Cannabinoid Receptor Signaling Directly Inhibits Thermogenesis and Alters Expression of Adiponectin and Visfatin

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Introduction

The endocannabinoid system has recently emerged as a critical component in the control of energy homeostasis. It is considered to be a general stress-recovery system and stimulates food intake [1]. Cannabinoid type 1 (Cb1) receptor-deficient mice are leaner than their wild-type littermates [2]. Furthermore, these animals are protected against diet-induced obesity [3]. The selective Cb1 receptor blocker Rimonabant promotes weight loss in large clinical trials in obese and insulin-resistant individuals [4,5]. Both central and peripheral actions of the endocannabinoid system contribute to the regulation food intake and energy expenditure [6]. The Cb1 receptor is expressed in adipocytes [7]. However, little is known to date about direct Cb1 receptor-mediated actions on important functional levels of adipose tissue implicated in the control of energy and glucose homeostasis. Here, we present the first evidence for direct Cb1 receptor-mediated actions in brown and white adipocytes. Cannabinoid signaling negatively impacts thermogenesis in brown adipocytes while favoring a positive energy balance by differentially regulating the novel adipokine visfatin as well as adiponectin in white adipocytes.

Materials and Methods

Materials

The Cb1 receptor agonist WIN 55212–2 was purchased from Tocris (Avonmouth, UK). The UCP-1 antibody was from Chemicon International (Temecula, CA, USA). Primers for expression analysis were ordered from Biometra (Göttingen, Germany). All other chemicals were from Sigma-Aldrich Co. (St. Louis, MO, USA) unless stated otherwise.

Cell culture

Immortalized murine epididymal and inguinal white and interscapular brown adipocytes were cultured as previously described [8]. In brief, cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Paisley, Scotland) with 4.5 g/l glucose, supplemented with 20 nmol/l insulin, 1 nmol/l triiodothyronine, 20% fetal bovine serum, and penicillin/streptomycin (Bio Whittaker, Vervier, Belgium). Upon reaching confluence, differentiation was induced for 24 h with 500 μmol/l isobutylmethylxanthine, 250 μmol/l indomethacine, and 2 μg/ml dexamethasone. Cell culture was continued for five more days after this induction period. Prior to the experiments, cells were starved overnight in serum-free medium.

Western blotting

Proteins were isolated in whole-cell lysis buffer containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mmol/l vanadate and 2 mmol/l phenylmethylsuphonyl fluoride (Fluka Chemie, Neu-Ulm, Germany). Protein content was measured using the Bradford method with dye from Bio-Rad (Hercules, CA, USA). Proteins were separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked overnight with 10 mmol/l Tris, 150 mmol/l NaCl, and 0.05% Tween 20, pH 7.2, supplemented with 3% bovine serum albumin, and incubated with the antibody for 1.5 h. Protein bands were visualized using chemiluminescence (Roche Molecular Biochemicals, Mannheim, Germany) and enhanced chemiluminescence film (Amersham Pharmacia Biotech, Freiburg, Germany).

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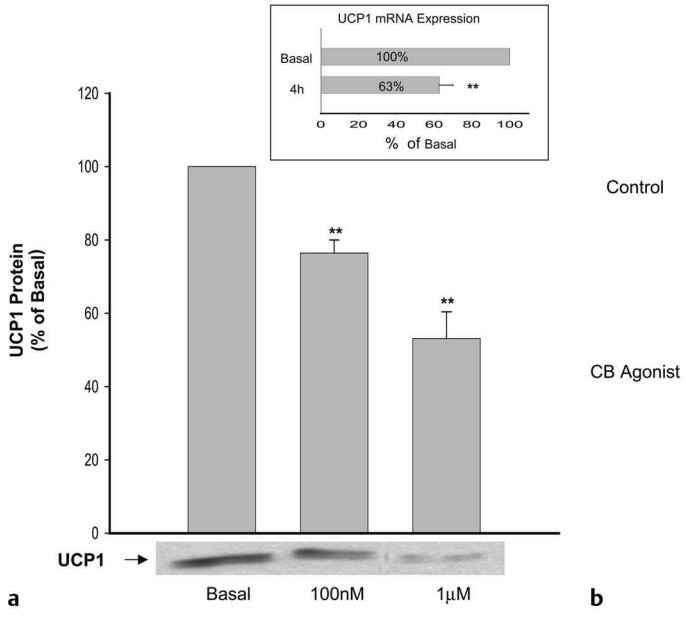


