

Negative Regulation of Leptin-induced Reactive Oxygen Species (ROS) Formation by Cannabinoid CB₁ Receptor Activation in Hypothalamic Neurons

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Background: In hypothalamic neurons, leptin induces ROS production via PPAR- γ inhibition.

Results: CB₁ agonism prevents leptin-induced ROS accumulation by reversing PPAR- γ and catalase inhibition. Inhibition of endocannabinoid inactivation also counteracts leptin effects.

Conclusion: CB₁ inhibits effects of leptin that underlie part of its anorexic actions.

Significance: During conditions of increased endocannabinoid tone CB₁ might reduce leptin activity in the hypothalamus.

The adipocyte-derived, anorectic hormone leptin was recently shown to owe part of its regulatory effects on appetite-regulating hypothalamic neuropeptides to the elevation of reactive oxygen species (ROS) levels in arcuate nucleus (ARC) neurons. Leptin is also known to exert a negative regulation on hypothalamic endocannabinoid levels and hence on cannabinoid CB₁ receptor activity. Here we investigated the possibility of a negative regulation by CB₁ receptors of leptin-mediated ROS formation in the ARC. Through pharmacological and molecular biology experiments we report data showing that leptin-induced ROS accumulation is 1) blunted by arachidonyl-2'-chloroethylamide (ACEA) in a CB₁-dependent manner in both the mouse hypothalamic cell line mHypoE-N41 and ARC neuron primary cultures, 2) likewise blocked by a peroxisome proliferator-activated receptor- γ (PPAR- γ) agonist, troglitazone, in a manner inhibited by T0070907, a PPAR- γ antagonist that also inhibited the ACEA effect on leptin, 3) blunted under conditions of increased endocannabinoid tone due to either pharmacological or genetic inhibition of endocannabinoid degradation in mHypoE-N41 and primary ARC neuronal cultures from MAGL^{-/-} mice, respectively, and 4) associated with reduction of both PPAR- γ and catalase activity, which are reversed by both ACEA and troglitazone. We conclude that CB₁ activation reverses leptin-induced ROS formation and hence possibly some of the ROS-mediated effects of the hormone by preventing PPAR- γ inhibition by leptin, with subsequent increase of catalase activity. This mechanism might underlie in part CB₁ orexigenic actions under physiopathological conditions accompanied by elevated hypothalamic endocannabinoid levels.

In the central nervous system the hypothalamus, originally recognized as the brain “feeding center,” is the main regulator

of body weight. Hormonal and nutrient signals are processed in this brain area and inform the rest of the brain and the body about the free and stored levels of fuel available for the organism (1). In turn, hypothalamic neuronal circuits use this information to regulate caloric intake, energy consumption, and peripheral lipid and glucose metabolism. Among the chemical mediators involved in this regulation, the endocannabinoids (ECs)³ are master controllers of the fast (*i.e.* non-genomic) and stress-related fine-tuning of energy intake and processing. The endocannabinoid system (ECS) is composed of two, mostly G_{i/o} protein-coupled “cannabinoid” receptors, CB₁ and CB₂; their lipid ligands, *i.e.* the ECs anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and the enzymatic machinery for EC biosynthesis and degradation. Lately, we and others have demonstrated that the ECS plays a pivotal role in the regulation of energy balance through interactions with the anorexigenic adipokine hormone, leptin. In particular, this hormone reduces hypothalamic EC levels (2). Leptin is produced and secreted predominantly from the adipose tissue into the circulation. Circulating leptin levels positively reflect adipose tissue size and communicate energy storage status to the brain (3, 4). The central action of leptin is linked to the interaction with its receptor, which is strongly expressed in the hypothalamic arcuate nucleus (ARC), and coupled with the stimulation and inhibition of anorexic and orexigenic signals, respectively.

Recently it was shown that in hypothalamic neurons leptin produces its anorexic effects partly through an increase in reactive oxygen species (ROS) levels and subsequent activation of anorexic pro-opiomelanocortin (POMC) neurons, whereas diminishing ROS levels decrease POMC neuron activity but increase the activity of orexigenic neurons co-producing neuropeptide Y and agouti-related peptide (5). In these neurons the

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³ The abbreviations used are: EC, endocannabinoid; ECS, endocannabinoid system; ACEA, arachidonyl-2'-chloroethylamide; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; ARC, arcuate nucleus; DHR, dihydorhodamine 123; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; PPAR- γ , peroxisome proliferator-activated receptor- γ ; ROS, reactive oxygen species; ANOVA, analysis of variance.

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proliferation of peroxisomes mediated by a peroxisome proliferator-activated receptor- γ (PPAR- γ) agonist resulted in the decrease of ROS levels. ROS are a group of highly reactive molecules, such as singlet oxygen, hydroxyl radicals, superoxide, and hydrogen peroxides. Most ROS have extremely short half-lives (nanoseconds), whereas some others, such as hydrogen peroxide, have millisecond half-lives. Due to their high reactivity, ROS can oxidize cell constituents such as lipids, proteins, and DNA, thus damaging cell structures and compromising their function (6). Because of these potentially noxious effects, cells maintain ROS at a tolerable level by means of antioxidants such as the redox system, superoxide dismutase, and catalase (7). Catalase, predominantly located in peroxisomes, catalyzes the conversion of hydrogen peroxide into water and molecular oxygen (8). The transcription of this enzyme is regulated by PPAR- γ . A putative functional PPAR response element was identified at the promoter region of the rat catalase gene (9). Activation of PPAR- γ by a specific agonist further enhances catalase activity and protects neurons from oxidative stress (10). Growing evidence indicates that endocannabinoids exhibit profound anti-inflammatory and neuroprotective properties in response to harmful insults, including oxidative stress (11–15). Some of these effects appear to be mediated by PPAR- γ activation (16–18).

