Inhibition of cannabinoid CB1 receptor upregulates \( \text{Slc2a4} \) expression via nuclear factor-\( \kappa \)B and sterol regulatory element-binding protein-1 in adipocytes

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Abstract

Evidences have suggested that the endocannabinoid system is overactive in obesity, resulting in enhanced endocannabinoid levels in both circulation and visceral adipose tissue. The blockade of cannabinoid receptor type 1 (CB1) has been proposed for the treatment of obesity. Besides loss of body weight, CB1 antagonism improves insulin sensitivity, in which the glucose transporter type 4 (GLUT4) plays a key role. The aim of this study was to investigate the modulation of GLUT4-encoded gene (\( \text{Slc2a4} \) gene) expression by CB1 receptor. For this, 3T3-L1 adipocytes were incubated in the presence of a highly selective CB1 receptor agonist (1 \( \mu \text{M} \) arachidonyl-2\(^\text{-} \)chloroethylamide) and/or a CB1 receptor antagonist/inverse agonist (0.1, 0.5, or 1 \( \mu \text{M} \) AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide). After acute (2 and 4 h) and chronic (24 h) treatments, cells were harvested to evaluate: i) \( \text{Slc2a4}, \text{Cnr1} \) (CB1 receptor-encoded gene), and \( \text{Srebf1} \) type a (SREBP-1a type-encoded gene) mRNAs (real-time PCR); ii) GLUT4 protein (western blotting); and iii) binding activity of nuclear factor (NF)-\( \kappa \)B and sterol regulatory element-binding protein (SREBP)-1 specifically in the promoter of \( \text{Slc2a4} \) gene (electrophoretic mobility shift assay). Results revealed that both acute and chronic CB1 receptor antagonism greatly increased (~2-5-fold) \( \text{Slc2a4} \) mRNA and protein content. Additionally, CB1-induced upregulation of \( \text{Slc2a4} \) was accompanied by decreased binding activity of NF-\( \kappa \)B at 2 and 24 h, and by increased binding activity of the SREBP-1 at 24 h. In conclusion, these findings reveal that the blockade of CB1 receptor markedly increases \( \text{Slc2a4}/\text{GLUT4} \) expression in adipocytes, a feature that involves NF-\( \kappa \)B and SREBP-1 transcriptional regulation.

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Introduction

Obesity has been described as a chronic multifactorial and complex genetic disorder, which is associated with several complications and related comorbidities. The increasing prevalence of obesity remains a major public concern. For this, the pharmacotherapy of obesity has been explored extensively in the past few decades. A new approach includes drugs that produce therapeutic effects by blocking the cannabinoid receptor type 1 (CB1).

The endocannabinoid system (ECS) consists of endogenous cannabinoids (endocannabinoids), cannabionoid receptors, and enzymes that synthesize and degrade the endocannabinoids (Howlett et al. 2002). CB1 receptor can be found in the brain and in tissues involved in energy metabolism such as liver, adipose tissue, and skeletal muscle (Cota et al. 2003, Liu et al. 2005, Osei-Hyiaman et al. 2005).

Hyperstimulation of the ECS has been described in obesity with elevated levels of endocannabinoids in blood and adipose tissue (Engeli et al. 2003, Matias et al. 2006, Vettor & Pagano 2009). In this condition, insulin resistance is currently associated, and the pharmacological treatment of obesity with SR141716 (\( N-(\text{piperidin-1-yl})-5-(4\text{-chloro-phenyl})-1-(2,4\text{-dichlorophenyl})-4\text{-methyl-1H-pyrazole-3-carboxamide}) \), a selective CB1 receptor antagonist also known as rimonabant, has been shown to have beneficial effects on several components of the metabolic syndrome, such as waist circumference, LDL cholesterol, triglyceridemia, blood pressure, fasting glycaemia, and insulinenia (Pi-Sunyer et al. 2006, Van Gaal et al. 2008). Additionally, it has been reported that CB1 receptor blockers enhance insulin sensitivity in obese rats (Ravinet Trillou et al. 2003).