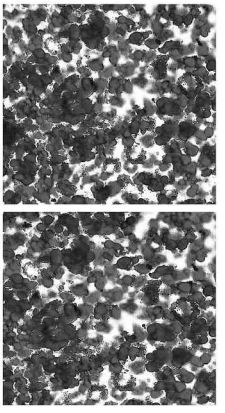
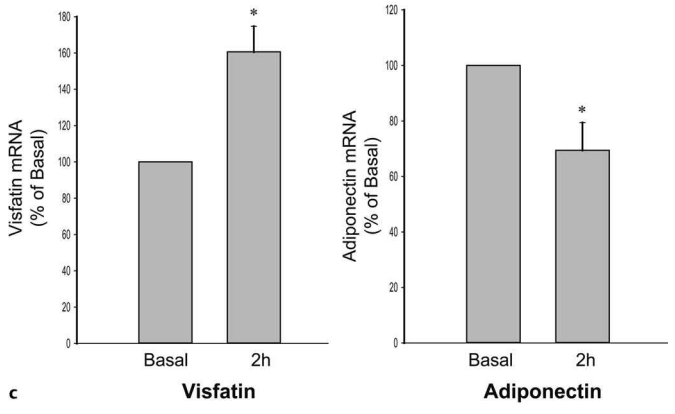


Fig. **1** Cannabinoid signaling directly inhibits thermogenic UCP-1 without affecting terminal differentiation, and differentially regulates visfatin and adiponectin in brown and white adipocytes. Brown adipocytes were treated for (**a**) 2 h, 4 h (insert), and (**b**) chronically (9 days) with selective cannabinoid Cb1 receptor agonist WIN 55 212 – 2 (CB Agonist) at the concentrations indicated (insert in **a** and **b**: 1 μM). UCP-1 immunoblots and quantitative mRNA analysis (**a**), and oil red O staining (**b**, microscopic digital image, 40 x) were performed. Quantitative mRNA analysis of visfatin (epididymal white adipocytes) and adiponectin (inguinal and epididymal white adipocytes) was carried out in fully differentiated cells (**c**, 100 nM WIN 55212–2). A bar graph analysis including the SEM and a representative immunoblot analysis (**a**) of 2 – 5 independent experiments are shown. * $p \leq 0.05$ and ** $p < 0.01$ comparing non-treated (Basal) to treated cells.



Oil red O staining

Cells were washed with PBS and fixed with 10% formalin for 30 min. After removing the formalin, 10 ml of the oil red O staining solution previously filtered (Schleicher & Schuell GmbH, Dassel Germany) were added for 1 h. Cells were rinsed several times with distilled water to remove excess stain and any precipitate that may have formed.

Analysis of gene expression

Expression of UCP1, visfatin, and adiponectin mRNA was determined by reverse transcription real-time polymerase chain reaction. Total RNA was isolated using TRIzol reagents (Invitrogen, Karlsruhe, Germany). RNA quality was tested by photometric analysis and RNA visualization on an agarose gel. RNA was reverse-transcribed using Superscript II (Invitrogen, Karlsruhe, Germany) and oligo $p(DT)_{15}$ primer (Roche Molecular Biochemicals, Mannheim, Germany) in the presence of RNase inhibitor (Roche Molecular Biochemicals). SYBR Green I fluorescence emissions were monitored after each cycle in an Abi Prism 7000 detection system (Applied Biosystems, Foster City, CA, USA). The following primers were used: UCP-1 (acc. no. NM_009 463) ATG GTG AAC CCG ACA ACT TCC GAA GTG (sense) GTA CTG GAA GCC TGG CCT TCA CCT TGG (antisense); visfatin (acc. no. NM_021 524) TCG GTT CTG GTG GCG CTT TGC TAC (sense) and AAG TTC CCC GCT GGT GTC CTG TGT (antisense); adiponectin (NM_009 605) CTT AAT CCT GCC CAG TCA TGC (sense) and CCA TCC AAC CTG CAC AAG TTC (antisense). PCR was performed as follows: initial denaturation at 95 °C for 15 min, 40 cycles with 95 °C for 30 s, 60 °C for 60 s, 72 °C for 60 s (visfatin) or 95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s (adiponectin). Specific amplification was confirmed using melting curve profiles. Optimized relative quantification was done based on the second derivative maximum method normalized to 36B4 as housekeeping gene.

Statistical analysis

Unpaired Student's *t*-test was performed for determining statistical significance of differences using "sigma plot"-software (SPSS Science; Chicago IL, USA). Data are presented as means ± SEM; values of $p \leq 0.05$ were considered significant, $p < 0.01$ highly significant.

Results

Cannabinoid Cb1 receptor stimulation inhibits UCP-1

Treatment of differentiated brown adipocytes with the selective Cb1 receptor agonist WIN 55212–2 decreased expression of the thermogenic UCP-1 by approximately 50%. This negative effect was dose-dependent with a highly significant 25% suppression of UCP-1 protein at 100 nM of the Cb1 agonist (Fig. **1 a**). Moreover, the cannabinoid receptor-mediated inhibition was maximal between 2 – 4 h of stimulation and was paralleled by similar decreases in UCP-1 mRNA levels (Fig. **1 a**, insert). UCP-1 is a marker of terminal brown fat differentiation. However, chronic stimulation of Cb1 receptor signaling did not impair lipid accumulation and terminal adipocyte differentiation, thus further corroborating the concept of an acute and differentiation-independent negative effect of cannabinoid signaling on thermogenic UCP-1 (Fig. **1 b**).