The present study was designed to investigate whether leptin-induced ROS formation could be controlled by activation of CB₁ receptors in hypothalamic neurons. We report that in a mouse hypothalamic neuronal cell line (mHypoE-N41) as well as in primary cultures of hypothalamic neurons, ACEA, a selective CB₁ receptor agonist, is able to prevent ROS formation induced by leptin in a manner sensitive to AM251, a CB₁ receptor antagonist/inverse agonist, and through PPAR- γ activation and subsequent enhancement of catalase activity. Furthermore, by using pharmacological tools and knock-out mice, we show that CB₁ inhibition of leptin-induced ROS formation is exerted tonically by ECs in hypothalamic neurons.

Experimental Procedures

Cell Cultures—mHypoE-N41 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% horse fetal bovine serum (Life Technologies), penicillin (50 units/ml), and streptomycin (50 μ g/ml), at 37 °C in 100-mm culture dishes (Life Technologies) gassed with an atmosphere of 95% air, 5% CO₂.

Primary cultures of hypothalamic ARC neurons, derived from neonatal 0- or 1-day-old C57BL/6 (Charles River) or monoacylglycerol lipase (MAGL) null mice, were obtained as described (19). Briefly, the ARC was quickly dissected and mechanically dispersed in Ca²⁺- and Mg²⁺-free buffered Hanks' balanced salt solution. Then tissues were dissociated enzymatically (0.125% trypsin solution, 37 °C for 20 min) and mechanically. Cells were plated at a density of 2 \times 10⁴ cells/cm² on polylysine-coated coverslips and grown in Neurobasal medium supplemented with 2% B27, 0.5 mM L-glutamine, penicillin (50 unit/ml) and streptomycin (50 μ g/ml). Cells were used between 6 and 8 days *in vitro*. More than 80% of primary cultured cells were positive for neuronal marker NeuN anti-

bodies, determined by immunocytochemistry (data not shown).

Quantitative PCR Analysis—RNA was isolated from mHypoE-N41 cells using TRIzol (Invitrogen), DNase-treated (Ambion) and reverse-transcribed with SuperScript III RT reaction kit (Invitrogen) according to the manufacturers' instructions. 10 ng of starting RNA was then used for quantitative PCR analysis in 10- μ l reactions using IQ SYBR Green Supermix on a CFX 384 optical thermal cycler (Bio-Rad). Data are expressed as raw Ct values, as determined by CFX Manager software (Bio-Rad).

Measurement of ROS—ROS formation was assayed using dihydrorhodamine 123 (DHR) or 2',7'-dichlorofluorescein diacetate, two cell-permeable oxidation-sensitive dyes. Briefly, the cells were preloaded with 10 μ M DHR (20 min), treated as detailed in the legend to the figures, and finally analyzed with a Leica DMI6000 fluorescence microscope equipped with a Leica DFC320 cooled digital CCD camera (Leica Microsystems). The excitation and emission wavelengths were 488 and 515 nm, respectively. Images were collected with exposure times of 100–400 ms, digitally acquired, and processed for fluorescence determination at the single cell level with Metamorph Imaging Software (Leica MetaMorph® AF). Mean fluorescence values were determined by averaging the fluorescence values of at least 50 cells/treatment condition/experiment.

Cnr1 Gene Silencing—Cnr1 silencing was obtained by transfecting mHypoE-N41 cells with endoribonuclease-prepared siRNA sequences (EMU088771; Sigma) using Lipofectamine 2000 (Life Technologies) and following the manufacturer's instructions. The siRNA silencing efficiency was determined 24 h after the initial transfection by measuring mRNA levels (data not shown) or the relative amount of the protein using Western blot analysis.

Detection of CB₁ Receptors—Immunocytochemical and Western blot techniques were used to detect the expression of CB₁ receptor protein. For immunocytochemical analysis, primary cultures of hypothalamic ARC neurons were seeded on polylysine-coated coverslips, washed 3 times with PBS, and finally fixed for 20 min with paraformaldehyde (4%; v/v). These preparations were finally rinsed with PBS and blocked in PBS-containing BSA (2%, w/v). The rabbit polyclonal anti-CB₁ (Calbiochem) or mouse monoclonal anti-NuN (Abcam) antibody was used as a primary antibody. After 18 h at 4 °C, the cells were washed and exposed to a fluorescein isothiocyanate-conjugated secondary antibody for 2 h in the dark. Stained cells were analyzed with a Leica DMI6000 fluorescence microscope equipped with a Leica DFC320 cooled digital CCD camera (Leica Microsystems).

For Western blot analysis mHypoE-N41 cell homogenate was subjected to electrophoresis in 10% polyacrylamide gel and transferred to PVDF membranes. Membranes were blocked with 3% BSA for 1 h and incubated overnight at 4 °C with a rabbit polyclonal anti-CB₁ antibody (Calbiochem; 1:1000 dilutions) or with a polyclonal anti-actin (1:1000, Sigma), whereas incubation with the secondary antibody (peroxidase-labeled) was for only 2 h. Actin was taken as the reference protein expression. Detection was performed using ECL Western blotting detection reagents (GE Healthcare Italia).

Measurement of 2-AG and AEA Levels—After treatment, the cells (each data point contained 1×10^5 and 0.5×10^5 cells/ml for N41 and primary neurons, respectively) and supernatant were homogenized in 50 mmol/liter Tris-HCl, pH 7.5, in chloroform/methanol (1:2:1, v/v) containing 10 pmol of [$^2\text{H}_8$]AEA and 5 pmol of 2- $[\text{H}_8]$ AG as internal standards and analyzed using liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry as previously described (20). 2-AG and AEA levels were calculated on the basis of their peak area ratio (in the SIM mode) with the internal deuterated standard peak areas, and their amounts (pmol) were normalized per ml of cells plus medium.

Catalase Activity—Catalase activity was measured using an Amplex Red Catalase Assay kit (Life Technologies) following the manufacturer's instructions. Briefly, cells treated as detailed in the figure legend were washed twice with PBS, scraped on ice, and finally sonicated 3 times with a Branson Sonifer operating at 20 watts for 15 s. The resulting homogenates were centrifuged for 5 min at $18,000 \times g$ at 4°C . The supernatant was used to measure catalase activity. The decomposition of hydrogen peroxide ($40 \mu\text{M}$) by catalase was monitored by reaction with $50 \mu\text{M}$ Amplex red reagent in the presence of 0.2 unit/ml horseradish peroxidase. Fluorescence was measured in a fluorescence microplate reader using excitation at 530 nm and emission at 590 nm. Catalase activity was normalized to protein concentration, which was quantified by the Lowry protein assay.

