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WJD mainly publishes articles reporting research results and findings obtained in the field of diabetes and covering a wide range of topics including risk factors for diabetes, diabetes complications, experimental diabetes mellitus, type 1 diabetes mellitus, type 2 diabetes mellitus, gestational diabetes, diabetic angiopathies, diabetic cardiomyopathies, diabetic coma, diabetic ketoacidosis, diabetic nephropathies, diabetic neuropathies, Donohue syndrome, fetal macrosomia, and prediabetic state.

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Basic Study

Metabolic and inflammatory functions of cannabinoid receptor type 1 are differentially modulated by adiponectin

Qiong Wei, Jong Han Lee, Chia-Shan Wu, Qun S Zang, Shaodong Guo, Hui-Chen Lu, Yuxiang Sun

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Abstract

BACKGROUND

Antagonists of cannabinoid type 1 receptor (CB1) have been shown to promote body weight loss and improve insulin sensitivity. Cannabinoids decrease adiponectin, and CB1 blocker increase adiponectin. However, the mediators of CB1 actions are not well defined.

AIM

To investigate whether the beneficial effects of CB1 inhibition are, at least in part, mediated by adiponectin.

METHODS

We compared metabolic and inflammatory phenotypes of wild-type (WT) mice, CB1-null (CB1^{-/-}) and CB1/adiponectin double-knockout (DKO) mice. We assessed the insulin sensitivity using insulin tolerance test and glucose tolerance test, and inflammation using flow cytometry analysis of macrophages.

Institutional Animal Care and Use Committee at Baylor College of Medicine, Houston, TX, United States).

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RESULTS

CB1^{-/-} mice exhibited significantly reduced body weight and fat mass when compared to WT mice. While no significance was found in total daily food intake and locomotor activity, *CB1*^{-/-} mice showed increased energy expenditure, enhanced thermogenesis in brown adipose tissue (BAT), and improved insulin sensitivity compared to WT mice. DKO showed no difference in body weight, adiposity, nor insulin sensitivity; only showed a modestly elevated thermogenesis in BAT compared to *CB1*^{-/-} mice. The metabolic phenotype of DKO is largely similar to *CB1*^{-/-} mice, suggesting that adiponectin is not a key mediator of the metabolic effects of *CB1*. Interestingly, *CB1*^{-/-} mice showed reduced pro-inflammatory macrophage polarization in both peritoneal macrophages and adipose tissue macrophages compared to WT mice; in contrast, DKO mice exhibited increased pro-inflammatory macrophage polarization in these macrophages compared to *CB1*^{-/-} mice, suggesting that adiponectin is an important mediator of the inflammatory effect of *CB1*.

CONCLUSION

Our findings reveal that *CB1* functions through both adiponectin-dependent and adiponectin-independent mechanisms: *CB1* regulates energy metabolism in an adiponectin-independent manner, and inflammation in an adiponectin-dependent manner. The differential effects of adiponectin on *CB1*-mediated metabolic and inflammatory functions should be taken into consideration in *CB1* antagonist utilization.

Key Words: Cannabinoid type 1 receptor; Adiponectin; Thermogenesis; Macrophages; Inflammation; Insulin resistance

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Core Tip: Antagonists of cannabinoid type 1 receptor (*CB1*) have been shown to promote body weight loss and improve insulin sensitivity. Cannabinoids have been shown to regulate adiponectin. However, it is unclear whether adiponectin is a key mediator of the functions of *CB1*. We compared metabolic and inflammatory phenotypes of *CB1*-null vs *CB1*/adiponectin double-knockout mice. Our findings reveal that *CB1* functions through both adiponectin-dependent and adiponectin-independent mechanisms: *CB1* regulates energy metabolism in an adiponectin-independent manner, and inflammation in an adiponectin-dependent manner.

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INTRODUCTION

The incidence of obesity has increased rapidly during recent decades, particularly in developed/industrialized countries. Obesity increases the incidences of hyperinsulinemia, insulin resistance, type 2 diabetes, dyslipidemia, atherosclerosis, hypertension, inflammation, and cancer[1,2]. Endocannabinoids are key regulators of food intake and energy metabolism, and the effects are mediated through the activation of the cannabinoid type 1 receptor 1 (*CB1*)[3,4]. Recent studies have demonstrated that blocking the activity of the endogenous cannabinoid system might be a strategy for the treatment of obesity and metabolic syndrome[5-7].

Previous study demonstrated that *CB1* knockout mice consume less food and have reduced body weight[4,8]. Rimobabant, a specific antagonist of *CB1*, reduces food intake by blocking the orexigenic effect of cannabinoids[9]. There is also evidence that endogenous cannabinoids regulate energy expenditure[10]. It has been shown that

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virally-induced hypothalamic *CB1* knockout mice showed no change in food intake, but did show less body weight gain over time due to increased energy expenditure; and the mRNA expression of β 3-adrenergic receptor and uncoupling protein-1 (UCP-1) was elevated in the brown adipose tissue (BAT)[10]. There are also data showing that rimonabant alleviates dyslipidemia and obesity *via* a BAT thermogenesis-mediated increase of energy expenditure[9]. It has been shown that peripheral *CB1* blockade is effective in activating thermogenesis in BAT to mitigate dyslipidemia and obesity[11], which suggests that the function of *CB1* in BAT can be peripherally mediated and is not necessarily dependent on its central action. These results suggest that endocannabinoids may regulate energy metabolism by binding to *CB1* expressing cells in peripheral white adipose tissue (WAT)[8] and/or BAT[9].

Adiponectin, an adipokine with insulin-sensitizing functions, has been reported to be relevant in many metabolic diseases such as obesity, and with associated complications such as diabetes, hyperinsulinemia, insulin resistance, dyslipidemia, hypertension, and inflammation[12]. Adiponectin treatment reduces body weight, improves hyperglycemia, ameliorates hyperinsulinemia and insulin resistance, and increases fatty acid oxidation and lipid clearance, in animal models of obesity and diabetes[13,14]. One of the most intriguing consequences of rimonabant treatment is increased adiponectin gene expression in adipose tissue of diet-induced obese (DIO) mice[9] and in cultured adipocytes[15]. However, the rimonabant-treated adiponectin- and leptin-deficient mice exhibit significantly ameliorated insulin resistance, which suggests that rimonabant reduces insulin resistance *via* both adiponectin-dependent and adiponectin-independent mechanisms[16]. These results suggest that rimonabant may regulate adiponectin expression in adipocytes, and the metabolic effects of rimonabant, at least in part, could be due to enhanced adiponectin secretion.

To determine whether adiponectin is indeed required for the peripheral functions of *CB1*, we used a genetic approach by breeding *CB1*^{-/-} mice with adiponectin-deficient mice to generate a mouse model lacking both *CB1* and adiponectin, *aka* double KO (DKO). We studied metabolic regulation such as thermogenesis and insulin sensitivity in these mice. The link between inflammation and obesity is now increasingly recognized and inflammation is considered a culprit of insulin resistance. Thus, we also characterized macrophage polarization in peritoneal macrophages and adipose tissue macrophages to elucidate whether *CB1* acts through adiponectin to modulate CB-1 mediated inflammation.

