

Cannabinoid type 1 receptor blockade promotes mitochondrial biogenesis through eNOS expression in white adipocytes

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Abbreviations: AMPK, adenosine monophosphate activated protein kinase; COX-IV, cytochrome c oxidase; CT, threshold cycle; Cyt c, cytochrome c; eNOS, endothelial nitric oxide synthase; HFD, high-fat diet; mtDNA, mitochondrial DNA; NO, nitric oxide; NRF-1, nuclear respiratory factor-1; PGC-1 α , proliferator-activated receptor γ coactivator 1 α ; SR141716, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-pyrazole-3-carboxamide, hydrochloride; STD, standard diet; Tfam, mitochondrial transcription factor A; WAT, white adipose tissue; WT, wild-type.

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Objective: Cannabinoid type 1 (CB1) receptor blockade decreases body weight and adiposity in obese subjects, however the underlying mechanism is not yet fully understood. Nitric oxide (NO) produced by endothelial NO synthase (eNOS) induces mitochondrial biogenesis and function in adipocytes. This study was undertaken to test the hypothesis whether CB1 receptor blockade increases the expression of eNOS and mitochondrial biogenesis in white adipocytes.

Research Design and Methods: We examined the effects on eNOS and mitochondrial biogenesis of selective pharmacological blockade of CB1 receptors by SR141716 (rimonabant) in mouse primary white adipocytes. We also examined eNOS expression and mitochondrial biogenesis in white adipose tissue (WAT) and isolated mature white adipocytes of CB1 receptor deficient (CB1^{-/-}) and chronically SR141716-treated mice on either standard (STD) or high-fat diet (HFD).

Results: SR141716 treatment increased eNOS expression in cultured white adipocytes. Moreover, SR141716 increased mitochondrial DNA amount, mRNA levels of genes involved in mitochondrial biogenesis, and mitochondrial mass and function through eNOS induction, as demonstrated by reversal of SR141716 effects by small interfering RNA-mediated decrease in eNOS. While HFD-fed wild-type mice showed reduced eNOS expression and mitochondrial biogenesis in WAT and isolated mature white adipocytes, genetic CB1 receptor deletion or chronic treatment with SR141716 restored these parameters to the levels observed in wild-type mice on STD, an effect linked to the prevention of adiposity and body weight increase.

Conclusions: CB1 receptor blockade increases mitochondrial biogenesis in white adipocytes by inducing the expression of eNOS. This is linked to the prevention of HFD-induced fat accumulation, without concomitant changes in food intake.

The adipose tissue is not merely an energy store but rather an endocrine organ playing an important role in fuel metabolism (1, 2). Recent studies have demonstrated that mitochondrial biogenesis increases during adipocyte differentiation (3), and this is presumably due at least in part to the presence of the endothelial nitric oxide synthase (eNOS). In fact, eNOS protein is not expressed in either undifferentiated 3T3-L1 cells or preadipocytes, whereas its expression markedly increases with adipocyte differentiation (4). Noteworthy, nitric oxide (NO) increases mitochondrial biogenesis in white adipocytes via cyclic GMP (cGMP)-dependent pathways, including peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) gene expression (5, 6). Consistently, mitochondrial biogenesis is reduced in fat of eNOS null mutant mice (eNOS^{-/-}), with decreased energy expenditure and increased body weight (5, 6). This suggests that the eNOS-dependent mitochondrial biogenesis is relevant to adipocyte maturation and fuel metabolism.

Cannabinoid type 1 (CB1) receptors participate in the physiological modulation of many central and peripheral functions related to the control of energy metabolism (7). They are the most abundant G protein-coupled receptors expressed in the brain, where CB1 receptor activation promotes feeding and modulates the rewarding properties of food (7-9). CB1 receptors also control metabolic functions by acting on peripheral organs, including white adipose tissue (WAT) (7-9). Notably, in adipocytes, CB1 receptor expression increases with cell differentiation (10, 11). Moreover, CB1 receptor-deficient mice (CB1^{-/-}) are lean (12) and resistant to high fat diet (HFD) (13). Similarly, the selective CB1 receptor antagonist SR141716 (rimonabant) persistently reduces body weight during the treatment of obese animals, although food intake renormalizes after an

initial 1-2 week weight reduction (14, 15), suggesting that CB1 receptor blockade stimulates fat metabolism, leading to a decreased fat content.

