < 0.05$ was considered to be statistically significant.

3. Results

To investigate the expression pattern of CB1R and CB2R in fully differentiated C2C12 myotubes, an RT-PCR experiment was performed. The results indicated that both CB1R (Fig. 1A) and CB2R (Fig. 1C) were expressed in differentiated C2C12 myotubes. Mouse brain samples were used as a positive control. Expression of CB2R was stronger than expression of CB1R in C2C12 myotubes based on RT-PCR. The protein expression of CB2R in C2C12 myotubes was confirmed by western blot analysis (Fig. 1D). However, the expression of CB1R in C2C12 myotubes was not detectable by western blot analysis (Fig. 1B). Therefore, we speculated that CB2R might have a more important role in C2C12 myotubes.

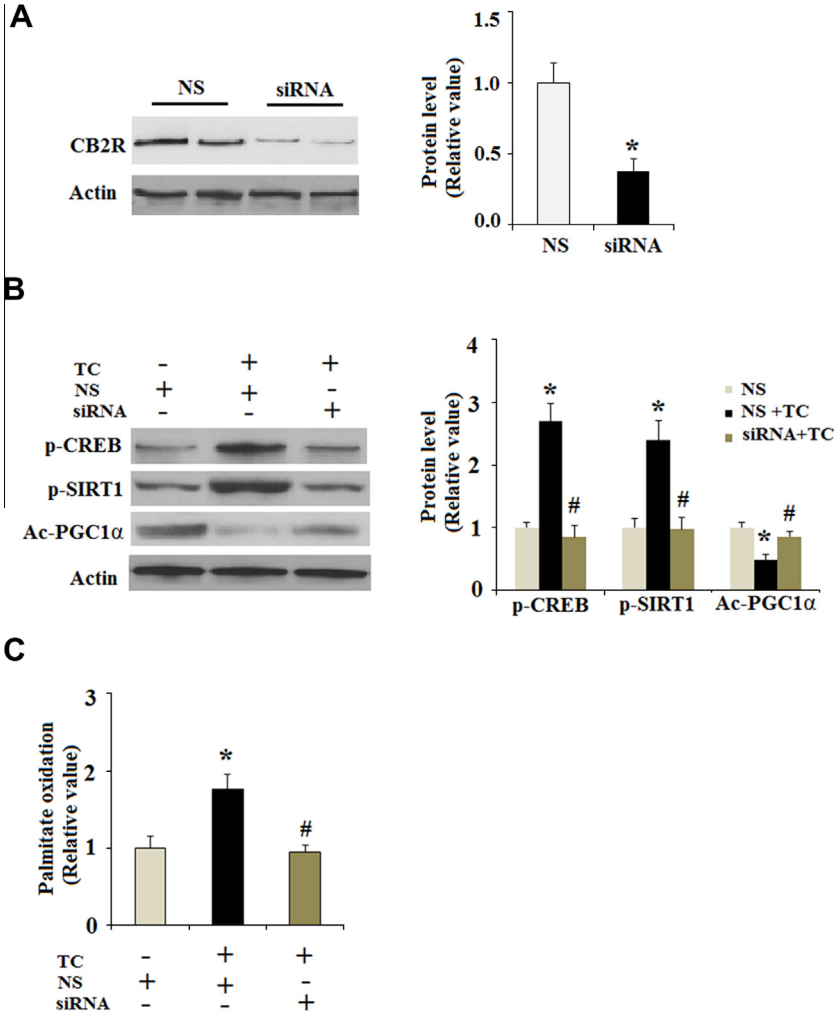

Fig. 4. The effects of TC on promoting fatty acid oxidation are dependent on CB2R. (A) The expression of CB2R was knocked down using small RNA interferences, and western blot analysis confirmed the successful knockdown of CB2R (*$p < 0.01$ vs. non-specific (NS) group); (B) Representative western blot and quantification analysis reveals that CB2R silencing abolishes the effects of TC on CREB phosphorylation, SIRT1 phosphorylation, and PGC-1α deacetylation (*$p < 0.01$ vs. control group; #$p < 0.01$ vs. TC treatment group); (C) CB2R silencing completely abolishes the increased rate of fatty acid oxidation induced by TC (*$p < 0.01$ vs. control group; #$p < 0.01$ vs. TC treatment group).

It is unknown whether activation of CB2R affects PGC-1α acetylation. To test this, we treated skeletal muscle cells with trans-caryophyllene (TC), a CB2R-selective agonist, and the results demonstrated that TC promoted PGC1α deacetylation in a dose-dependent manner (Fig. 2A). Endogenous SIRT1 deacetylase activity is a main regulator controlling the level of PGC-1α acetylation. To assess whether or not TC-induced PGC-1α deacetylation is dependent on SIRT1, we used a specific SIRT1 inhibitor (EX-527) [14]. Fig. 2B shows that TC-mediated PGC-1α deacetylation was completely blocked by EX-527, which is consistent with SIRT1 playing a critical role in PGC-1α deacetylation induced by TC. Since phosphorylation at Ser434 is part of the known mechanism of the deacetylase activity of SIRT1 [15], we then tested if TC could induce SIRT1 phosphorylation at Ser434. Fig. 2C shows that TC strongly induces SIRT1 phosphorylation at Ser434, which is consistent with changes in PGC-1α deacetylation. A previous study showed that phosphorylation of SIRT1 at Ser434 was regulated by PKA/CREB pathway [15]. H89, a specific inhibitor for PKA [16], does indeed abolish the effects of TC on SIRT1 phosphorylation of SIRT1 at Ser434 and PGC-1α deacetylation (Fig. 2C).