MATERIALS AND METHODS

Animals

All procedures using animal experiments were approved by the Institution of Animal Care and Use Committee at Baylor College of Medicine. All mice used in this study were congenic male mice. All mice were on a pure C57/6J background. To generate mice lacking both *CB1* and adiponectin, *CB1*^{-/-} mice and *adiponectin*^{-/-} mice were bred to each other to create compound heterozygotes that were *CB1*^{+/-}/*adiponectin*^{+/-}. In the second cross, compound heterozygotes were further bred to each other to yield homozygous *CB1*^{-/-}/*adiponectin*^{-/-} (*aka* double-knockout DKO mice); *CB1*^{-/-}/*adiponectin*^{+/+} mice (*aka* *CB1*^{-/-}), and *CB1*^{+/+}/*adiponectin*^{+/+} (*aka* WT mice). Age-matched male WT, *CB1*^{-/-} and DKO were used in the studies. There were three groups of mice used in the study: (WT) control group, *CB1*^{-/-} group, DKO group. Animals were housed under controlled temperature and lighting (75 ± 1 °F; 12 h light-dark cycle). The diet was from Harlan-Teklad (2920X) and the diet compositions are as follows: 16% of calories from fat, 60% from carbohydrates, and 24% from protein. All experiments were approved by the Animal Care Research Committee of the Baylor College of Medicine.

Metabolic characterizations

Magnetic Resonance Imaging analysis of body composition was also carried out using an EchoMRI Whole Body Composition Analyzer (Echo MRI®, United States). Metabolic parameters were obtained using an OxyMax open-circuit indirect calorimetry Comprehensive Lab Animal Monitoring System (CLAMS) from Columbus Instruments (Columbus, OH, United States). Energy expenditure (EE) was calculated as the product of the value of oxygen (3.815 + 1.232 × RQ) and the volume of O₂ consumed. Respiratory quotient (RQ) ratio of VCO₂/VO₂ was then calculated[17]. Energy expenditure was normalized to both body weight and lean mass. Locomotor activity was measured using infrared beams to count the number of beam breaks

during the recording period. The CLAMS data was the average of 3 d of data that were collected after 3 d of acclimation.

Insulin tolerance test and glucose tolerance test

The Insulin tolerance test (ITT) and glucose tolerance test (GTT) were carried out on WT, *CB1*^{-/-} and DKO mice. For ITT, after being fasted for 6 h, glucose of mouse tail blood was measured using One Touch Ultra glucose meter (lifeScan, New Brunswick, NJ, United States). It can detect glucose concentrations from 20 to 600 mg/dl using an electrochemical biosensor technology based on glucose oxidase chemistry. Mice then received an *i.p.* injection of human insulin (Eli Lilly Indianapolis, IN, United States) at a dose of 1.0 U kg⁻¹ of body weight. Tail blood glucose concentration was measured at 0, 30, 60, 90 and 120 min after *i.p.* insulin injection. The GTT was carried out after the mice were fasted for 18 h overnight. The mice received *i.p.* injection of glucose (Sigma-Aldrich, St. Louis, MO, United States) at a dose of 2.0 g kg⁻¹ body weight. The tail blood glucose was measured at 0, 15, 30, 60 and 120 min after glucose injection, and blood was collected for ELISA insulin analysis at 0, 15, 30 and 120 min after glucose injection.

Flow cytometry analysis

Peritoneal macrophage and stromal vascular (SV) cells of epididymal adipose tissues were fractionated as described[18,19]. Briefly, to get peritoneal macrophage, 5 ml of cold phosphate buffer saline (PBS) was injected into mouse peritoneal cavities immediately after anesthesia. After shaking the mice for 2-3 min, peritoneal fluid was harvested and spun down for peritoneal macrophages at 500 g for 5 min at 4 °C. The stromal vascular cells were isolated from the equal mass of epididymal adipose tissues using the collagenase digestion method. For flow cytometry analysis, same quantity cells (1 × 10⁶) were subsequently re-suspended and stained with appropriate antibodies (F4/80 and CD11c for M1 type macrophage, or F4/80 and CD206 for M2 type macrophage) as described in our previous study[20]. Antibody information used in flow cytometry analysis is as follows: PE anti-mouse F4/80 antigen (eBioscience, San Diego, CA), FITC anti-mouse CD11c antigen (BD Bioscience, San Jose, CA), purified CD16/CD32 antigen (BD Bioscience, San Jose, CA), and APC anti-mouse CD206 antigen (BD Bioscience, San Jose, CA). All data were collected using FACScan and analyzed using CellQuest software (BD Biosciences, San Jose, CA).

Analysis of gene expression

BAT and WAT were snap-frozen in liquid nitrogen and stored at -80 °C. Total RNA was extracted from frozen tissue samples using TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA was subsequently treated with DNase (Ambion, Austin, TX). RNA quality was assessed on 1.5% agarose gel electrophoresis in the presence of formaldehyde, and RNA concentration was determined by NanoDrop. The cDNA was synthesized from 1g RNA using the Superscript III First-Strand Synthesis system for reverse transcription-polymerase chain reaction (RT-PCR) (Invitrogen). Quantitative real-time RT-PCR was performed on an ABI7900 using the SYBR Green PCR Master Mix or the Taqman gene expression Master Mix (Applied Biosystems, Carlsbad, CA, United States). After amplification, the PCR product was subjected to 2% agarose gel electrophoresis. 18S RNA and -actin were used as internal controls. The primer sequences of quantitative RT-PCR are listed in Table 1 below.

Data analysis

Data are expressed as means ± SEM. Two groups were compared by *t*-test. *P* < 0.05 was considered statistically significant. All statistical analyses were carried out with SPSS 23.0 statistical software (IBM, Armonk, NY, United States).