We now demonstrate a prominent role for the CB1 receptors to modulate eNOS gene expression and mitochondrial biogenesis in white adipocytes. Collectively, these data suggest that targeting the endocannabinoid system improves mitochondrial function, implying a novel, unrecognized mechanism of action for the anti-obesity drug rimonabant.

RESEARCH DESIGN AND METHODS

Cell culture. White fat precursor cells were enzymatically isolated from epididymal WAT of wild-type C57BL/6J mice, and cultured as described (16). SR141716 [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-pyrazole-3-carboxamide, hydrochloride from National Institute of Mental Health (Chemical Synthesis and Drug Supply Program, Supplier RTI, Contact person: Dr. George A. Brine, Contract N01-MH-32005 WA 08928.019, NIMH number S-705)] stock solutions (50 mM in DMSO) were diluted in the culture medium to the required final concentrations containing up to 0.02% DMSO. At day 3-4 from seeding, cells were exposed to the culture medium containing SR141716 (0.1 – 10 μ M) for 72 h or different periods of time when specified. The medium was discarded, the wells were washed twice with 2 ml of ice-cold phosphate-buffered solution (PBS) (Sigma-Aldrich) and the cells were harvested as reported below for the different assays.

eNOS siRNA. White adipocytes were transfected at day 3-4 after seeding with 100 nM eNOS siRNA SMARTpool (Dharmacon, Lafayette, Colorado) or *siCONTROL* non-targeting siRNA using Dharmafect 3 transfection reagent. Cells were treated for 72 h with 1 μ M SR141716, and RNA and protein

were harvested. Efficacy of transfection was determined using *siGLO*-RISC-free non-targeting siRNA and estimation of siRNA uptake by fluorescence detection (absorbance/emission 557/570 nm).

Animals and treatments. Animals were treated according to protocols approved by Milan and Bologna University Institutional Animal Care and Use Committee. Eight week-old male $CBI^{-/-}$ mice (17) and their wild-type littermates (WT) ($n = 10$ per group) were fed either a standard mouse diet (STD) (8 kcal% fat, 19 kcal% protein, 73 kcal% carbohydrate) or a high-fat diet (HFD) (D12492, Research Diets, New Brunswick, NJ, 60 kcal% fat, 20 kcal% protein, 20 kcal% carbohydrate) for 12 weeks. Moreover, four-week-old male C57BL/6J mice (Harlan Nossan) were fed either STD or HFD for 6 weeks before treatments. While fed on STD or HFD, these mice ($n = 10$ per group) were further treated with either vehicle or SR141716 (at 10 mg/kg orally in distilled water with 0.1% Tween 80) for 11 weeks. Body weight and food intake were recorded weekly. Adiposity (wet weight of visceral and subcutaneous fat), cumulative food intake (for the time of the experiment), and feed efficiency were measured (5). On the day of the experiments, animals were sacrificed by cervical dislocation and epididymal WAT was immediately isolated, frozen in liquid nitrogen and stored at -80°C before processing for mRNA, protein, and mtDNA analysis or citrate synthase and adenosine monophosphate activated protein kinase (AMPK) activity.

Isolation of mouse mature adipocytes. Wild-type and $CBI^{-/-}$ mice were fed STD or HFD ($n = 6$ per group), and wild-type mice either on STD or HFD were treated with either vehicle or SR141716 ($n = 6$ per group) as described above. Mice were sacrificed and epididymal WAT excised. Fat pads from two mice were pooled and mature adipocytes were acutely isolated in Hank's balanced salt

solution (HBSS) containing 4% bovine serum albumine (BSA) and 1.5 mg/ml collagenase (Calbiochem) as described (18).