The effects of CB1 receptor antagonism could be a consequence of not only body weight loss but also a direct action on glucose metabolism, a fact that could involve the regulation of the expression of glucose transporters. The insulin-sensitive glucose transporter type 4 (GLUT4), codified by the \( \text{Slc2a4} \) gene, is primarily expressed in adipose tissue and skeletal muscle and plays a fundamental role on glucose homeostasis. Insulin binding to its receptor evokes several intracellular phosphorylations that culminate into GLUT4 translocation to the plasma membrane,
thus acutely increasing glucose uptake (Thorens et al. 1990). Importantly, changes in GLUT4 content are related to changes in glucose homeostasis. Decreased GLUT4 expression has been associated with insulin-resistant conditions since a long time ago (Machado et al. 1993), and treatments that enhance GLUT4 expression are reported to show improved glycemic homeostasis (Machado et al. 1993, 1994, Papa et al. 1997). Furthermore, GLUT4 overexpression in adipocytes is known to prevent the development of insulin resistance in adipocytes and to protect the animals from developing further obesity when fed a high-fat diet (Gnudi et al. 1996).

Considering the above, it is expected that to modulate insulin sensitivity the ECS could regulate Slc2a4/GLUT4 expression. Regarding this, a chronic in vivo blockade of CB1 was reported to increase the expression levels of GLUT4 in adipose tissue (Jbilo et al. 2005), but a 2-day CB1 agonistic stimulus in differentiating 3T3-L1 adipocytes was shown to enhance insulin-induced GLUT4 translocation (Motaghedi & McGraw 2008), evincing that the ECS could play a role in the control of Slc2a4/GLUT4 expression. However, the true effects of ECS on Slc2a4 gene regulation, as well as potentially involved mechanisms, are completely obscure.

We hypothesize that to modulate glycemic homeostasis the ECS could regulate Slc2a4 gene expression. To address this issue, the present study investigated in 3T3-L1 adipocytes the effects of the selective CB1 receptor agonist, ACEA (arachidonyl-2'-chloroethylamine) and the CB1 receptor antagonist/inverse agonist, AM251 (1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide) on Slc2a4 gene expression and the potentially involved transcriptional mechanisms.

**Materials and methods**

**Cell culture**

3T3-L1 murine cells obtained from Rio de Janeiro Cell Bank (Rio de Janeiro, Brazil) were propagated and differentiated according to the protocol described by Chamberlain (2001), with modifications. In brief, cells were grown in growth medium containing DMEM (Vitrocell Embriolife, Campinas, Brazil), 10% fetal bovine serum (FBS; Vitrocell Embriolife), and penicillin/streptomycin (100 units/ml each; Sigma–Aldrich) and allowed to reach confluence. After 2 days, the fibroblasts were differentiated and maintained in DMEM containing 10 µg/ml insulin, 1 µmol/l dexamethasone, and 0.5 µmol/l 3-isobutyl-1-methylxanthine (Sigma) for 6 days. Medium was replaced every 2 days. Cells were switched back to growth medium for 2 days. Prior to treatment, cells were starved for 20 h in DMEM containing 0.02% FBS. The differentiated cells were incubated with 1 µmol/l ACEA (Sigma) and/or 0.1, 0.5, or 1 µmol/l AM251 (Sigma) for 2, 4, or 24 h. Control groups were incubated with vehicle (DMSO). All groups including control were incubated with the same final concentration of 0.007% DMSO.

**Western blotting analysis**

3T3-L1-treated cells were homogenized in buffer (10 mmol/l Tris, 1 mmol/l EDTA, 250 mmol/l sucrose, pH 7.4) and centrifuged at 1000 g and 4 °C for 15 min. The fat-free supernatant was centrifuged at 150 000 g and 4 °C for 75 min. The pellet was resuspended in the same buffer and used for the detection of GLUT4 content in a total membrane fraction. Thirty micrograms of protein were subjected to SDS–PAGE and electrotransferred onto nitrocellulose membrane. The membrane was blocked in PBS containing 8% (w/v) dried milk and probed with polyclonal anti-GLUT4 antibody (Chemicon, Temecula, CA, USA; 1:4000 in PBS containing 8% (w/v) BSA), followed by HRP-linked anti-rabbit immunoglobulin (Amersham Biosciences).

The intensity of chemiluminescence for the corresponding blots was quantified by densitometry (Image-Scanner III; GE Healthcare, Uppsala, Sweden). GLUT4 content was expressed in arbitrary units per microgram of applied protein, considering the mean of control animals 100%. Protein-loaded content was confirmed by analyzing posttransferring Coomassie-stained gels (Ferguson et al. 2005).