RESULTS

***CB1* ablation increases energy expenditure, reduces adiposity, and improves insulin sensitivity**

The body weights of *CB1*^{-/-} mice were significantly lower than WT littermates; the analysis of body composition showed a markedly decreased percentage of fat mass in *CB1*^{-/-} mice compared to WT mice (Figure 1A). We then assessed food intake, locomotor activity, and energy expenditure using CLAMS. Our data showed there was a trend of reduction but no significant difference in total daily food intake by *CB1*^{-/-}

Table 1 The sequences of reverse transcription-polymerase chain reaction primers

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
UCP-1	GTGAAGTCAAGATGCAAGC	AGGGCCCCCTTCATGAGGTC
PGC-1 α	CATTGATGCACTGACAGATGGA	CCGTCAGGCATGGAGGAA
IR	CAAAAGCACAATCAGAGTGAGTATGAC	ACCACGTTGTGCAGGTAATCC
IRS1	GCCTGGAGTATTATGAGAACGAGAA	GGGGATCGAGCGTTTGG
PPAR γ 2	GCCTATGAGCACTTCACAAGAAATT	TGCGAGTGGTCTTCCATCAC
GLUT4	GCCTTGGGAACACTCAACCA	CACCTGGGCAACCAGAATG
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
CD11C	CTGGATAGCCTTTCTCTGCTG	GCACACTGTGTCCGAAGTC
CD206	TGATTACGAGCAGTGGAAGC	GTTACCGTAAGCCCAATT
TNF α	GAGAAAGTCAACCTCCTCTCTG	GAAGACTCCTCCAGGTATATG
IL-1 β	TGTTCTTTGAAGTTGACGGACCC	TCATCTCGGAGCCTGTAGTGC
IL-6	CCAGAGATACAAAGAAATGATGG	ACTCCAGAAGACCAGAGGAAAT

mice compared to WT mice (Figure 1B). To further determine whether there is a difference in locomotor activity, we analyzed spontaneous locomotor activity of these mice. Neither total daily locomotor activity nor the locomotor activity during light or dark cycles was altered (Figure 1C). We next calculated energy expenditure and found that energy expenditure of *CB1*^{-/-} mice was higher compared to their WT counterparts when normalized to body weight but no difference when normalized to lean mass (Figure 1D). Together, the results indicate that while *CB1* ablation reduces body weight and fat deposition, it may be a due to a combination of changes in food intake and energy expenditure.

Next, we assessed the glycemic phenotype. ITT showed that *CB1*^{-/-} mice were more responsive to insulin challenge than WT mice (Figure 1E). During GTT, there was no difference in glucose clearance following a glucose load in WT and *CB1*^{-/-} mice; but remarkably, the insulin levels of *CB1*^{-/-} mice were significantly lower, indicative of increased insulin sensitivity (Figure 1F). These results indicate that *CB1*^{-/-} mice have improved insulin sensitivity, which is in line with reduced body weight and body fat.

Collectively, these data suggest that *CB1* is an important regulator of energy homeostasis and insulin sensitivity.

Adiponectin has little impact on *CB1*-mediated overall metabolic profile

To determine whether the metabolic effects of *CB1* are mediated by adiponectin, we compared the metabolic phenotypes of *CB1*^{-/-} and DKO mice. The body weights of *CB1*^{-/-} mice were similar to their age-matched DKO (Figure 2A). There were also no differences in fat mass and lean mass between *CB1*^{-/-} and DKO mice. Indirect calorimetry analysis showed similar total food intake (Figure 2B) and locomotor activity (Figure 2C) between DKO and *CB1*^{-/-} mice. Interestingly, compared to *CB1*^{-/-} mice, DKO mice had increased energy expenditure when corrected either by total body weight or by lean weight (Figure 2D).

Furthermore, there was no difference in insulin sensitivity assessment of ITT between *CB1*^{-/-} and DKO mice (Figure 2E). We further assessed glucose response during GTT: No difference was detected in glucose response, but interestingly, the insulin of DKO was lower at 15 min but higher at 120 min as compared to that of *CB1*^{-/-} mice (Figure 2F). These data suggest that DKO mice have mostly similar metabolic profile, insulin sensitivity and glycemic response as *CB1*^{-/-} mice, despite there are some varied insulin responses to glucose. Taken together, the effects of *CB1* on metabolism are dominant; adiponectin is not essential in mediating the metabolic effects of *CB1*.

***CB1* ablation activates thermogenesis in BAT**

To determine the underlying mechanisms of the increased energy expenditure observed in *CB1*^{-/-} mice, we subsequently analyzed BAT collected from the mice. *CB1*^{-/-} mice showed a decreased ratio of BAT: Body weight as compared to WT mice (Figure 3A). Mitochondrial uncoupling protein 1 (UCP1) is the hallmark regulator of mitochondrial biogenesis and thermogenesis; when activated, UCP1 dissipates the