RNA analysis. RNA was isolated from tissue or cells using the RNeasy[®] Lipid Tissue Mini Kit (Qiagen). cDNA was synthesized using iScript[™] cDNA Synthesis Kit (Bio-Rad Laboratories). Primers were designed using Beacon Designer 2.6 software from Premier Biosoft International. Triplicate PCR reactions were carried out with the intercalating dye SybrGreen as previously described (5, 16). PCR cycles were programmed on an iCycler iQ[™] Real Time PCR detection system (Bio-Rad Laboratories). The cycle number at which the various transcripts were detectable (threshold cycle, CT) was compared to that of β -actin, referred to as ΔCT . The gene relative level was expressed as $2^{-(\Delta\Delta\text{CT})}$, in which $\Delta\Delta\text{CT}$ equals ΔCT of either the knockout or drug-treated mice (or treated cells) minus ΔCT of the control mice (or untreated cells).

Immunoblot Analysis. Protein extracts were obtained by harvesting cultured adipocytes in M-PER Mammalian Protein Extraction Reagent or homogenizing WAT tissue in T-PER Tissue Protein Extraction Reagent (both from Pierce) as indicated by the manufacturer, in the presence of 1 mM NaVO_4 , 10 mM NaF and a cocktail of protease inhibitors (Sigma Aldrich). Protein content was determined by the bicinchoninic acid protein assay (Pierce) and 50 μg of proteins were run on SDS-PAGE under reducing conditions. The separated proteins were then electrophoretically transferred to a nitrocellulose membrane (Pierce). Proteins of interest were revealed with specific antibodies: anti eNOS (Transduction Labs), anti COX IV (Molecular Probes), anti Cyt c (BD Bioscience), at 1:500 dilution each one; anti-phospho-AMPK α (Thr172), anti-AMPK α (both at 1:1000 dilution, Cell Signaling Technology); anti β -actin (1:10000, Sigma-Aldrich). The immunostaining was detected

using horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin for 1 h at room temperature. After the visualization of phospho-AMPK α , filters were stripped with the Re-Blot Western blot recycling kit (Chemicon International) and further used for the visualization of total AMPK α . Bands were revealed by the SuperSignal Substrate (Pierce), and quantitated by densitometry using a Quick Image densitometer (Canberra Packard) and a Phoretix 1D, version 3.0, software image analyser.

FACS analysis. White adipocytes were treated or not with 1 μ M SR141716 for 72 h. Cells ($\sim 1 \times 10^6$ /sample) were harvested by trypsinization, fixed with 4% paraformaldehyde in PBS for 10 min, and incubated 1 h at room temperature in a permeabilization buffer containing 1% BSA, 0.1% saponin and either anti COX IV or anti Cyt c antibody. After incubation with labeled secondary antibody, the fluorescence was analysed by flow cytometry (FACScan, Becton Dickinson, Sunnyvale, California, USA) and Cell Quest software (Becton Dickinson). Data are expressed as relative fluorescence intensity (RFI) of the mean fluorescent signal versus unstained samples.

Mitochondrial DNA. Mitochondria were isolated from tissue and cells (19). The mtDNA was extracted and analyzed as described (5, 16). Briefly, an aliquot of mtDNA was loaded on ethidium bromide-stained agarose gel (1.2%) and quantified using the QuickImage densitometer (Packard). To determine mtDNA levels, the signal intensities were normalized to cell numbers in experiments on cultured cells or to protein content in experiments using tissues from wild-type and knockout mice. Alternatively, mtDNA copy number was measured by means of quantitative PCR from the cytochrome C oxidase II mtDNA gene compared to the UCP2 nuclear gene in WAT of CB1^{-/-} and wild-type mice as described (20).

Oxygen consumption. White adipocytes were harvested by trypsinization, centrifuged at 500 g for 5 min at 4°C and resuspended in HBSS. Cell samples (4×10^6 /ml) were analyzed at 37°C in a gas-tight vessel equipped with a Clark-type oxygen electrode (Rank Brothers) connected to a chart recorder. Cellular oxygen consumption was measured as described (5, 16). The oxygen electrode was calibrated, assuming the concentration of oxygen in the incubation medium at 37°C to be 200 μ M. Protein content in both cell and tissue samples was determined by the bicinchoninic acid protein assay (Pierce).