PPAR- γ DNA Binding Activity—The DNA binding activity of PPAR- γ was assessed using a commercially available PPAR- γ transcription factor assay kit (Abcam) according to the manufacturer's instructions. Briefly, the nuclear extracts prepared from treated cells were added to the provided wells coated with specific oligonucleotide sequences. A primary polyclonal anti-PPAR- γ antibody was then added followed by the addition of horseradish peroxidase-conjugated antibody and the substrate 3,3',5,5'-tetramethylbenzidine. The absorbance of the developed color was read at 450 nm using a microplate reader.

Results

CB $_1$ Receptor Activation Mitigates Leptin-induced ROS Formation in mHypoE-N41 Cells and in Primary Cultures of Hypothalamic ARC Neurons—Recently, it was reported that the mechanism whereby leptin produces its anorexic effects might involve ROS formation in ARC neurons (5). We used DHR, a cell-permeable fluorogenic probe that is useful for the detection of ROS formation, to determine if the treatment of murine hypothalamic mHypoE-N41 cells with increasing concentrations of leptin results in ROS accumulation. As shown in Table 1, mHypoE-N41 cells express high levels of CB $_1$ receptor mRNA together with the mRNAs encoding for the major biosynthetic enzymes of the ECS, including the anandamide hydrolyzing enzyme, fatty acid amide hydrolase-1 (FAAH-1), the anandamide biosynthesizing enzyme, *N*-acylphosphatidylethanolamine-specific phospholipase D, and the 2-AG biosynthesizing enzyme, diacylglycerol lipase- α . Very low levels of CB $_2$ receptor mRNA and MAGL were instead found. As illustrated in Fig. 1A, a 4.5-h treatment with leptin induced a dose-dependent formation of ROS with the maximum effect at 100

TABLE 1

mHypoE-N41 cells express PPAR- γ , unlike PPAR- α , as well as the major enzymes of the endocannabinoid system

Real time quantitative PCR results from mHypoE-N41 cells cultured in standard growth medium to 90% confluence in 24-well plates. Data are expressed as threshold cycle (Ct), and S.D. of reactions performed in triplicate. DAGL- α , diacylglycerol lipase- α ; NAPE-PLD, *N*-acylphosphatidylethanolamine-specific phospholipase D.

Genes	Ct
<i>Pparg</i>	25.67 \pm 0.14
<i>Ppara</i>	35.19 \pm 0.21
<i>Mgl</i>	31.90 \pm 0.28
<i>Dagla</i>	25.66 \pm 0.13
<i>Nape-pld</i>	25.94 \pm 0.03
<i>Cnr1</i>	27.88 \pm 0.11
<i>Cnr2</i>	30.09 \pm 0.90
<i>Faah-1</i>	26.76 \pm 0.09

ng/ml. The leptin (100 ng/ml) response was reduced by pre-exposure for 15 min to ACEA, a specific CB $_1$ receptor agonist, in a dose-dependent manner (Fig. 1B). The inhibitory effect of ACEA ($0.5 \mu\text{M}$) was abolished by treating the cells with AM251 ($0.5 \mu\text{M}$), a CB $_1$ receptor antagonist/inverse agonist (Fig. 2A). In the same experimental conditions, treatment with AM630, a CB $_2$ receptor antagonist, was ineffective. Similar results were obtained using 2',7'-dichlorofluorescein diacetate, another fluorogenic probe to detect ROS formation (data not shown). *In vivo* studies have demonstrated that the accumulation of ROS induced by leptin were mitigated by the proliferation of peroxisomes, induced by PPAR- γ activation (5). This might be the case also for mHypoE-N41 cells treated with leptin, because, as shown in Fig. 2, a short pre-exposure (15 min) to troglitazone ($1 \mu\text{M}$), a PPAR- γ agonist, prevented the formation in a manner sensitive to T0070907, a specific PPAR- γ antagonist. It is important to note that in the same experimental conditions, T0070907 also abolished the inhibitory effect of ACEA on leptin. Accordingly, mHypoE-N41 cells express high levels of the mRNA encoding for PPAR- γ but not PPAR- α (Table 1). Interestingly, leptin-induced ROS formation, although insensitive to heat-inactivated catalase, was prevented by enzymatically active catalase (10 unit/ml; Fig. 2A). These findings are, therefore, consistent with the possibility that H $_2$ O $_2$ is the major reactive molecule produced by the cells after treatment with leptin. Additionally, both ACEA and troglitazone were also able to prevent the H $_2$ O $_2$ -induced DHR oxidation. Again the inhibitory effect of ACEA was prevented by both AM251 and T0070907 but not by AM630 (Fig. 2B).

The results obtained with leptin were confirmed in primary cultures of ARC neurons. Using immunocytochemical techniques, we detected the expression of CB $_1$ receptors in these cells (Fig. 3A). Leptin was able to induce ROS formation in primary neurons (Fig. 3, B and C), and again, this effect was prevented by both ACEA (Fig. 3, B and C) and troglitazone (Fig. 3C) in a manner sensitive, in the case of ACEA, to AM251 (Fig. 3, B and C), and in the case of both compounds by T0070907 (Fig. 3C).