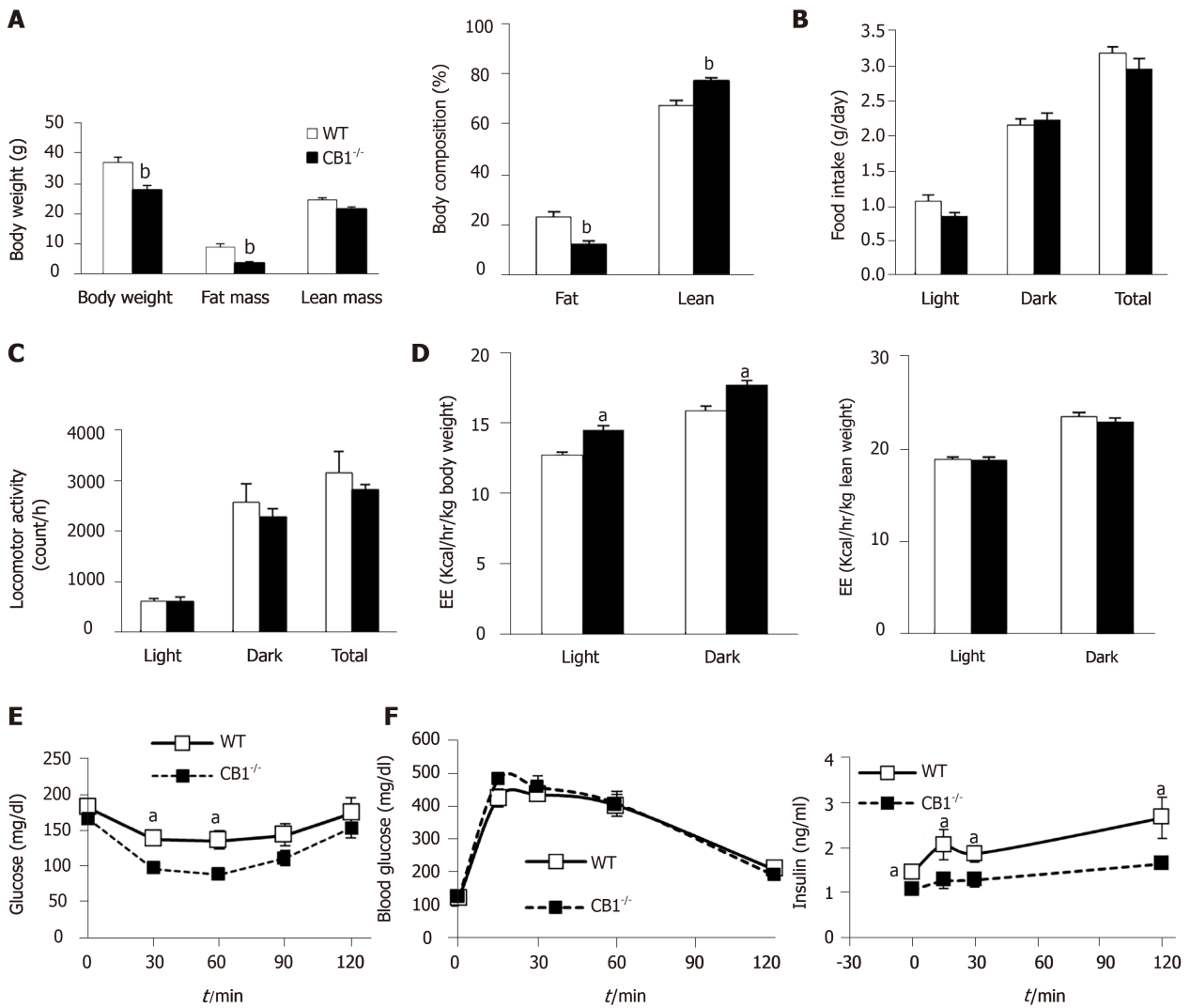


Figure 1 Cannabinoid type 1 receptor-null mice have reduced adiposity and improved insulin sensitivity. Wild-type (WT) and cannabinoid type 1 receptor-null (CB1^{-/-}) male mice at 4 mo of age. A: Body weight and body composition; B: Daily food intake; C: Locomotor activity; D: Energy expenditure adjusted by body weight or lean mass; E: Insulin tolerance tests; F: Glucose tolerance tests at 5 mo of age. *n* = 5-7. ^aP < 0.05, ^bP < 0.001, WT vs CB1^{-/-}.

transmembrane proton gradient and generates heat[21]. UCP1 mRNA was increased in CB1^{-/-} mice as compared to WT controls (Figure 3B). Peroxisome proliferator-activated receptor-coactivator-1 (PGC-1) is an upstream regulator of UCP1[22]. Indeed, PGC-1 expression was also increased in the CB1^{-/-} mice when compared to that of WT mice (Figure 3B).

Our result in Figure 1 showed CB1^{-/-} mice have higher insulin sensitivity compared to WT mice. Consistently, the gene expression of insulin receptor (IR) and insulin receptor substrate 1 (IRS-1) were increased in BAT of CB1^{-/-} mice. Peroxisome proliferator-activated receptorsγ2 (PPARγ2) is an important master adipogenic regulator [11]. Here we found that PPARγ2 was higher in BAT of CB1^{-/-} mice (Figure 3B). Glucose transporter type 4 (GLUT4) is a key mediator of glucose uptake in the adipose tissues[23]. As expected, GLUT4 expression in BAT of CB1^{-/-} mice was increased (Figure 3B), supporting increased glucose uptake and consistent with increased heat production. Together, ablation of CB1 increased BAT thermogenic activity, likely by modulating mitochondrial function, insulin signaling, adipogenesis, and glycose uptake signaling pathways in BAT.

We have reported that adiponectin has an important role in body temperature maintenance and thermogenesis. Here, we compared the weight of BAT depots in CB1^{-/-} and DKO mice. There was also no difference in total weight or BAT percentage between CB1^{-/-} and DKO mice (Figure 3C). The expression of thermogenic regulators UCP1 and PGC-1 was increased in BAT of DKO mice compared to that of CB1^{-/-} mice, while the expression of IR and IRS-1, GLUT4, and PPARγ2 were unchanged (Figure 3D). These results suggest that while adiponectin may be an important mediator for the effect of CB1 on mitochondrial genes in BAT, it is not critical for the

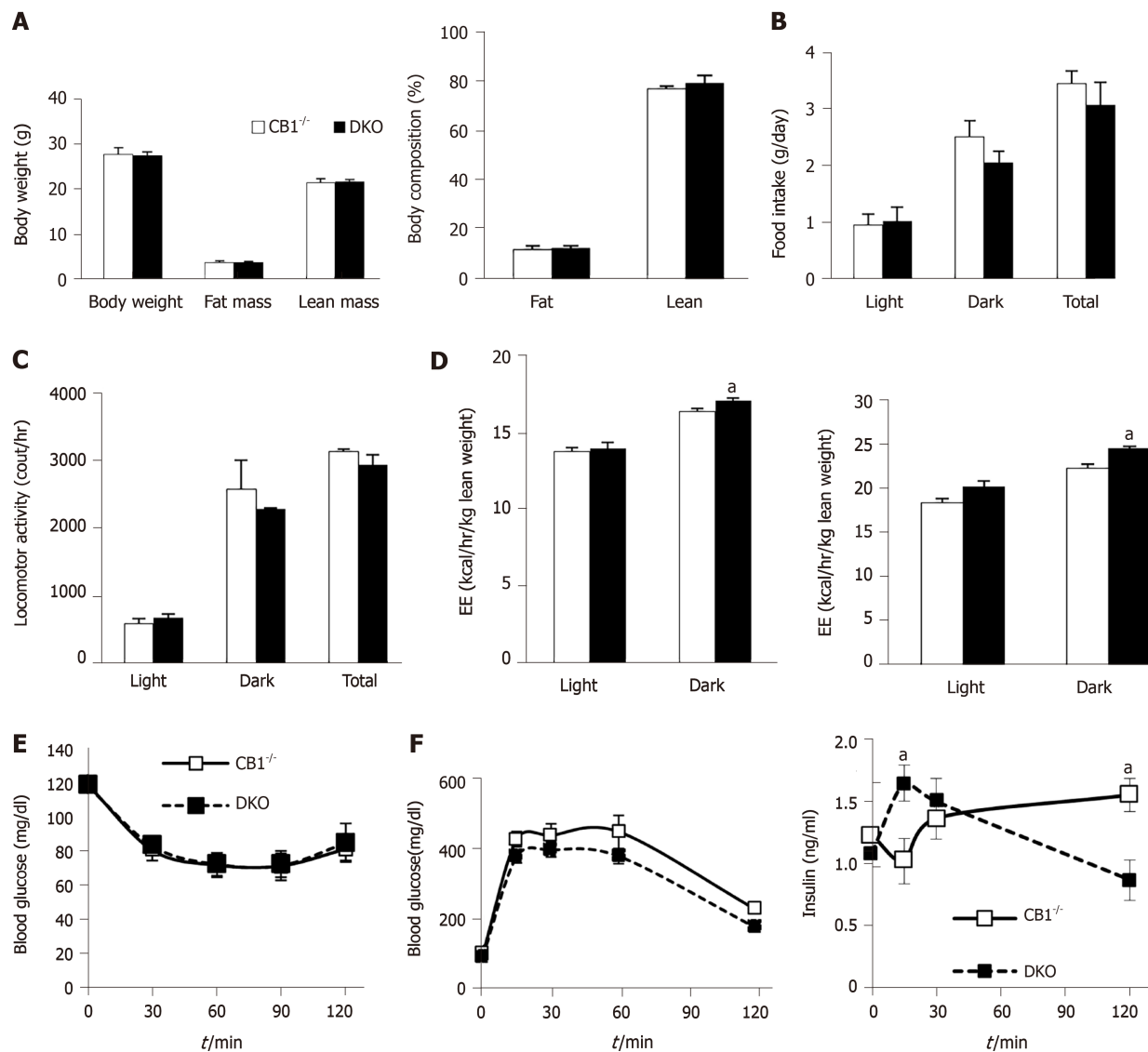


Figure 2 Cannabinoid type 1 receptor-null mice have similar adiposity and insulin sensitivity compared to double-knockout mice. Cannabinoid type 1 receptor-null (*CB1*^{-/-}) and double-knockout (DKO) male mice at 4 mo of age. A: Body weight and body composition; B: Daily food intake; C: Locomotor activity; D: Energy expenditure adjusted by body weight or lean mass; E: Insulin tolerance tests; F: Glucose tolerance tests at 5 mo of age. *n* = 5-7. ^a*P* < 0.05, *CB1*^{-/-} vs DKO.

regulation of *CB1* in insulin signaling, adipogenesis, and glucose uptake in BAT.