ATP levels. The whole amount of ATP in cells was measured by using the ATP determination kit from Molecular Probes. Cells were harvested by trypsinization and centrifuged at 500 g for 5 min at 4°C. ATP was extracted by incubating cell pellets with 1% trichloroacetic acid (TCA)/4 mM EDTA solution for 10 min on ice. Cell extracts were centrifuged at 12,000 g for 10 min at 4°C and used for ATP determination as indicated by the manufacturer.

Mitochondrial mass. White adipocytes seeded onto glass coverslips were incubated with medium containing 200 nM Mitotracker red 580 (Invitrogen). After incubation for 30 min at 37°C, coverslips were rinsed two times with medium and two times with PBS and fixed at room temperature for 10 min in 4% paraformaldehyde in PBS. Coverslips were mounted onto glass slides, and were then observed with a Bio-Rad confocal microscope MRC1024.

Citrate synthase activity. The activity was measured spectrophotometrically at 412 nm at 30°C in either tissue or whole cell extracts (21). Tissue or cell homogenates were added to buffer containing 0.1 mM 5,5-dithio-bis-(2-nitrobenzoic) acid, 0.5 mM oxaloacetate, 50 μ M EDTA, 0.31 mM acetyl CoA, 5 mM triethanolamine hydrochloride, and 0.1 M Tris-HCl, pH 8.1. Citrate synthase

activity was expressed as nmol citrate produced per min per mg of protein.

AMPK assay. Adipocytes were scraped in ice-cold lysis buffer (22) in the presence of 1 mM NaVO₄, 10 mM NaF and protease inhibitors (Sigma Aldrich). Epididymal fat pads from wild-type or CB1^{-/-} mice were homogenized in ice-cold lysis buffer. AMPK activity was determined on protein samples immunoprecipitated with anti-AMPK α (Cell Signaling Technology) as described (22). The immunocomplexes were collected and washed with AMPK reaction buffer (22). AMPK immunoprecipitates were incubated with 20 μ l of AMPK reaction buffer, 5 μ l of SAMS substrate peptide (1 mM), and 2 μ Ci of [γ -³²P]ATP (MP Biomedicals) for 30 min at 37 °C. At the end of the incubation, an aliquot was removed and spotted onto Whatman P81 paper. The P81 paper was washed three times with 0.75% (v/v) phosphoric acid and once with acetone, and radioactivity was determined by scintillation counting.

Statistical methods. Raw data from each experiment were analyzed using either an analysis of variance with Newman-Keuls' multiple comparison post hoc test or t test.

RESULTS

CB1 receptor antagonist increases eNOS expression and mitochondrial biogenesis in white adipocytes. CB1 receptor antagonist SR141716 affects eNOS expression in mouse white adipocytes in culture. In fact, the drug significantly increased eNOS mRNA (Fig. 1A) and protein levels (Fig. 1B) in a dose-dependent manner (0.1 – 10 μ M for 72 h).

The effect of SR141716 was further tested on several markers of mitochondrial biogenesis. The amount of mitochondrial DNA (mtDNA, a marker of mitochondrial content), and the mRNA levels of PGC-1 α , nuclear respiratory factor-1 (NRF-1) and mitochondrial DNA transcription factor A

(Tfam) (three master regulators of mitochondrial biogenesis) (23, 24) were higher in 1 μ M SR141716-treated than in vehicle-treated cells (Fig. 2A). Protein levels of cytochrome c oxidase IV (COX IV) and cytochrome c (Cyt c) (two mitochondrial proteins involved in oxidative phosphorylation) were also increased by SR141716 treatment as assessed either by immunoblot (Fig. 2B) or by flow cytometry (mean fluorescence intensity \pm SEM of SR141716- vs. vehicle-treated cells: 20.1 \pm 3.4 vs. 9.6 \pm 2.3 for COX IV and 15.6 \pm 3.2 vs. 9.9 \pm 1.9 for Cyt c, n = 3 experiments, P < 0.001). Both activity of citrate synthase and oxygen consumption were markedly increased by SR141716 treatment (Fig. 2C and D). Consistently, the ATP levels in the SR141716-treated cells were significantly higher than those in the vehicle-treated cells (1.73 \pm 0.19 vs. 1.34 \pm 0.16, n = 3 experiments, P < 0.05). Finally, white adipocytes treated with SR141716 showed a significant increase of Mitotracker red signal, indicating the increase of mitochondrial mass compared to vehicle-treated cells (Figure 2E). Altogether, these findings demonstrate that pharmacological blockade of the CB1 receptor increases eNOS expression and mitochondrial biogenesis and function in white adipocytes.