**Real-time PCR analysis**

Total RNA (1 µg) was extracted from cells using Trizol (Invitrogen Life Technologies). RNA was further treated with DNase I (Invitrogen) and reverse transcribed (Promega). cDNA amplification was performed using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) in the Rotor Gene 3000 (Corbett Research, Sydney, NSW, Australia). Real-time PCR conditions were 50 °C for 2 min, 95 °C for 2 min, then 40 cycles at 95 °C for 20 s, followed by 60 °C for 1 min and 72 °C for 15 s (extension). To amplify mouse Slc2a4 the following primer pairs were used: 5'-CTG-TGCCATCTTGTAGACCGTG-3' forward (fw) and 5'-GTTGGAGAAACCAGCGCAAG-3' reverse (rv). Primers for mouse Srebf1 type a (fw: 5'-TAGTCGAAG-CCGGGTGGCAGGCGCAGG-3', rv: 5'-GATGTCGTCTAAAACCGCTGTGTCG3'), Srebf1 type c (fw: 5'-ATCGGGCGGAAGCCTGTCCGGAGCTG-3', rv: 5'-ACTGTCTTGGTTGTGCAGGCTAG-3').
and mouse Cnr1 (fw: 5′-TGGTTCATCTGCTGTGGTTGTGTTTGAAGCG, rv: 5′-TTCAGCAGGAGACATCAGACTACAGA-3′) were previously described (Shimomura et al. 1997, Belloccio et al. 2008). As housekeeping genes, the following primer pairs were used: mouse 36b4 (Rplp0) (fw: 5′-GAGGAAATCGATGAGGATATGGGA-3′, rv: 5′-AAAGAACCCAGTAGACTCCACCGA-3′) (Dalen et al. 2003) and mouse Gapdh (fw: 5′-GAAGGCGGTGTGAACGGATT-3′, rv: 5′-AAGACACCAGTAGACTCCACCGA-3′) (Furuya et al. 2010).

Electrophoretic mobility shift assay

Extraction of nuclear protein was performed as previously reported (Silva et al. 2005), and the obtained nuclear extract supernatant was stored at −80 °C. The following double-stranded oligonucleotides were end labeled using T4 Polynucleotide Kinase (Invitrogen) and [γ-32P]ATP (Amersham): Slc2a4–nuclear factor (NF)-κB (−83), GTGAAATCAGATGAGGATATGGGA, corresponding to the −83 to −62 bp κB site of Slc2a4 gene 5′-flanking sequence, Slc2a4–NF-κB (−134), GGGTGGGGCGTGCTGGTTGGGG, corresponding to −134 to −113 bp κB site of Slc2a4 gene 5′-flanking sequence, and Slc2a4-sterol regulatory element-binding protein (SREBP1), GCCCTTTGCGGTGGTGCCTTG, containing the sterol response element (SRE) in the promoter region of Slc2a4. Nuclear proteins were bound to labeled oligonucleotide probe in a final binding buffer (60 mmol/l HEPES, pH 7.6, 150 mmol/l KCl, 10% glycerol, 0.6 mmol/l EDTA, 1.93 mg/ml BSA, 2.3 mmol/l dithiothreitol, and 0.25 μg/μl polydeoxyinosinic-deoxyctydilic acid (poly[dI-dC]; Amersham)) for 20 min at room temperature. Competitive binding assays were conducted under the same conditions, with the addition of an excess of unlabeled competitor oligonucleotides. For confirmation of specific SREBP-1 binding, 2 μg of SREBP-1 antibody (Santa Cruz Biotechnology, Inc.) were added to the final mixture. The DNA–protein complexes were electrophoresed on a 4% nondenaturing polyacrylamide gel at 4 °C in 45 mmol/l Tris, 45 mmol/l borate, and 1 mmol/l EDTA buffer. The gels were dried and subjected to autoradiography. The blots were analyzed by scanner densitometry and the results of the binding activity were expressed as arbitrary units.

Results

Acute and chronic CB1 receptor antagonisms upregulate Slc2a4 expression

To assess the role of CB1 receptor on Slc2a4 gene expression, 3T3-L1 adipocytes were treated either acutely (Fig. 1) or chronically (Fig. 2) with CB1 receptor agonist (ACEA) and/or CB1 receptor antagonist/inverse agonist (AM251). For all mRNA analysis, 36b4 mRNA was used for normalization. The housekeeping gene Gapdh was also tested, but its expression showed alteration with the treatment (data not shown), probably related to reduction in oxidative stress induced by CB1R antagonism (Comelli et al. 2010), pointing out that oxidative stress could increase Gapdh mRNA expression (Ito et al. 1996).