To provide further evidence that the effect of ACEA was due to CB $_1$ activation, experiments were performed in mHypoE-N41 cells transfected with CB $_1$ receptor-specific siRNA sequences. These cells showed an $\sim 50\%$ decrease (measured by Western blot assays) in CB $_1$ expression with respect to cells transfected with a scrambled siRNA control (Fig. 4A), which in

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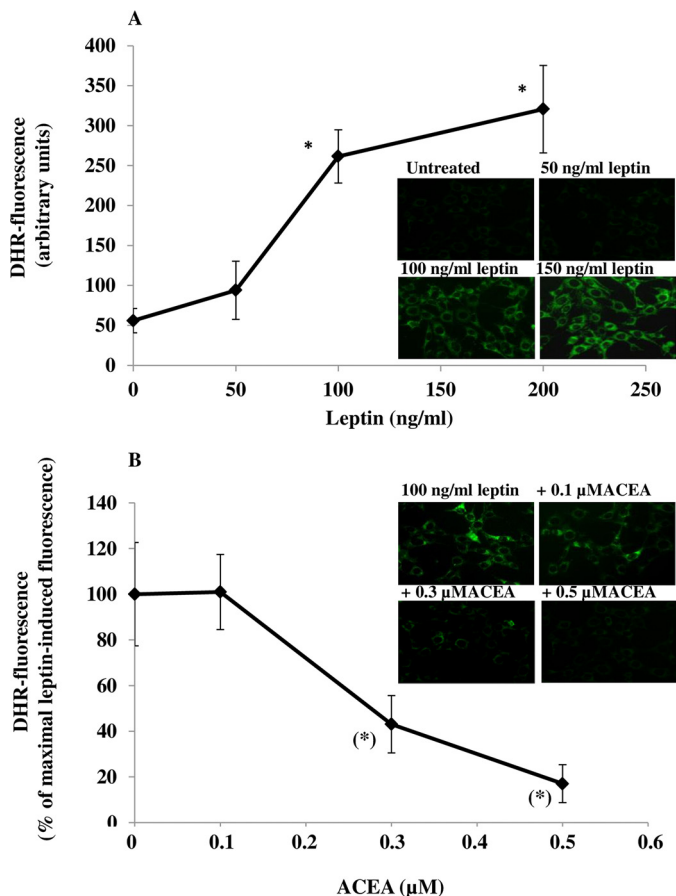


FIGURE 1. The CB₁ receptor agonist ACEA prevents leptin-induced ROS formation in mHypoE-N41 cells. *A*, DHR-loaded cells were incubated for 4.5 h with increasing concentrations of leptin. After treatments, the cells were observed with a Leica DMI6000 fluorescence microscope equipped with a Leica DFC320 cooled digital CCD camera (Leica Microsystems). The resulting images were analyzed to quantify the mean fluorescence of individual cells using Metamorph Imaging Software (Leica MetaMorph AF). Representative micrographs of ROS accumulation in cells treated with different concentrations of leptin are also shown. Scale bar: 20 μ m. Results are expressed as arbitrary units and represent the means \pm S.E. calculated from three to five separate experiments, each performed in duplicate. *B*, the cells, loaded with DHR, were incubated for 30 min with increasing concentrations of ACEA and then treated for an additional 4.5 h with 100 ng/ml leptin. After treatments, the cells were analyzed with a fluorescence microscope as described above. Representative micrographs of the dose-dependent inhibition of ROS formation by ACEA in cells treated with leptin (100 ng/ml) are also shown. Results represent the mean \pm S.E. of three separate experiments, each performed in duplicate. *, $p < 0.01$ compared with untreated cells; (*), $p < 0.01$ compared with leptin-treated cells (one-way ANOVA followed by Bonferroni's test).

turn displayed levels of CB₁ expression identical to those observed in non-transfected cells (not shown). As shown in Fig. 4*B*, in CB₁ siRNA-transfected cells, ACEA failed to prevent leptin-induced ROS formation, and this lack of effect was not modified by AM251.

A CB₁-mediated Tone of ECs Controls Leptin-induced ROS Formation and Is under the Negative Control of EC Hydrolyzing Enzymes—2-AG is the most abundant EC in the brain (21–23) where it is hydrolyzed primarily by MAGL (24). Pharmacological or genetic inhibition of MAGL results in an increase of 2-AG levels in the brain (25, 26). To provide evidence for a tonic action of ECs on leptin-induced ROS formation and because mHypoE-N41 cells express only very low levels of MAGL

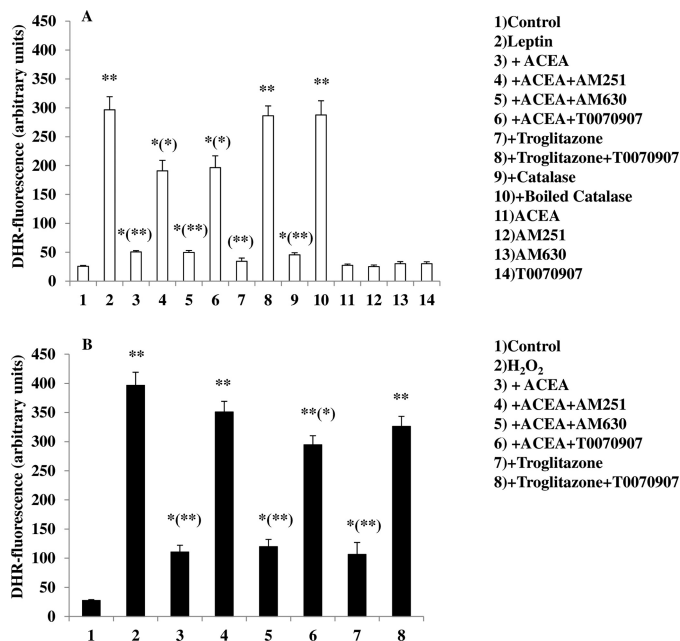


FIGURE 2. A CB₁ as well as a PPAR- γ agonist reduces leptin (A)- or H₂O₂ (B)-induced DHR oxidation in mHypoE-N41 cells. DHR-loaded cells were incubated with AM251 (0.5 μ M), AM630 (1 μ M), or T0070907 (1 μ M). After 30 min, cells were exposed to ACEA (0.5 μ M) or troglitazone (1 μ M) for an additional 30 min and, finally, treated with leptin (100 ng/ml; 4.5 h) or H₂O₂ (0.1 mM; 4.5 h). In other experiments the cells were incubated with catalase (10 unit/ml; 30 min) or boiled catalase and then treated with leptin (100 ng/ml; 4.5 h). After treatments, the cells were analyzed with a fluorescence microscope as described in Fig. 1*A*. Results represent the mean \pm S.E. of three separate experiments, each performed in duplicate. *, $p < 0.05$; **, $p < 0.01$ compared with untreated cells; (*), $p < 0.05$; (**), $p < 0.01$ compared with leptin-treated cells (one-way ANOVA followed by Bonferroni's test).