***CB1* ablation promotes macrophage anti-inflammatory polarization**

Macrophages have an important role in inflammation and insulin resistance[20]. To determine the underlying mechanisms of improved insulin sensitivity in *CB1*^{-/-} mice, we conducted flow cytometry analysis on peritoneal macrophages and adipose tissue macrophages. M1-like macrophages are pro-inflammatory and M2-like macrophages are anti-inflammatory[20]. Peritoneal M1-like macrophages, as well as the M1/M2 ratio as a readout of inflammation, were significantly decreased in *CB1*^{-/-} mice compared to WT mice; this suggests that *CB1* ablation reduces systemic inflammation (Figure 4A). Since insulin resistance is closely linked to adipose tissue mass and adipose macrophages (ATM), we next assessed epididymal white adipose tissue. As expected, both the weight and the ratio of epididymal fat/body weight was lower in *CB1*^{-/-} mice (Figure 4B). To assess the effect of *CB1* on ATM polarization, we isolated the stromal vascular fraction from epididymal adipose tissues. Our flow cytometry studies revealed that while M1 was slightly decreased, M2 was significantly increased in epididymal fat of *CB1*^{-/-} mice (Figure 4C). The M1/M2 ratio of ATM was decreased in epididymal fat of *CB1*^{-/-} mice (Figure 4C). Next, we studied the gene expression of macrophage markers of F4/80, CD11c, CD206, as well as proinflammatory cytokines of tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1), and interleukin-6 (IL-6) in epididymal fat. The expression levels of F4/80, CD11c, CD206, TNF, IL-1, and IL-6

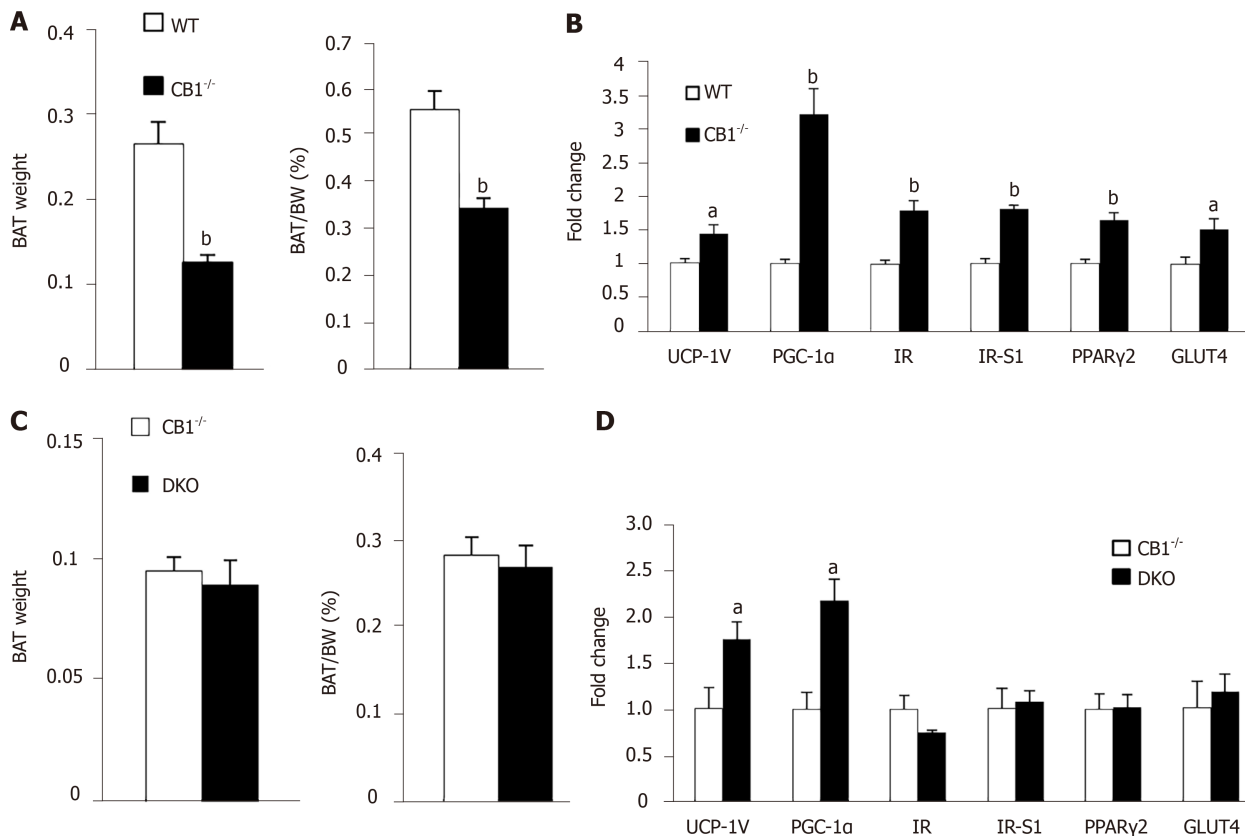


Figure 3 Double-knockout mice have similar expression of thermogenic genes compared to cannabinoid type 1 receptor-null mice. Wild-type (WT) and cannabinoid type 1 receptor-null (*CB1*^{-/-}) male mice at 8 mo of age. A: Brown adipose tissue (BAT) weight and percentage of BAT depot; B: Quantitative real-time RT-PCR analysis of gene expression in BAT. Cannabinoid type 1 receptor-null (*CB1*^{-/-}) and double-knockout (DKO) male mice at 8 mo of age. C: BAT weight and percentage of BAT depot; D: Quantitative real-time RT-PCR analysis of gene expression in BAT. *n* = 6–8. ^a*P* < 0.05, ^b*P* < 0.001, WT vs *CB1*^{-/-} or *CB1*^{-/-} vs DKO.

were significantly decreased in the epididymal fat of *CB1*^{-/-} mice compared to WT mice (Figure 4D), which is in agreement with the reduced inflammation revealed by flow cytometry analysis of ATM.