Acute treatment consisted of 2 h for mRNA (Fig. 1A) and 4 h for protein analysis (Fig. 1B). Acutely, ACEA alone affected neither Slc2a4 mRNA nor GLUT4 protein expression; however, AM251 alone at all concentrations resulted in a massive upregulation of Slc2a4 mRNA and GLUT4 protein. Furthermore, the coincubation of ACEA with AM251 (0.5 and 1 μmol/l) also significantly upregulated the expression of Slc2a4 mRNA and GLUT4 protein. Similarly, chronic (24 h) coincubation of ACEA with 0.5 or 1 μmol/l AM251 and all concentrations of AM251 alone resulted in the upregulation of Slc2a4 mRNA (Fig. 2A) and GLUT4 protein (Fig. 2B). Interestingly, 24-h incubation with AM251 in the presence of ACEA revealed a clear dose–response effect; 0–1 mmol/l of AM251 correlated with both Slc2a4 mRNA (Pearson r=0.9569, P<0.05) and GLUT4 protein (Pearson r=0.9606, P<0.05) content. Furthermore, the results (Fig. 2B) show that ACEA exerted an inhibitory effect on the GLUT4 protein expression (P<0.001, C vs Ac1 and P<0.05, Ac1 vs Ac1/Am0.1, Student’s t-test).

Acute CB1 receptor antagonism upregulates Cnr1

The CB1 receptor is encoded by Cnr1 gene. Acute incubation with ACEA did not change Cnr1 expression in adipocytes (Fig. 3A). However, acute treatment with 0.5 μmol/l AM251 either with or without ACEA upregulated Cnr1 gene expression (P<0.05 vs control and ACEA) (Fig. 3A). However, chronic CB1 agonist
promoter, two oligonucleotides containing specific kB sites in the mouse Slc2a4 promoter region were used: the −83 to −62 bp sequence, named Slc2a4–NF–kB (−83); and the −113 to −134 sequence, named Slc2a4–NF–kB (−134). Both Slc2a4–NF–kB (−83) and Slc2a4–NF–kB (−134) formed two complexes with nuclear proteins of 3T3-L1 adipocytes named complexes A and B (Fig. 4, lanes 2 and 6). Complex A often seems to be subdivided into complex A1 and A2. The oligonucleotides’ binding specificity was checked by competition with their respective unlabeled oligonucleotides. The addition of 100-fold molar excess of the unlabeled specific competitor drastically reduced

or antagonist treatment did not impact the Cnr1 mRNA content (Fig. 3B).

**Chronic and acute CB1 antagonism decreases NF–kB binding activity**

To further investigate the mechanisms involved in Slc2a4 upregulation by the CB1 receptor, an electrophoretic mobility shift assay (EMSA) was performed. For the analysis of NF–kB binding to the Slc2a4

**Figure 1** Effect of acute ACEA and/or AM251 treatment on Slc2a4 mRNA and GLUT4 protein content. 3T3-L1 adipocytes were treated with vehicle (control, C) or 1 μmol/l ACEA (Ac1) and/or 0-1, 0-5, and 1 μmol/l AM251 (Am0-1, Am0-5, and Am1, respectively) for either 2 h (for mRNA analysis) or 4 h (for protein analysis). (A) Relative values of Slc2a4 mRNA content. 36b4 gene was used for normalization. (B) Top: image of a typical experiment; and bottom: relative value of GLUT4 total protein (30 μg protein). Results represent (A) three to five and (B) three independent experiments. (A) $P=0.0063$ and (B) $P=0.0025$, (B) Kruskal–Wallis. $*P<0.05$, $**P<0.01$, $***P<0.001$ vs C; $†P<0.01$, $‡P<0.001$ vs Ac1; $§§§P<0.001$ vs Ac1/Am0-1, $†P<0.05$, $††P<0.01$ vs Ac1/Am0-5, $‡‡P<0.01$, $‡‡‡P<0.001$ vs Ac1/Am1, ANOVA (Student–Newman–Keuls).