Being that PGC-1α is a main activator of fatty acid oxidation, we then tested the ability of TC to modulate PGC-1α target gene expression with a particular focus on those genes that promote fatty acid oxidation. Firstly, as shown in Fig. 3A, fatty acid oxidation transcriptional regulatory genes ERRα and NOR1 are induced by TC treatment, an effect that is abolished by EX-527. Secondly, we investigated the expression of the fatty acid transport gene CD36, and we found that TC increases the expression of CD36, an effect that was eliminated by EX-527 (Fig. 3B). Thirdly, TC causes a complete change in expression of all three mitochondrial β-oxidation genes, CPT1, MCAD, ACO, all of which were inhibited by EX-527 (Fig. 3C). Finally, we measured the complete rate of fatty acid oxidation in the presence and absence of TC. Findings show that cellular fatty acid oxidation rates are indeed markedly increased following treatment with TC as measured by radioactive CO_2 derived from radiolabelled ^{14}C-palmitate (Fig. 3D).

In order to determine whether or not the effect of TC on fatty acid oxidation is dependent on CB2R, we inhibited the expression of CB2R using CB2R siRNA. Successful suppression of CB2R is shown in Fig. 4A by western blot analysis. The effect of TC on the PGC-1α deacetylation pathway is abolished when C2C12 myotubes are transfected with CB2R siRNA (Fig. 4B). Most importantly, the radiolabelled ^{14}C-palmitate assay revealed that the effect of TC on cellular fatty acid oxidation rates is abolished by muting the CB2 receptor (Fig. 4C).

4. Discussion

In these studies, we have identified a new function of the CB2R pathway by finding that it can increase the rate of fatty acid oxidation and contribute to lipid homeostasis. Our data demonstrate that using a selective agonist to modulate CB2 receptor function can regulate the rate of fatty acid oxidation. This modulation works through activating the cAMP/PKA pathway, which in turn leads to phosphorylation of SIRT1 Ser434 and increases its deacetylase activity. Activated SIRT1 deacetylates PGC-1α and increases the expression of fatty acid oxidation genes which leads to increases in the complete oxidation of fatty acids. This study is the first to implicate a role for the CB2 receptor in the function of skeletal muscle cells and suggests that CB2 receptor activation influences fatty acid oxidation.

Previous studies have shown that specific activation through transgenic expression of PGC-1α in skeletal muscle tissue provides strong beneficial effects and protects against age-associated diseases [17,18]. The increase of PGC-1α activity has been considered to be a valid therapeutic target in the amelioration of these metabolic diseases, but identifying small molecules that bind directly to and activate PGC-1α has proven to be difficult. Alternatively, targeting pathways that lead to PGC-1α activation may be more feasible. Increasing the deacetylation and activation of PGC-1α by promoting SIRT1 activity using various means has been viewed as an efficient way to ultimately promote downstream target functions, including mitochondrial oxidative metabolism [19,20]. The work reported here suggests that trans-caryophyllene, a CB2R-selective agonist, could be used to activate PGC-1α in skeletal muscle. We chose a CB2R-selective agonist but not a CB1R-selective agonist because the expression level of CB1R is low in C2C12 myotubes. CB2R belongs to the G-protein coupled receptor superfamily and is coupled to the inhibitory Gi/Go-subtype of G-proteins. It was previously held that CB2 receptors are primarily expressed by immune and haematopoietic cells [21]. Intriguingly, recent studies have provided evidence for the presence of CB2 receptors in the brain [9], myocardium [22], and endothelial cells [23]. Here, we report expression of CB2 and/or CB1 receptors in C2C12 cells of mice under basal cell culture conditions. CB2R has recently been shown to mediate cell proliferation and neuroprotection. TC was reported to decrease neuronal injury and mitochondrial depolarization caused by oxygen-glucose deprivation/re-oxygenation (OGD/R) by way of its enhancement of AMPK and CREB phosphorylation [11], which is in agreement with our study. Moreover, a previous study reported that modulating the activity of CB1R in L6 skeletal muscle regulates both insulin-dependent mitogen-activated protein (MAP) kinase (ERK1/2) and the canonical PI3-kinase/PKB signaling pathways, implying a potential role for muscle growth and differentiation as well as regulation of glucose and lipid metabolism [24]. Activation of CB2R in different tissues might result in coupling to several principal signaling pathways, including MAP kinase (e.g., ERK1/2, p38, and P42/44), c-Jun N-terminal kinase, ERK, and PI3/Akt pathways [25]. Whether these pathways are recruited in the CB2R agonist-specific action that results in PGC-1α deacetylation and fatty acid oxidation is still unknown. At present, CB2R signaling pathways in muscle cells are poorly understood and therefore, further systematic study must be carried out to enhance our understanding of this issue.

In conclusion, our data indicate that CB2R activation by TC promotes fatty acid oxidation through activating the PGC-1α pathway. To our knowledge, the data presented in this study provides the first evidence which supports a previously unappreciated role for CB2R in fat metabolism: to increase activity of SIRT1 and stimulate PGC-1α deacetylation. Furthermore, we demonstrate PKA/CREB involvement in CB2R-mediated fatty acid oxidation. Abnormal fatty acid oxidation has been involved in causing obesity and type 2 diabetes [2]. Because TC both appears to maintain its CB2R agonist activity when administered orally and is a common ingredient found in many food additives and folk medicines [11], our study suggests that further investigation is warranted to establish the clinical usefulness of TC as a preventative or therapeutic agent for obesity and insulin resistance.

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