(Table 1), we adopted a dual experimental strategy: 1) to investigate the role of AEA, we treated mHypoE-N41 cells with a selective FAAH-1 inhibitor, URB597, and 2) we performed experiments in primary cultures of hypothalamic neurons isolated from wild-type or MAGL null mice; neurons from the former mice were also investigated in the presence of a selective MAGL inhibitor, JZL184. As shown in Fig. 5*A*, in mHypoE-N41 cells, URB597 (1 μ M) reproduced the effects described above with ACEA in terms of the AM251-sensitive inhibition of leptin-induced ROS formation and induced a significant increase of AEA levels (Table 2). Like AEA, ACEA was suggested to be metabolized by FAAH (previously known as anandamide amidohydrolase; see Ref. 27). Inhibition of FAAH activity with a low concentration (0.1 μ M) of URB597 rendered a non-effective dose of ACEA (0.1 μ M) effective against leptin-mediated ROS formation. As shown in Fig. 5*B*, in primary neurons isolated from MAGL null mice, treatment with leptin failed to induce ROS formation. This lack of effect was also observed in primary neuronal cultures from wild-type mice pretreated for 30 min with 1 μ M JZL184 (untreated, 66.86 \pm 21.86; 100 ng/ml leptin, 406.45 \pm 50.36; 100 ng/ml leptin after JZL184, 110 \pm 45.63; JZL184 only, 55.38 \pm 18.22; DHR fluorescence is expressed as arbitrary units \pm S.E.). The lack of effect of leptin in primary neurons from MAGL null mice was most likely due to the increase of basal activation of CB₁ by high levels of 2-AG in these neurons as compared with wild-type mouse neurons (Table 2). Accordingly, the treatment with increasing concen-