**Figure 2** Effect of chronic ACEA and/or AM251 treatment on Slc2a4 mRNA and GLUT4 protein content. 3T3-L1 adipocytes were treated with vehicle (control, C) or 1 μmol/l ACEA (Ac1) and/or 0-1, 0-5, and 1 μmol/l AM251 (Am0-1, Am0-5, and Am1, respectively) for 24 h. (A) Relative values of Slc2a4 mRNA. 36b4 was used for normalization. (B) Top: image of a typical experiment; and bottom: relative value of GLUT4 total protein (30 μg protein). Results represent (A) three and (B) four independent experiments. (A) $P=0.0453$ and (B) $P=0.008$, Kruskal–Wallis. $*P<0.05$, $**P<0.01$, $***P<0.001$ vs C; $‡‡P<0.01$, $‡‡‡P<0.001$ vs Ac1; $§P<0.01$, $§§P<0.001$ vs Ac1/Am0-1,age 0-5, and Am1, ANOVA (Student–Newman–Keuls).
the formation of the A1, A2, and B complexes for both oligonucleotides (Fig. 4, lane 3 for Slc2a4–NF-κB (K83) and lane 5 for Slc2a4–NF-κB (K134)). Neither ACEA nor AM251 altered the Slc2a4–NF-κB binding activity in both acute (Fig. 5A) and chronic (Fig. 6A) treatments. Inversely, 0.5 mmol/l AM251 alone acutely (Fig. 5B) and chronically (Fig. 6B) reduced NF-κB binding to the Slc2a4–NF-κB (K134) oligonucleotide. Besides, ACEA chronic treatment increased NF-κB binding to Slc2a4–NF-κB (K134), which was reversed by the addition of 0.5 mmol/l AM251 (Fig. 6B), reinforcing the inhibitory effect of AM251.

Figure 3 Effect of acute and chronic ACEA and/or AM251 treatment on Cnr1 (CB1 receptor) mRNA content. 3T3-L1 adipocytes were treated with vehicle (control, C) or 1 μmol/l ACEA (Ac1) and/or 0.5 μmol/l AM251 (Am0.5) for either (A) 2 h or (B) 24 h and the relative values of Cnr1 mRNA content were calculated. 36b4 gene was used for normalization. Results represent (A) five and (B) four independent experiments. (A) P=0.0119, Kruskal–Wallis. *P<0.05 vs C, **P<0.05 vs Ac1, one-way ANOVA (Student–Newman–Keuls).

Neither ACEA nor AM251 altered the Slc2a4–NF-κB (−83) binding activity in both acute (Fig. 5A) and chronic (Fig. 6A) treatments. Inversely, 0.5 μmol/l AM251 alone acutely (Fig. 5B) and chronically (Fig. 6B) reduced NF-κB binding to the Slc2a4–NF-κB (−134) oligonucleotide. Besides, ACEA chronic treatment increased NF-κB binding to Slc2a4–NF-κB (−134), which was reversed by the addition of 0.5 μmol/l AM251 (Fig. 6B), reinforcing the inhibitory effect of AM251.

Chronic but not acute CB1 antagonism increases Srebf1 type a expression and SREBP-1 activation

To address the hypothesis that SREBP-1 may be involved in Slc2a4 upregulation by CB1 receptor, the described SRE site of Slc2a4 promoter was utilized in EMSA experiments (Im et al. 2006). To examine the specificity of the nuclear protein binding into the labeled oligonucleotide containing the SRE binding site, a competition with unlabeled SRE oligonucleotide was performed (Fig. 7). The addition of 10-, 50-, or 100-fold molar excess of unlabeled oligonucleotide (Fig. 7, lanes 2, 3, and 4 respectively) banished the formation of the upper complex. Besides, to confirm the identity of the upper band DNA–protein complex, SREBP-1 antibody was added to the nuclear extracts. The formation of the upper complex was drastically reduced with the addition of SREBP-1 antibody (Fig. 7, lane 7), showing that the upper complex contains the SREBP-1 protein.

Figure 8A and B shows that 2-h treatment with either ACEA or AM251 did not change SREBP-1 binding to Slc2a4 promoter. Differently, 24-h treatment with
Results represent three to five independent experiments.

Discussion

It has been reported that endocannabinoid levels are elevated in the circulation and visceral adipose tissue of obese subjects (Engeli et al. 2005), and that the treatment with CB1 receptor antagonists improves insulin sensitivity (Ravinet Trillou et al. 2003). Inversely, in vitro studies revealed that CB1 receptor agonists improve acute insulin-induced glucose uptake in adipocytes (Gasperi et al. 2007, Pagano et al. 2007), leading to an apparent controversy. In the present study, we are clearly showing that the 24-h treatment of adipocytes with CB1 antagonist enhances Slc2a4/GLUT4 expression, a key protein for glucose uptake, thus explaining in vivo effects of the CB1 antagonists. Besides, the present study shows that Slc2a4 gene regulation by CB1 antagonism involves a transcriptional effect of both NF-κB and SREBP-1 in the promoter region.