SR141716 effects on mitochondrial biogenesis are eNOS-dependent.

Coregulation of eNOS expression and mitochondrial biogenesis in the SR141716-treated adipocytes suggests that these processes might be mechanistically linked. To assess whether eNOS expression is relevant in the drug-induced mitochondrial biogenesis, white adipocytes were transfected with small interference RNA (siRNA) against eNOS or a scrambled RNA sequence. siRNA against eNOS reduced basal eNOS mRNA and protein levels by 82% and 85%, respectively, in WAT cells (Fig. 1C and D), with a moderate, but statistically significant

decrease of all measured parameters of mitochondrial biogenesis in vehicle-treated cells (Fig. 2A-E). Importantly, however, siRNA against eNOS strongly decreased mitochondrial biogenesis induced by 1 μ M SR141716 treatment, indicating that the effect of SR141716 depends, at least in part, on the presence of eNOS (Fig. 2A-E).

Mitochondrial biogenesis is increased in WAT of CB1^{-/-} mice. To assess whether the CB1 receptor blockade increases mitochondrial biogenesis in WAT *in vivo*, we examined the eNOS expression and markers of mitochondrial biogenesis in epididymal fat pads derived from eight week-old male CB1^{-/-} mice and their wild-type littermates either on STD or on HFD for 12 weeks. As expected (16), HFD reduced eNOS expression and mitochondrial biogenesis in wild-type mice (Fig. 3A-D). Endothelial NOS mRNA levels in WAT of CB1^{-/-} mice were significantly higher than those of wild-type mice both on STD and HFD (Fig. 3A). Furthermore, CB1^{-/-} mice displayed increased mRNA levels of PGC-1 α and Tfam (Fig. 3A), COX IV and Cyt c (Fig. 3C), increased mtDNA amounts (Fig. 3B) and citrate synthase activity (Fig. 3D) as compared to wild-type littermates. These increments were evident in animals either on STD or HFD. To confirm that the mitochondrial biogenesis induced by CB1 gene deletion occurs in white adipocytes *in vivo*, mature white adipocytes were isolated from epididymal fat pad of CB1^{-/-} and wild-type littermates after STD or HFD. Although we do not rule out that other cell types present in whole WAT (i.e., endothelial cells, macrophages, or others) may be involved, we found that CB1 gene deletion increases eNOS expression and mitochondrial biogenesis and function in white adipocytes (Supplementary Fig. 1, available in an online appendix).

As previously described (12), CB1^{-/-} mice on STD had a lower body weight and adiposity (Supplementary Fig. 2A) than wild-

type controls, having showed a significantly reduced body weight as compared to wild-type throughout the whole period of observation (data not shown). Most importantly, CB1^{-/-} mice maintained a leaner phenotype under HFD as evidenced by lower body weight and adiposity (Supplementary Fig. 2A and B) (13). Cumulative food intake was comparable between the two groups of mice on STD or HFD (Supplementary Fig. 2C), although CB1^{-/-} mice showed a smaller feed efficiency (weight gain/food intake, an indirect measurement of increased energy expenditure) than their wild-type littermates (Supplementary Fig. 2D).

Defective mitochondrial biogenesis is restored in WAT of HFD-fed SR141716-treated mice. CB1 receptor effects on mitochondrial biogenesis were further confirmed *in vivo* by a long-term treatment with the CB1 receptor antagonist. Four-week-old male mice were put on either STD or HFD. Starting at week 10, STD and HFD mice were treated either with SR141716 (10 mg/kg/day per os) or vehicle for 11 weeks (13-15). Consistent with the data from CB1^{-/-} mice, eNOS mRNA and protein levels and mitochondrial biogenesis were significantly higher in WAT of the SR141716-treated HFD mice than in those of vehicle-treated HFD mice, although as expected (16) they were lower in the vehicle-treated HFD mice than in the vehicle-treated STD mice (Fig. 5). These results suggest that SR141716 treatment amends the HFD-induced impairment of NO generating system, restoring the mitochondrial function at levels observed in wild-type mice on STD. Treatment with SR141716 induced only a slight, but not statistically significant increase of eNOS expression and mitochondrial biogenesis in mice on STD (Fig. 5). Superimposable results were obtained in isolated mature white adipocytes from SR141716-treated mice (Supplementary Fig. 3).