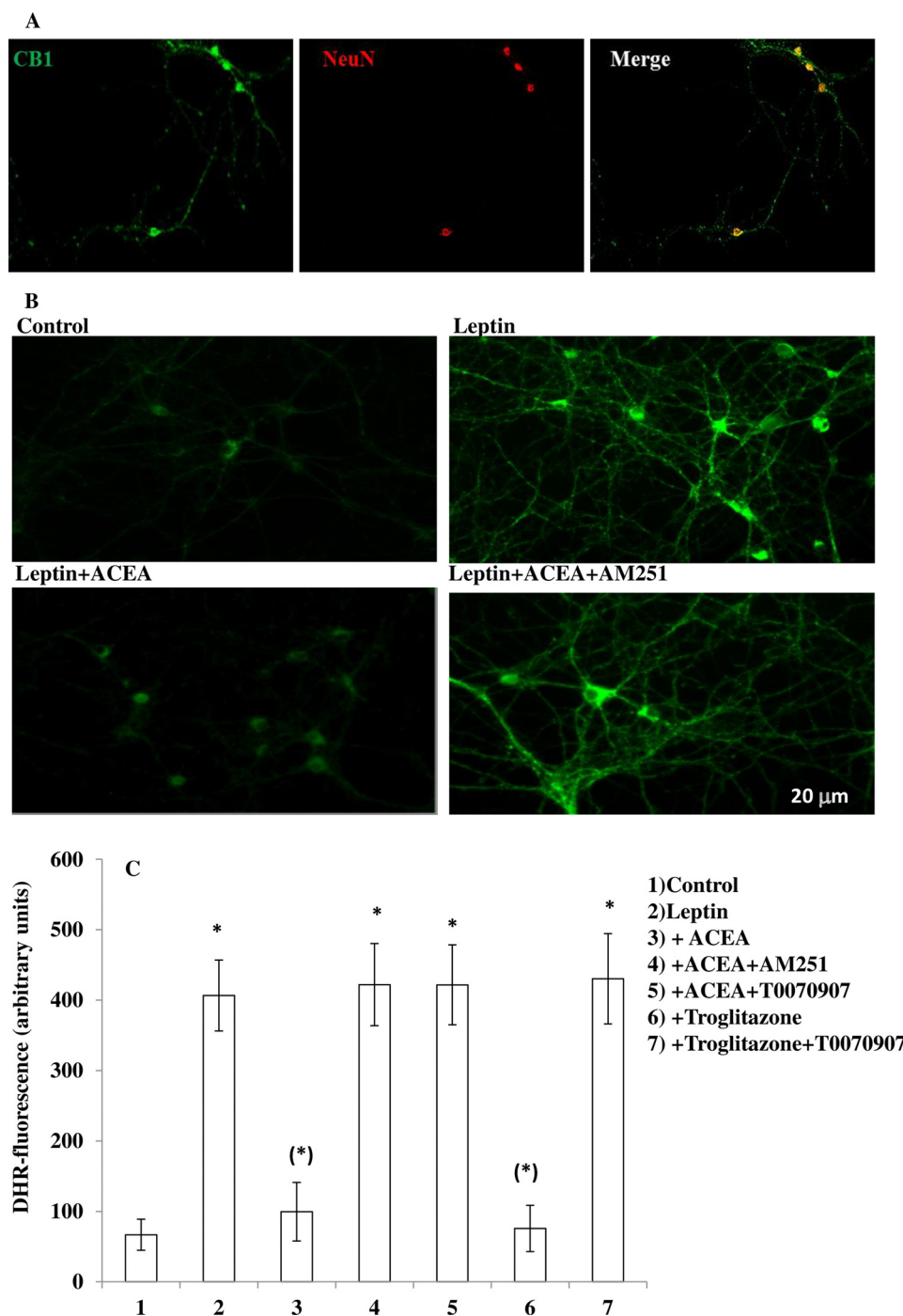


FIGURE 3. A CB₁ as well as a PPAR- γ agonist decrease leptin-induced ROS levels in primary cultures of hypothalamic ARC neurons. *A*, immunocytochemical staining of the CB₁ (green signal) receptor in primary culture of hypothalamic neurons. Neuronal-specific nuclear protein (NeuN) antibody (red signal) was used as marker of neuronal cells. *B*, representative micrographs of ROS accumulation in primary culture of hypothalamic ARC neurons exposed to 100 ng/ml leptin for 4.5 h after a 30-min preincubation with DMSO or 0.5 μ M ACEA with or without 0.5 μ M AM251. A representative micrograph of control cells is also shown. *C*, DHR-loaded cells were incubated with AM251 (0.5 μ M) or T0070907 (1 μ M). After 30 min, cells were exposed to ACEA (0.5 μ M) or troglitazone (1 μ M) for an additional 30 min and, finally, treated with leptin (100 ng/ml; 4.5 h). After the treatments, the cells were analyzed with a fluorescence microscope as described in Fig. 1A. Results represent the mean \pm S.E. of three separate experiments, each performed in duplicate. *, $p < 0.01$ compared with untreated cells; (*), $p < 0.01$ compared with leptin-treated cells (one-way ANOVA followed by Bonferroni's test).

trations of AM251 restored the leptin-induced ROS formation. These data strongly suggest that ECs may tonically inhibit leptin-induced ROS formation, at least *in vitro*, and that this inhibition is under the negative control of EC degrading enzymes.

PPAR- γ Activation by CB₁ and Its Impact on Leptin-induced ROS Formation in mHypoE-N41 Cells—To provide additional evidence of the involvement of PPAR- γ activation in ROS

inhibition by ACEA, we measured PPAR- γ DNA binding activity in the above experimental conditions. As shown in Fig. 6A, a 4.5-h treatment of mHypoE-N41 cells with leptin (100 ng/ml) resulted in the reduction of PPAR- γ activity. This response was prevented by preincubation with both ACEA and troglitazone. T0070907 alone resulted in the decrease of PPAR- γ activity.

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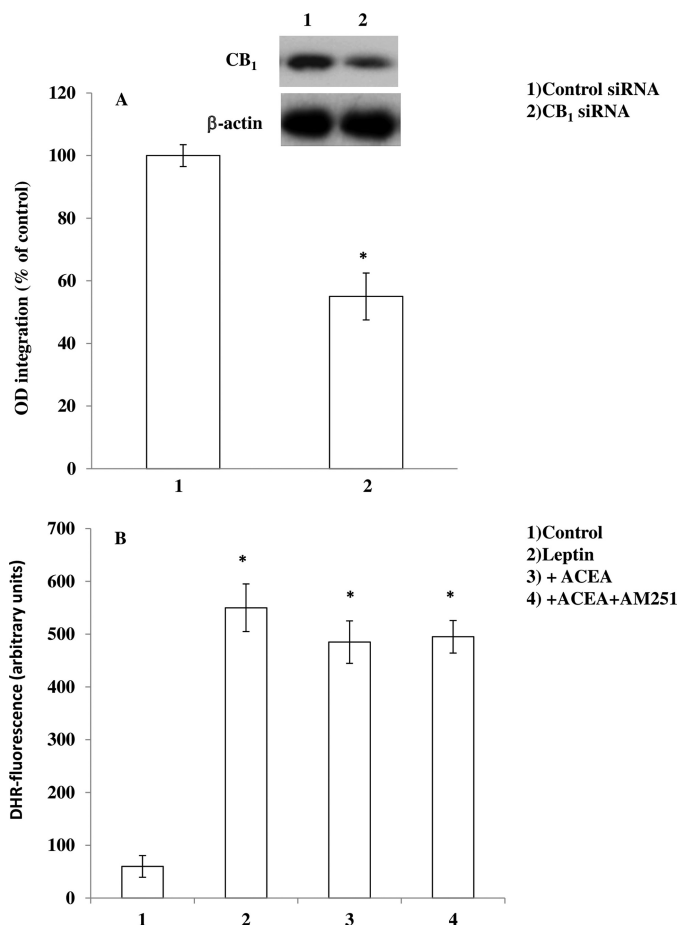


FIGURE 4. ACEA fails to prevent leptin-induced ROS formation in mHypoE-N41 cells transfected with CB₁ receptor siRNA. *A*, Western blot quantification of CB₁ protein levels in cells transfected with scrambled or CB₁ siRNAs for 24 h. The blots shown are representative of three separate experiments with similar outcomes. *B*, siRNA-transfected cells were loaded with DHR and then incubated with AM251 (0.5 μM). After 30 min, cells were exposed to ACEA (0.5 μM) for an additional 30 min and, finally, treated with leptin (100 ng/ml; 4.5 h). After the treatments, the cells were analyzed with a fluorescence microscope as described above. Results represent the mean ± S.E. of three separate experiments, each performed in duplicate. *, $p < 0.01$ compared with untreated cells (one-way ANOVA followed by Bonferroni's test).

The Effect of CB₁ Activation on Leptin-induced ROS Formation Is Due to PPAR-γ-mediated Catalase Activation—It is well known that PPAR-γ regulates a large number of enzymes, including catalase, the most important enzyme for antioxidant defense. We, therefore, investigated the effect of leptin on catalase activity. As illustrated in Fig. 6*B*, exposure of cells to 100 ng/ml leptin for 4.5 h resulted in a reduction of catalase activity. Under the same conditions, a pretreatment with ACEA (15 min) as well as troglitazone prevented this effect in a manner sensitive to both AM251 and T0070907, respectively (Fig. 5*B*). It is important to note that ACEA alone was able to increase catalase activity. Taken together, these results strongly suggest that the mechanism whereby ACEA counteracts leptin-mediated ROS formation involves PPAR-γ activation and subsequent activation of catalase.