The presently used AM251 is a structural analog of SR141716, with similar efficacy and potency (Hildebrandt et al. 2003) but with higher selectivity for CB1 receptor (Lan et al. 1999). The concentration of ACEA in our experiments (1 μmol/l) is in accordance with previous studies in adipocytes, which have verified the effects of CB1 agonists on glucose metabolism after 3 h (Pagano et al. 2007) or 2 h (Eckardt et al. 2009). Concentrations for the CB1 antagonists range from 0.1 to 10 μmol/l in the literature (Matias et al. 2006, Gasperi et al. 2009).

Figure 5 Effect of acute ACEA and/or AM251 treatment on NF-κB binding activity to the Slc2a4 promoter. 3T3-L1 adipocytes were treated with vehicle (control, C) or 1 μmol/l ACEA (Ac1) and/or 0.5 μmol/l AM251 (Am0.5) for 2 h. EMSA was performed as described in Materials and methods. Nuclear extracts of 3T3-L1 were incubated with the following radiolabeled probes: (A) Slc2a4–NF-κB (–83) or (B) Slc2a4–NF-κB (–134). Left: relative binding intensity; and right: image of a typical experiment. Results represent three to five independent experiments. (B) P=0.0391, Kruskal–Wallis. *P<0.05 vs C, †P<0.05 vs Ac1/Am0.5, one-way ANOVA (Student–Newman–Keuls).

0.5 μmol/l AM251 alone increased SREBP-1 binding activity (Fig. 8C and D).

To assess the possibility that CB1 antagonism might change Srebf1 expression, quantitative PCR was performed for Srebf1 type a (Fig. 9) and Srebf1 type c. Srebf1 type c was undetectable in 3T3-L1 cells (data not shown). Interestingly, Srebf1 type a was found to be upregulated by 0.5 μmol/l AM251 alone after the 24-h treatment (Fig. 9B). No effect on the Srebf1 type a mRNA was observed in the 2-h treated cells (Fig. 9A).

Figure 6 Effect of chronic ACEA and/or AM251 treatment on NF-κB binding to the Slc2a4 promoter. 3T3-L1 adipocytes were treated with vehicle (control, C) or 1 μmol/l ACEA (Ac1) and/or 0.5 μmol/l AM251 (Am0.5) for 24 h. EMSA was performed as described in Materials and methods. Nuclear extracts of 3T3-L1 were incubated with the following radiolabeled probes: (A) Slc2a4–NF-κB (–83) or (B) Slc2a4–NF-κB (–134). Left: relative binding intensity of NF-κB to Slc2a4 promoter; and right: image of a typical experiment. Results represent three independent experiments. (B) P=0.0151, Kruskal–Wallis. *P<0.05, **P<0.01, ***P<0.001 vs C, #P<0.001 vs Ac1, †††P<0.001 vs Ac1/Am0.5, one-way ANOVA (Student–Newman–Keuls).
Pagano et al. 2007, Eckardt et al. 2009), and we tested concentrations from 0.1 to 1 \( \text{mmol/l} \). Since clear effects on \( \text{Slc2a4} \)/GLUT4 expression were observed with the intermediary concentration of the antagonist AM251 (0.5 \( \text{mmol/l} \)), this was the chosen concentration for the experiments of transcriptional regulation.

Our findings showed acute (2–4 h) and chronic (24 h) upregulation of \( \text{Slc2a4} \) mRNA and GLUT4 protein by AM251-induced CB1 receptor antagonism/inverse agonism. Similarly, a chronic in vivo effect of CB1 antagonism on \( \text{Slc2a4} \) mRNA was described in adipose tissue of obese mice (Jbilo et al. 2005).

Interestingly, 24-h treatment with all concentrations of AM251 alone induced a maximal response of \( \text{Slc2a4} \)/GLUT4 expression, revealing a very sensitive inverse agonistic effect. It is known that the CB1 receptor, an inhibitory G protein-coupled receptor, has a constitutive activity (Howlett et al. 2002). Consequently, it is reasonable to suppose that AM251 acts as an inverse agonist by inhibiting the constitutive activity of CB1.