Adiponectin ablation abolishes the anti-inflammatory effect of *CB1* deficiency

Adipose tissue releases adiponectin, which plays an important role in the regulation of energy metabolism and inflammation[24]. Intriguingly, our flow cytometry analysis showed increased pro-inflammatory peritoneal M1 macrophages and increased ratio of M1/M2 in DKO mice; this suggests that the adiponectin deletion abolishes the anti-inflammatory effect of *CB1* knockout (Figure 5A). Subsequently, we analyzed epididymal white adipose tissues of *CB1*^{-/-} and DKO mice. There was no difference in the percentage of fat depot: Body weight between *CB1*^{-/-} and DKO mice (Figure 5B). Our flow cytometry studies further revealed that the M1/M2 ratio of ATM was increased in epididymal fat of DKO mice compared to *CB1*^{-/-} mice (Figure 5C). To investigate the effect of adiponectin ablation of *CB1* on ATM-mediated inflammation, gene expressions of proinflammatory cytokines were evaluated in epididymal fat. The expression levels of F4/80, CD11c, CD206, TNF-α, IL-1, IL-6 and MCP-1 were significantly increased in epididymal fat of DKO mice as compared to *CB1*^{-/-} mice (Figure 5D), in line with increased inflammation observed by flow cytometry.

Collectively, the data indicate that the *CB1* deficiency-induced anti-inflammatory effect on macrophage polarization is adiponectin-dependent, suggesting that adiponectin is a key mediator for the effect of *CB1* on inflammation.

DISCUSSION

The *CB1* blockade has been shown to ameliorate metabolic abnormalities of obese animals and to promote weight loss and improved insulin sensitivity[25]. Adipokine adiponectin is an insulin-sensitizer, and it has many beneficial effects that phenocopy

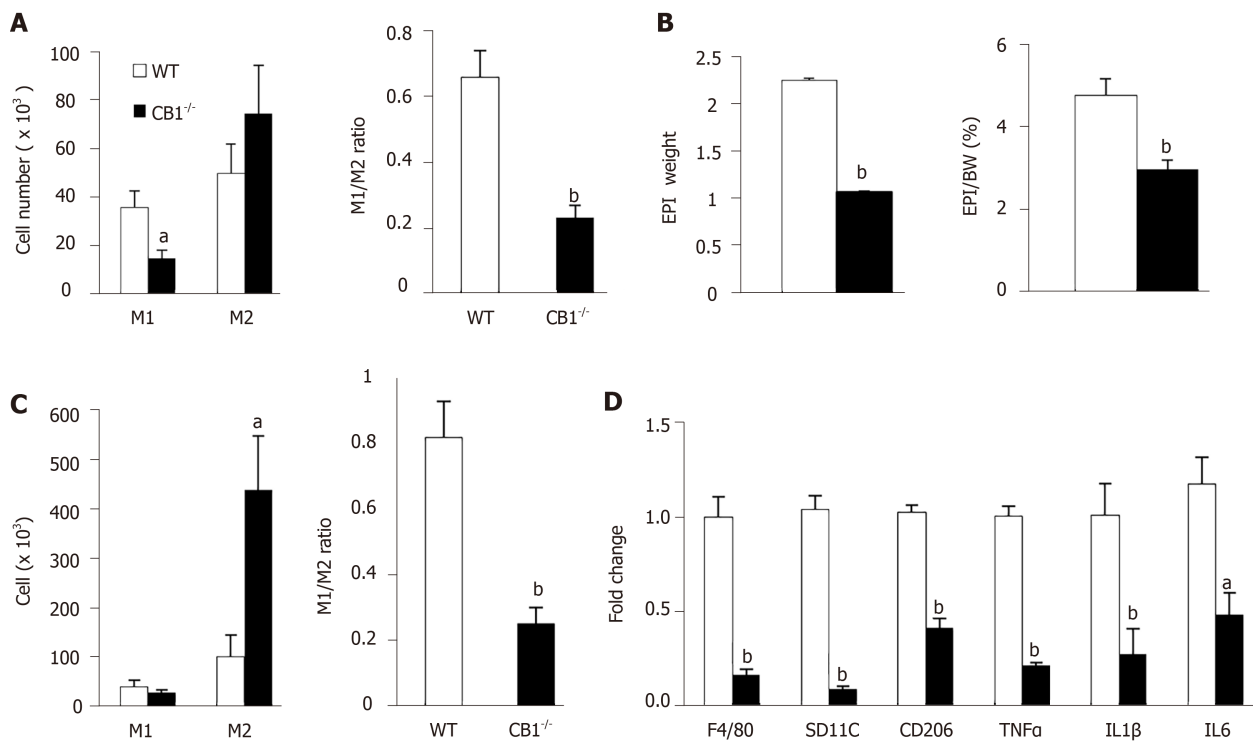


Figure 4 Cannabinoid type 1 receptor-null mice have reduced peritoneal and adipose tissue inflammation. Wild-type (WT) and cannabinoid type 1 receptor-null (CB1^{-/-}) male mice at 8 mo of age. A: Flow cytometry analysis of M1 and M2 in peritoneal macrophages; B: Percentage of epididymal fat depot; C: Flow cytometry analysis of M1 and M2 in epididymal fat; D: Pro-inflammatory cytokines expression in epididymal fat. $n = 6-8$. ^a $P < 0.05$, ^b $P < 0.001$, WT vs CB1^{-/-} mice.

CB1 antagonists[12]. It has been shown that cannabinoids decrease adiponectin[26]. Moreover, CB1 blocker rimonabant has been reported to increase the plasma adiponectin levels in obese and diabetic animal models[6,27,28]. Thus, adiponectin is thought to be a mediator of the effects of CB1 antagonists such as rimonabant. However, the functional relationship between adiponectin and the endocannabinoid system is not fully defined. To determine whether CB1 and adiponectin are functionally dependent on each other, we conducted a comparative study of the CB1^{-/-} and DKO mice to investigate whether the adiponectin deletion abolishes the healthy phenotype of CB1^{-/-} in metabolism and inflammation.

As expected in CB1^{-/-} mice, we observed decreased body weight:fat mass, increased thermogenic activation in BAT, and improved whole-body insulin sensitivity. Interestingly, DKO mice showed changes similar to CB1^{-/-} mice in the body weight:fat mass ratio, BAT thermogenic regulation, and insulin sensitivity. These results suggest that the beneficial metabolic effects of CB1 blockage are not mediated by adiponectin. Our findings are mostly consistent with previous reports in literature, but with some differences which could be due to models of choice and/or diet variations. Watanabe *et al*[16] reported that rimonabant improved hepatic insulin resistance in both *ob/ob* and *adiponectin^{-/-}ob/ob* mice. Migrenne *et al*[29] reported that adiponectin is not required for body weight loss in diet-induced obese mice, but is required in rimonabant-induced improvement of insulin sensitivity. Our experiment was conducted with a genetic approach of loss-of-function with CB1 knockout, not with CB1 antagonist; under regular diet-feeding, not diet-induced obesity. It is possible that the impact of adiponectin on CB1 metabolic regulation differs under different metabolic states. Indeed, Tam *et al*[30] reported a reversal of the HFD-induced hepatic steatosis and fibrosis by chronic administration of CB1 blocker or adiponectin, but the reduction of adiposity and improved glycemic control are not affected by adiponectin, which is similar to our results.