Interestingly, the mean body weight and adiposity of the SR141716-treated mice were markedly lower as compared to that of vehicle-treated mice on HFD (Supplementary Fig. 4A and B). Cumulative food intake was comparable between the drug- and vehicle-treated animals on HFD (Supplementary Fig. 4C), whereas SR141716-treated mice showed a reduced feed efficiency as compared to their vehicle-treated HFD controls (Supplementary Fig. 4D). Only slight, but not statistically significant decreases in body weight and adiposity were seen in the SR141716-treated mice on STD (Supplementary Fig. 4A and B).

CB1 receptor blockade stimulates AMPK phosphorylation and activity. The AMPK cascade has been described to activate eNOS and NO production (25). Thus, AMPK might be involved in the effect of CB1 receptor blockade on eNOS-mediated mitochondrial biogenesis. In fact, treatment of cultured white adipocytes with 1 μ M SR141716 induced a rapid and persistent AMPK phosphorylation (Fig. 5A) and significantly increased AMPK activity (Fig. 5B). Moreover, in line with previous reports (26) we found that AMPK activity was lower in WAT of wild-type mice fed a HFD compared to those fed a STD. Noticeably, while CB1 receptor deletion did not affect AMPK phosphorylation nor activity in WAT of STD-fed mice (Fig 5C and D), both AMPK phosphorylation (Fig 5C) and activity (Fig. 5D) were normalised in WAT of HFD-fed CB1^{-/-} mice, up to the levels measured in wild type STD-fed mice.

Taken together, these findings demonstrate that the CB1 receptor blockade *in vivo* concomitantly activates AMPK cascade and up-regulates eNOS expression and mitochondrial biogenesis, which are impaired by HFD in WAT, and these processes may be involved in the control of adiposity and body weight of animals on HFD.

DISCUSSION

The present study demonstrates that genetic and pharmacological blockade of the cannabinoid CB1 receptor increases mitochondrial biogenesis in white adipocytes. Treatment of adipocytes with SR141716, a selective antagonist of CB1 receptors (14, 15, 27), increases mitochondrial biogenesis genes, including PGC-1 α and Tfam. This possibly implies that SR141716 is antagonizing an endocannabinoid tone present in cultured fat cells. It has been recently reported by us and others that rodent and human adipocytes produce and release endocannabinoids, including 2-arachidonoylglycerol, anandamide, and other cannabimimetic compounds (28, 29). Thus, our results suggest that SR141716 increases mitochondrial biogenesis by antagonizing the endocannabinoids produced in cell culture. In turn, this strongly implies that the CB1 receptor agonists might down-regulate the eNOS-dependent mitochondrial biogenesis in white adipocytes. Consistently, cannabinoids, including Δ^9 -tetrahydrocannabinol, inhibit the respiratory enzyme system in brain, liver, skeletal muscle, and heart (30) and, consequently, reduce the rates of oxygen consumption in rodents (31, 32).

We have recently found that eNOS-dependent NO production induces mitochondrial biogenesis in different cell types, including white adipocytes, by cGMP-dependent pathways and that the newly synthesized mitochondria are functionally active, ensuing high oxygen consumption and ATP production (5, 6, 33). This suggests that the SR141716-dependent increase in eNOS expression is mechanistically linked to the mitochondrial biogenesis induced by the CB1 receptor blockade. This was confirmed by the observation that gene silencing of eNOS by RNAi markedly impairs the SR141716 stimulatory effects on mitochondrial

biogenesis and function in cultured adipose cells.