Discussion

The results reported in the present study establish for the first time the existence of a negative control by CB₁ receptor

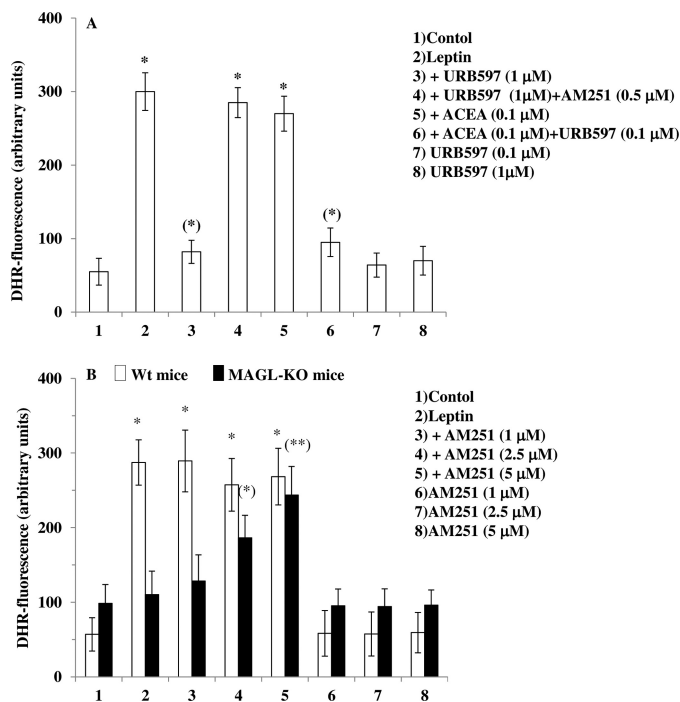


FIGURE 5. FAAH inhibition and MAGL knock out mitigates leptin-induced ROS formation. *A*, a FAAH-1 inhibitor, URB597, prevents the leptin-induced ROS formation in mHypoE-N41 cells. DHR-loaded cells were incubated for 30 min with AM251 (0.5 μM), then with URB597 (0.1 or 1 μM) for an additional 30 min without or with ACEA (0.1 μM) and, finally, treated with leptin (100 ng/ml; 4.5 h). After treatment, the cells were analyzed with a fluorescence microscope as described above. Results represent the mean ± S.E. of three separate experiments, each performed in duplicate. *, $p < 0.01$ compared with untreated cells; (*), $p < 0.01$ compared with leptin-treated cells (one-way ANOVA followed by the Bonferroni's test). *B*, leptin fails to induce ROS formation in primary cultures of hypothalamic neurons isolated from MAGL null mice. Primary neurons isolated from wild-type or MAGL null mice were incubated for 30 min with increasing concentration of AM251 and then treated with leptin (100 ng/ml; 4.5 h). After the treatments, the cells were analyzed with a fluorescence microscope as described above. Results represent the mean ± S.E. of three separate experiments, each performed in duplicate. *, $p < 0.01$ compared with WT untreated cells; (*), $p < 0.05$, (**), $p < 0.01$ compared to MAGL-KO untreated cells (one-way ANOVA followed by Bonferroni's test).

activation over an action by leptin, *i.e.* the capability of the hormone to elevate ROS levels in hypothalamic neurons, which is emerging as an important signal mediating leptin anorectic effects. We found that in both mHypoE-N41 cells and primary cultures of hypothalamic ARC neurons, leptin was able to induce ROS formation (Figs. 1–3) and that this effect was blunted by ACEA, a specific CB₁ receptor agonist, in a manner sensitive to AM251, a CB₁ receptor antagonist/inverse agonist. The CB₁-mediated mechanism of action of ACEA was further confirmed using CB₁ siRNA-mediated knockdown in mHypoE-N41 cells. We also report the mechanism whereby CB₁ receptors control leptin action. In fact, it was recently demonstrated that the ROS-mediated anorectic effect of leptin can be counteracted by the induction of peroxisome proliferation mediated by PPAR-γ activation. The latter effect occurs also in our experimental conditions, as a PPAR-γ agonist, troglitazone, was able to prevent leptin-mediated ROS formation in both mHypoE-N41 cells and in primary hypothalamic neurons. As expected, the effect of troglitazone was prevented by the pretreatment with T0070907, a PPAR-γ antagonist. The observa-

TABLE 2**AEA and 2-AG levels in leptin-treated mHypoE-N41 cells and in primary hypothalamic neurons**

Endogenous levels of AEA and 2-AG were quantified by LC-atmospheric pressure chemical ionization-MS in mHypoE-N41 cells treated with leptin (100 ng/ml) with or without URB597 (1 μ M) or in primary cultures of hypothalamic neurons isolated from C57BL/6 or MAGL null mice. AEA and 2-AG levels are normalized per ml of cell + medium. Each sample contained 1×10^{-5} and 0.5×10^{-5} cells/ml for N41 and primary neurons, respectively. Results represent the means \pm S.E. of three separate experiments and were compared by using ANOVA followed by the Bonferroni's test.

	AEA	2-AG
	pmol/ml	pmol/ml
mHypoE-N41 cells		
Untreated	0.15 \pm 0.05	2.91 \pm 0.55
Leptin	0.20 \pm 0.01	3.13 \pm 0.005
Leptin+ URB597	0.89 \pm 0.04 ^a	2.43 \pm 0.31
Primary hypothalamic neurons		
<i>Wt</i>		
Untreated	0.07 \pm 0.01	5.51 \pm 0.26
<i>MAGL</i> ^{-/-}		
Untreated	0.04 \pm 0.01	11.23 \pm 0.54*
Leptin	0.12 \pm 0.03	11.45 \pm 0.96*

^a, $p > 0.05$.