The findings from our current study and others[4,8] support the idea of increased energy expenditure induced by CB1 suppression, either by CB1 blocker such as rimonabant or by CB1 gene ablation. It is well known that BAT plays an important role in adaptive thermogenesis, and that thermogenic activation of BAT can directly affect metabolic rate through the function of mitochondrial protein UCP1. UCP1 is a key regulator of thermogenesis; it recruits free fatty acid into the mitochondrial matrix to dissipate as heat, depleting circulating lipids and increasing energy expenditure[31]. Previous studies demonstrated that rimonabant treatment increased the expression of

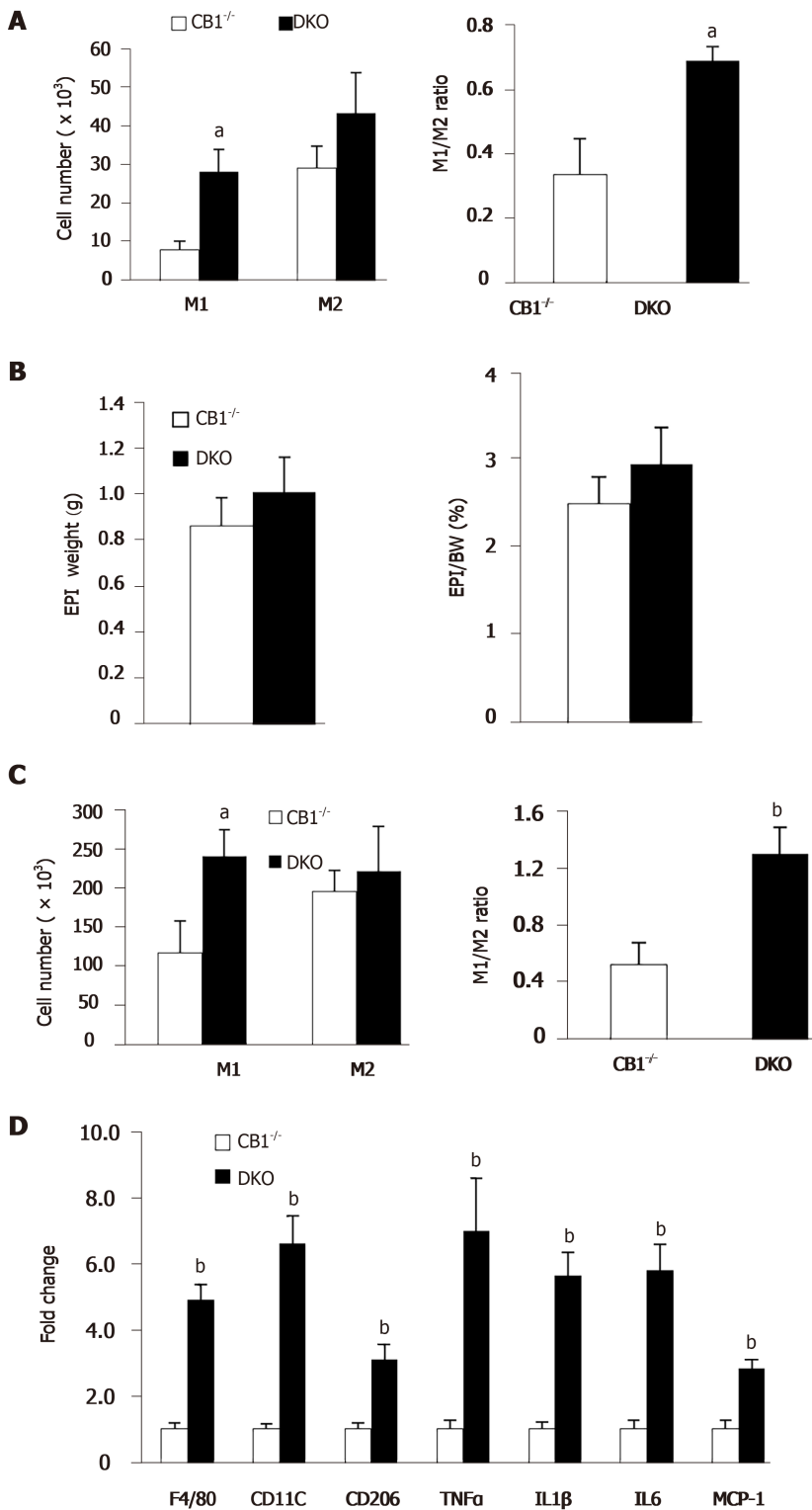


Figure 5 Double-knockout mice have increased peritoneal and adipose tissue inflammation compared to cannabinoid type 1 receptor-null mice. Cannabinoid type 1 receptor-null (CB1^{-/-}) and double-knockout (DKO) male mice at 7 mo of age. A: Flow cytometry analysis of M1 and M2 macrophages of peritoneal macrophages; B: Epididymal weight and percentage of epididymal depot; C: Flow cytometry analysis of M1 and M2 in stromal vascular fraction of epididymal fat; D: Pro-inflammatory cytokines expression of epididymal fat. *n* = 6-7. ^a*P* < 0.05, ^b*P* < 0.001, CB1^{-/-} vs DKO mice.

UCP1 mRNA in BAT[32]. In metabolic profiling, DKO mice showed even higher energy expenditure than CB1^{-/-} mice. Similarly, UCP-1 expression in BAT was higher in DKO mice than in CB1^{-/-} mice. These results suggest that adiponectin deletion not diminishes the CB1 deficiency-induced thermogenic activation in BAT. In the current study, we found that insulin signaling IR and IRS-1 gene expression in BAT was increased in CB1^{-/-} mice, and the expression of these genes was no different between DKO and CB1^{-/-} mice. Our thermogenic gene expression data in DKO showed that

adiponectin deletion further enhanced the thermogenic activation compared to *CB1*^{-/-} mice, implying that the effect of *CB1* on thermogenesis is largely independent of adiponectin. The effect of adiponectin on thermogenesis is an area of ongoing debate currently. Qiao *et al*[33] reported that adiponectin suppresses thermogenic action in BAT to reduce energy expenditure. We reported that the core body temperature of adiponectin-null mice was not affected under normal housing temperature but reduced under cold temperature, supporting that adiponectin is required for maintaining body temperature in cold[24]. Different from our previous report, our current study was conducted under room temperature, so it is not surprising that the effect of adiponectin on thermogenic activation of *CB1*^{-/-} mice is minimal.