We observed that genetic CB1 receptor blockade *in vivo* increases eNOS expression and mitochondrial biogenesis both in whole WAT and isolated mature white adipocytes, and this is accompanied by prevention of HFD-induced fat accumulation. Most importantly, the pharmacological CB1 receptor blockade fully counteracts the down-regulation of eNOS-dependent mitochondrial biogenesis due to HFD in mice. This might indicate a novel, previously unrecognized mechanism of action of SR141716 as an anti-obesity drug. Not only it would transiently suppress food intake (34, 35) and reduce lipogenesis (12), but it also would increase the oxidative metabolism in white adipocytes by counteracting the inhibitory effects of endocannabinoids, whose levels are increased in fat tissues of obese rodents and humans (28). Thus, we propose that the overactivity of endocannabinoids might contribute to down-regulate mitochondrial biogenesis in white adipocytes of obese mice, and that the blockade of adipose CB1 receptor might revert this process, thus preventing the HFD-induced increase in adiposity and body weight gain.

An open question regards the mechanisms that link CB1 receptor blockade to the eNOS expression. Further, it remains to be clarified how the recovery of mitochondrial biogenesis and function in WAT could counteract the development of obesity. We found that SR141716 enhances AMPK activity in cultured white adipocytes, and that the WAT AMPK activity, which is reduced by HFD, is normalized in WAT from HFD-fed CB1^{-/-} mice to the levels measured in wild type STD-fed mice. Since AMPK is known to enhance NO production by activating eNOS (25), the AMPK cascade might operate upstream of the NO generating system, possibly contributing to the CB1-blockade-mediated mitochondrial biogenesis.

Accordingly, induction of AMPK activity has been found to increase mitochondrial content in adipocytes (36, 37). Thus, the enhanced β -oxidation of free fatty acids elicited by AMPK activation (36, 38) and by eNOS-dependent mitochondrial biogenesis (39) might functionally link SR 141716 treatment to its anti-obesity effects.

The mechanisms that link eNOS-derived NO to the gene expression machinery involved in mitochondrial biogenesis also remain to be elucidated. Studies of eNOS^{-/-} mice have demonstrated an obligatory role of eNOS in mitochondrial biogenesis (5, 6). Noticeably, eNOS, NO and its intracellular messenger cGMP are located in both the cytoplasm and the nucleus, and several transcription factors possess heme moieties that can bind NO (6), but further investigation is needed to understand whether eNOS-mediated responses involve the nuclear NO/cGMP.

It is now widely accepted that obesity is causally linked to a chronic, low-grade inflammation of visceral adipose tissue, characterized by infiltrating macrophages, abnormal cytokine production and activation of a network of inflammatory signalling pathways, including tumor necrosis factor (TNF)- α and inducible NOS (iNOS) (40, 41). It has been observed that up-regulation of iNOS (which is induced in inflammatory conditions) often correlates with a down-regulation of eNOS (16). Corroborating this, TNF- α increases iNOS expression in different cells and tissues, including fat (42) and inhibits eNOS expression, mitochondrial function and energy production in WAT (16). Interestingly, SR141716 has been shown to reduce plasma TNF- α levels in inflammatory conditions (43). Thus, we can speculate that the anti-inflammatory effects of SR141716 could help counteracting the increase of iNOS expression and the reduction of eNOS expression and mitochondrial biogenesis in HFD-induced obesity.

Endogenous cannabinoids and CB1 agonists promote vasodilation and decrease blood pressure, particularly in rodent models of hypertension (44, 45). A variety of putative mechanisms could explain these effects, including the increased production of vasodilator NO in vessels. Although we have not analyzed the possible effects of SR141716 on eNOS expression in the vasculature, a recent analysis of the impact of rimonabant on blood pressure in the pooled population from four large trials with similar design – the Rimonabant-In-Obesity (RIO) programme – demonstrates that there is no effect of rimonabant on blood pressure beyond the modest reduction mediated by weight loss (46).

In conclusion, our results demonstrate that mitochondrial biogenesis and function, which are impaired by HFD, are restored to normal levels in white adipocytes by the CB1 receptor antagonist SR141716, and this is accompanied by prevention of adiposity and body weight increase in high-fat fed obese mice. In view of the present findings,

targeting eNOS expression and mitochondrial biogenesis might offer therapeutic gains and simplify the drug approach to clinical situations as complex as obesity and related disorders.