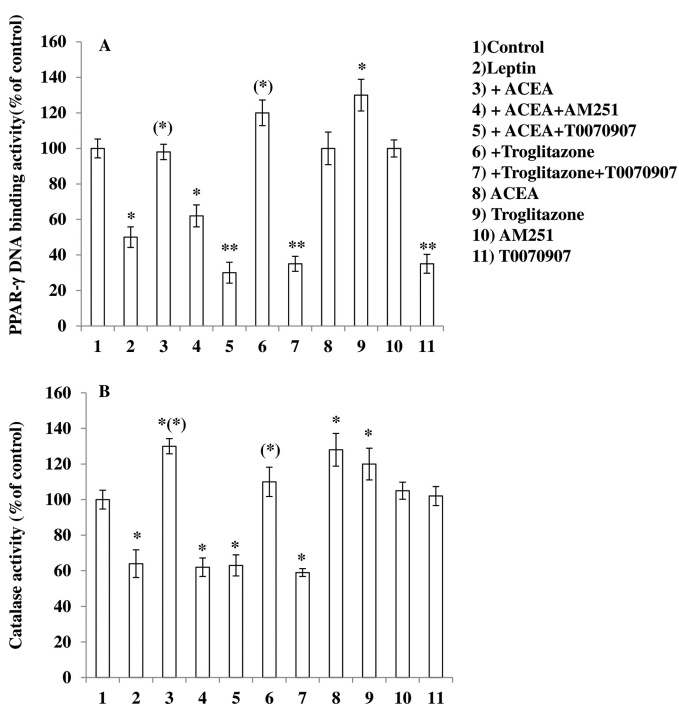


FIGURE 6. Effect of CB₁ activation on leptin-induced decrease of PPAR- γ and catalase activity in mHypoE-N41 cells. Cells were incubated with AM251 (0.5 μ M) or T0070907 (1 μ M). After 30 min, the cell were exposed to ACEA (0.5 μ M) or troglitazone (1 μ M) for an additional 30 min and, finally, treated with leptin (100 ng/ml; 4.5 h). After treatments, the cells were analyzed for both PPAR- γ (A) and catalase (B) activity as described under "Experimental Procedures." Results represent the mean \pm S.E. of three separate experiments, each performed in duplicate. *, $p < 0.05$; **, $p < 0.01$ compared with untreated cells; (*), $p < 0.01$ compared with leptin-treated cells (one-way ANOVA followed by Bonferroni's test).

tion that T0070907 was able to also prevent the effect of ACEA suggests a possible involvement of PPAR- γ activation in ACEA inhibition of leptin-mediated ROS stimulation. In further support of this hypothesis, we found that treatment with leptin results in a reduction of PPAR- γ DNA binding activity and that preincubation with ACEA leads to the prevention of this effect of the hormone. This observation is consistent with the hypothesis that the CB₁ receptor agonist controls leptin action at least

in part through PPAR- γ activation. This nuclear receptor can directly regulate the expression of a large number of antioxidant enzymes, including catalase (9, 28, 29), which is ubiquitously expressed in the CNS and is mainly located in peroxisomes (8). In agreement with the report that PPAR- γ activation by a specific agonist enhances catalase activity, thereby resulting in the protection of neurons from oxidative stress (10), we found here that ACEA also prevented the inhibition of catalase induced by leptin in a PPAR- γ -mediated manner.

In agreement with the present findings in neurons, the activation of CB₁ receptors was previously shown to lead to overexpression of PPAR- γ in adipocytes (30, 31). The underlying mechanism of this effect has never been investigated, but it is possible that the well known CB₁-induced activation of ERKs might cause phosphorylation of C/EBP β (32), a transcription factor that activates PPAR- γ , thus explaining why ACEA enhances PPAR- γ activity also in hypothalamic neurons, which express C/EBP β . Indeed, we have found that the effects of ACEA were inhibited by the MAPK inhibitor PD098059 (data not shown). However, ACEA differs from AEA only in the presence of a chlorine atom on the 2'-carbon atom of the ethanolamine moiety, and it possible that, like AEA (as well as other cannabinoids; for review, see Ref. 33); it also directly activates PPAR- γ .

Importantly, we have also shown that in the cell model mostly used in our experiments, the mHypoE-N41 cells, not only are CB₁ receptor mRNA and protein-expressed, but also the mRNAs encoding the enzymes responsible for EC biosynthesis and hydrolytic inactivation (except for MAGL) are abundant. This finding is in keeping with our data indicating that endogenous AEA might exert, under the negative control of FAAH-1, a CB₁-mediated inhibitory tone on leptin-induced ROS stimulation in mHypoE-N41 cells and in particular with our finding that a FAAH-1 inhibitor, at a concentration elevating anandamide levels in these cells, also inhibits leptin-induced ROS formation. Because we could not perform experiments with MAGL inhibitors in these cells, which express hardly measurable amounts of MAGL mRNA, we instead analyzed the capability of leptin to elevate ROS levels in hypothalamic neurons from MAGL null mice, which we show to contain significantly higher amounts of 2-AG than wild type mice. Again in agreement with the existence of an inhibitory CB₁-mediated tone over leptin action in the hypothalamus by ECs, we found that a concentration of leptin that showed high efficacy in cultures from wild-type mice did not produce any effect on ROS in cultures from MAGL^{-/-} mice and that a CB₁ antagonist could dose-dependently restore this effect of leptin in these cultures. Thus, we propose that pharmacologically or genetically elevated levels of endogenous AEA in mHypoE-N41 cells or endogenous 2-AG in primary cultures of mouse hypothalamic neurons, respectively, exert tonic inhibition over leptin induction of ROS, indicating that the findings of the first part of this study might have physiological relevance. In fact, it is tempting to speculate that during physiological or pathological conditions in which hypothalamic EC levels are likewise elevated, *i.e.* after short term food deprivation (34) or in either genetic or high fat diet-induced obesity (2, 35, 36), a similar negative control over leptin activity by CB₁ activation might

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occur. Interestingly, a recent report published during the reviewing process of the present study (37) showed that food deprivation-induced food intake is partly dependent on PPAR- γ -mediated enhancement of agouti-related protein/neuropeptide Y signaling (which is the major system expressed in mHypoE-N41 cells), and it is tempting to speculate that such a process might be due in part to CB₁ activation by ECs.

In conclusion, the results reported in this study may facilitate a better understanding of the role of ECs and CB₁ receptors in leptin-mediated effects on food intake and energy storage/expenditure as well as other physiopathological functions of this hormone, such as its role in neuroinflammation (38).

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