Since metabolism and insulin sensitivity are closely linked to inflammation, we further studied the role of *CB1* deficiency in macrophages. Remarkably, both systemic (peritoneal macrophages) and tissue macrophages (ATM) showed an anti-inflammatory polarization shift, supporting reduced inflammation in *CB1*^{-/-} mice. Especially, *CB1*^{-/-} mice exhibited decreased pro-inflammatory M1 macrophages in peritoneal macrophages, less epididymal fat mass, and reduced M1/M2 ratio and pro-inflammatory cytokine expression in the epididymal fat as compared to WT mice. The results indicate that *CB1*^{-/-} mice have reduced adiposity and adipose inflammation, which is consistent with improved systemic insulin sensitivity. Intriguingly, our study further revealed that DKO mice had an opposite profile of increased inflammation compared to *CB1*^{-/-} mice, which suggested that adiponectin deletion reversed the anti-inflammatory effect of *CB1* deletion. The DKO mice exhibited an increase in pro-inflammatory M1 macrophages and M1/M2 ratio for both peritoneal macrophages and ATM, as well as elevated pro-inflammatory cytokine expression in epididymal fat compared to *CB1*^{-/-} mice. The anti-inflammatory effect on *CB1*^{-/-} mice was reversed in the DKO mice clearly demonstrates that adiponectin is required for the anti-inflammatory benefit of *CB1* antagonism, and the inflammation phenotype of *CB1* is adiponectin-dependent. These exciting results suggest that adiponectin counters the pro-inflammatory effect of cannabinoids, and the beneficial anti-inflammatory effect of *CB1* antagonists is dependent on adiponectin. Indeed, data from a mouse model of adipocyte-specific deletion of the *CB1* gene lends support to our conclusion[34]. Plasma adiponectin levels were significantly increased in the adipocyte-specific *CB1*-deleted mice, and adipocyte-specific deletion of *CB1* was shown to be sufficient to protect against diet-induced obesity and promote anti-inflammatory polarization towards alternatively-activated M2 macrophages.

CONCLUSION

In conclusion, our study demonstrates that *CB1* deletion activates thermogenesis and suppresses inflammation *via* adiponectin-independent and adiponectin-dependent pathways, respectively (Figure 6). Based on our findings, we conclude that there are differential pathways and mechanisms by which *CB1* utilizes to regulate metabolism and inflammation; that the effects on metabolism are adiponectin-independent and the effects on inflammation are adiponectin-dependent. *CB1* deletion increases plasma adiponectin[30,35], which promotes anti-inflammatory polarization of macrophages, thereby promoting the beneficial anti-inflammatory effect. Adiponectin is not required for *CB1*-mediated metabolism, but is required for *CB1*-mediated inflammation. A better understanding of the signaling crosstalk between *CB1* and adiponectin would facilitate further therapeutic development of *CB1* antagonists. Our study provides new insights to the comprehensive connection between *CB1* and adiponectin for regulation of energy homeostasis, insulin sensitivity and inflammation.

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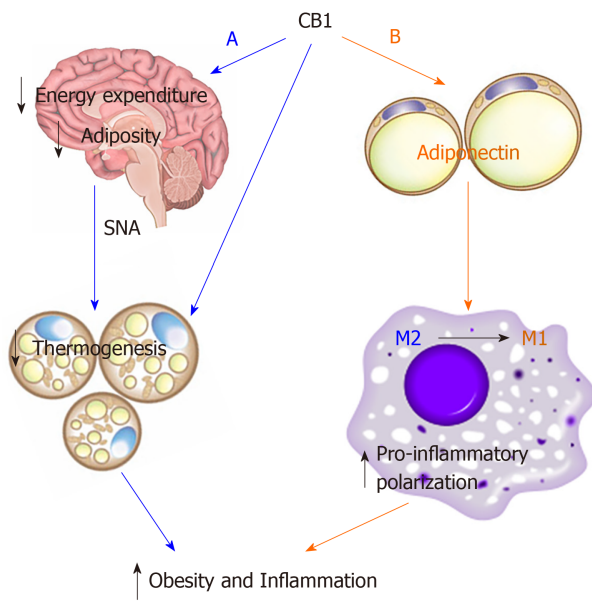


Figure 6 Schematic diagram of summary. Cannabinoid type 1 receptor (CB1) utilizes differential mechanisms in control of metabolism and inflammation. A: CB1 decreases thermogenesis in BAT through sympathetic nerve activity to reduce energy expenditure and adiposity. So the effect of CB1 on metabolism is adiponectin-independent; B: CB1 suppresses adiponectin in adipose tissue, which diminishes the anti-inflammatory effect of adiponectin, thus promoting macrophage pro-inflammatory polarization. So the effect of CB1 on inflammation is adiponectin-dependent. Thus, CB1 utilizes differential mechanisms in control of metabolism and inflammation: its effect on metabolism is adiponectin-independent while effects on inflammation are adiponectin-dependent. Thus, adiponectin is not required for CB1-mediated metabolism, but is required for CB1-mediated inflammation.

ARTICLE HIGHLIGHTS

Research background

Antagonists of cannabinoid type 1 receptor (CB1) have been shown to promote body weight loss and improve insulin sensitivity.

Research motivation

Cannabinoids is implicated in regulation of adiponectin. However, the mediators of CB1 actions are not fully defined, specifically in regard to adiponectin signaling *in vivo*.

Research objectives

To determine whether adiponectin is indeed required for the peripheral functions of CB1.

Research methods

We compared metabolic and inflammatory phenotypes of CB1-null (CB1^{-/-}) vs. CB1 / Adiponectin double-knockout (DKO) mice. We investigated the insulin sensitivity using insulin tolerance test and glucose tolerance test, and inflammation using flow cytometry analysis of macrophages.

Research results

CB1^{-/-} mice significantly reduced body weight and fat mass without change of total daily food intake and locomotor activity compared to wild-type (WT) mice. CB1^{-/-} mice showed increased energy expenditure and improved insulin sensitivity compared to WT mice. DKO showed no difference in body weight, adiposity, or insulin sensitivity, only showed a modestly elevated thermogenesis in BAT compared to CB1^{-/-} mice. CB1^{-/-} mice showed reduced pro-inflammatory macrophage polarization in both peritoneal macrophages and adipose tissue macrophages compared to WT mice; in contrast, DKO mice exhibited elevated pro-inflammatory macrophage polarization in these macrophages compared to that of CB1^{-/-} mice.

Research conclusions

Our findings reveal that CB1 functions through both adiponectin-dependent and adiponectin-independent mechanisms: CB1 regulates energy metabolism in an

adiponectin-independent manner, and inflammation in an adiponectin-dependent manner.

Research perspectives

Adiponectin is not required for CB1-mediated metabolism but is required for CB1-mediated inflammation. To fully understand the direct interactions and regulatory mechanisms between CB1 and adiponectin, further dissemination in co-culture system to might be beneficial.

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