Cannabinoid Cb1 receptor stimulation alters expression of glucostatic adipokines

Visfatin, a novel insulin-mimetic adipokine, and adiponectin have been implicated in the regulation of glucose and energy

Table **1** Role of adipose cannabinoid signaling in white (WAT) and brown adipocytes (BAT)

Adipose Modulators of Energy Homeostasis	*Biologic Actions*	*Effect of Adipose Cannabinoid Signaling*
Mediators of Mitochondrial Proton Leak		
UCP-1 (BAT)	Adaptive Thermogenesis	Downregulation
Glucostatic Adipokines		
Visfatin (WAT)	Increased in Obesity Insulin-Mimetic Promoting Obesity?	Upregulation
Adiponectin (WAT)	Decreased in Obesity Insulin-Sensitizing Antiatherosclerotic Promoting Weight Loss	Downregulation

homeostasis in mice and humans. Direct stimulation of differentiated white adipocytes with the Cb1 receptor agonist (100 nM) for 2 h increased visfatin mRNA levels by approximately 60% compared to non-treated control cells. In contrast, adiponectin mRNA levels were decreased by 30% relative to controls (Fig. **1 c**).

Discussion

This study demonstrates a direct role for cannabinoid signaling to control thermogenesis and endocrine activity of adipose tissue. Cannabinoid signaling inhibits thermogenic UCP-1 in brown adipocytes and differentially regulates glucostatic adipokines visfatin and adiponectin in white adipocytes. These data provide the first evidence for direct cannabinoid receptor-mediated effects on two important functional levels of adipose tissue contributing to the control of energy and glucose homeostasis. The endocannabinoid system controls energy homeostasis by both central and peripheral mechanisms [6]. Cb1 receptors are expressed in adipocytes, and activation of adipocyte lipoprotein lipase has been reported by Cb1 receptor stimulation [2]. However, only indirect evidence using the Cb1 receptor antagonist SR141716 [7] exists for direct cannabinoid action on adipocyte function to date.

Brown adipose tissue-mediated thermogenesis importantly contributes to energy expenditure in small mammals. The role of brown adipose tissue is less clear in adult humans. Brown adipocytes are dispersed between white adipocytes, and a basal brown fat gene expression has been associated with insulin resistance in humans [9]. Physiological and pharmacological stimuli both appear capable of reactivating "dormant" brown fat cells and inducing transdifferentiation of white to brown adipocytes [10]. Similar to effects described for thiazolidinediones, Cb1 receptor inhibition induces a brown fat phenotype and may be an intriguing mechanism to mediate increases in energy expenditure. In support of this hypothesis, a very recent study describes the induction of genes coding for the β3-adrenergic receptor, which induces thermogenesis, and for enzymes involved in mitochondrial β-oxidation in mice treated with Cb1 receptor blocker Rimonabant [11].

On the level of endocrine activity, the present study describes a decrease in adiponectin levels by direct Cb1 receptor stimulation. This is consistent with the increase in adiponectin levels induced by Cb1 receptor blocker SR141716 (Rimonabant) in rat adipocytes *in vitro* [7] and *in vivo* in humans [12]. The present study is the first to describe a positive regulation of the novel adipokine visfatin. Visfatin levels are increased in obesity. Increased levels of this insulin-mimetic growth factor have been postulated to play a role in the pathogenesis of insulin resistance and obesity [13]. Conversely, adiponectin levels are decreased in obesity, and there is general agreement about the insulin-sensitizing and potential antiatherogenic properties of this adipocyte-derived factor [14]. Thus, our study demonstrates a direct divergent regulation of these glucostatic adipokines that favors insulin resistance and weight gain. In concert with the negative role of cannabinoid action on UCP-1 expression, the net effect of cannabinoid modulation of adipose endocrine function is compatible with the induction of a positive energy balance (Table **1**). In agreement with these findings, increasing numbers of observations demonstrate the induction of a negative energy balance by Cb1 receptor blockage in mice and humans [2 – 5].

Taken together, the present study reveals that cannabinoid signaling directly elicits thermogenic and differential endocrine responses in adipocytes. These adipotropic effects most likely constitute an important component of peripheral actions of the endocannabinoid system, and their inhibition can be postulated to underlie, in part, the beneficial actions of Cb1 receptor blockers in the treatment of obesity and associated cardiovascular complications.

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