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Figure 1

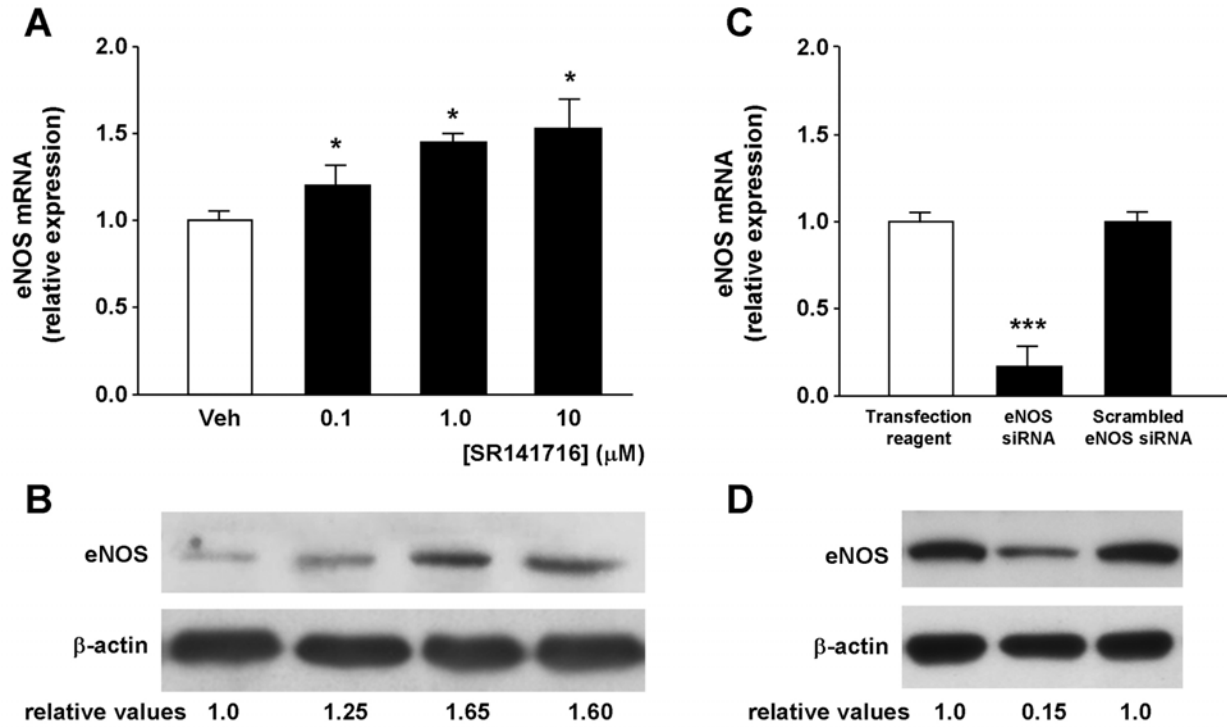


FIG. 1

The CB1 receptor antagonist SR141716 dose-dependently up-regulates eNOS expression in cultured white adipocytes. *A* and *B*: eNOS mRNA and protein, analyzed by quantitative RT-PCR and immunoblotting, respectively, in vehicle-treated cells (0.02% DMSO, Veh) or after exposure to different doses of SR141716 for 3 days. The cycle number at which the transcript were detectable was compared to that of β -actin, and expressed as relative expression *vs.* values in vehicle-treated cells taken as 1.0. ($n = 5$ experiments; $*P < 0.05$ *vs.* vehicle). eNOS protein was detected by immunoblot analysis (in *B*, one experiment representative of five reproducible ones). The numbers below the blots show the relative values from the densitometric analysis, relative to β -actin levels, when control (vehicle) measurement is given a value of 1.0. *C* and *D*: eNOS expression in white adipocytes transfected with either 100 nM scrambled control siRNA or eNOS siRNA and harvested 48 h later. eNOS mRNA and protein levels were measured by means of quantitative RT-PCR and immunoblotting ($n = 3$ experiments; $***P < 0.01$ *vs.* transfection reagent-treated cells). All data represent mean \pm SEM.