Differently, incubation with AM251 (0.1–1 \( \text{mmol/l} \)) in the presence of the agonist ACEA (1 \( \text{mmol/l} \)) showed a progressive dose–response enhancing effect on \( \text{Slc2a4} \)/GLUT4 gene expression, which characterizes a competitive antagonism. Additionally, it can be clearly observed that, in the 24-h treatment, the ACEA agonist exerts an inhibitory effect on GLUT4 protein expression.

On the whole, the present data clearly reveal that the inhibition of CB1 receptor activity in adipocytes increases \( \text{Slc2a4} \)/GLUT4 expression. Although the improvement of GLUT4 translocation and glucose uptake was attributed to CB1 agonists in isolated adipocytes (Gasperi et al. 2007, Pagano et al. 2007, Motaghedi & McGraw 2008), in vivo long-term treatment with CB1 antagonists improve peripheral glucose clearance (Jbilo et al. 2005, Van Gaal et al. 2008) which can be explained by the mechanisms we are reporting here.

Although 0.5 \( \text{mmol/l} \) AM251 acutely increased \( \text{Cnr1} \) mRNA expression, both the agonist and antagonist studied had no effect on \( \text{Cnr1} \) expression after 24-h incubation, pointing out that the 24-h effects observed in \( \text{Slc2a4} \)/GLUT4 expression were not related to changes in CB1R protein content. CB1R expression is known to increase during 3T3-L1 adipocyte differentiation (Gasperi et al. 2007, Pagano et al. 2007), but the

![Figure 7](image-url) Identification of nuclear proteins interaction with SRE site of \( \text{Slc2a4} \) promoter. EMSA was performed as described in Materials and methods. SRE radiolabeled probe was incubated in the absence (–), in lane 5, or in the presence, in other lanes, of 3T3-L1 nuclear extracts. For competition analysis, tenfold (lane 2), 50-fold (lane 3), or 100-fold (lane 4) excess amount of nonlabeled competitor was added. For identification of the formed complexes, SREBP-1 antibody was added to the binding mixture containing the nuclear extract (lane 7).

![Figure 8](image-url) Effect of acute and chronic ACEA and/or AM251 treatment on SREBP-1 binding activity to the \( \text{Slc2a4} \) gene promoter. 3T3-L1 adipocytes were treated with vehicle (control, C) or 1 \( \text{mmol/l} \) ACEA (Ac1) and/or 0.5 \( \text{mmol/l} \) AM251 (Am0.5) for either (A and B) 2 h or (C and D) 24 h. EMSA was performed as described in Materials and methods. Left: relative binding intensity of SREBP-1 to the \( \text{Slc2a4} \) promoter; and right: image of a typical experiment. Results represent three independent experiments. (C) \( P=0.0257, \) Kruskal–Wallis. **\( P<0.01 \) vs C. ###\( P<0.01 \) vs Ac1. ††\( P<0.01 \) vs Ac1/Am0.5, one-way ANOVA (Student–Newman–Keuls).
effects of the CB1 agonist or antagonist in mature adipocytes have never been reported. In immune cells, the cannabinoids stimulate CB1R expression, but in a CB2R-mediated pathway (Börner et al. 2007), remembering that the mature adipocyte does not express CB2 receptor (Gasperi et al. 2007).

The present data revealed that Slc2a4 upregulation by CB1 receptor antagonism in adipocytes starts rapidly (2 h) via NF-κB. Afterward, in addition to NF-κB, SREBP-1 also participates (24 h). The regulation of the activity of these transcriptional factors in adipocytes by CB1 receptor has never been reported before.

Considering that NF-κB represses the Slc2a4 gene (Ruan et al. 2002, Silva et al. 2005, Furuya et al. 2010), it is reasonable that the blockade of the CB1 receptor should enhance Slc2a4 gene expression by decreasing the binding activity of NF-κB. Two molecular complexes (Silva et al. 2005), as reported here, or more (Ruan et al. 2002), have been described in the literature. This is a consequence of the distinct homo/heterodimers that can be formed from the various isoforms of NF-κB, which include RelA (p65), NF-κB1 (p50/p105), NF-κB2 (p52/p100), c-Rel, and RelB (Perkins 2007). Furthermore, coactivators and corepressors involved in different gene transcriptions and biological systems can form distinct molecular complexes. In the present study, both complexes A and B showed the same regulation (data not shown), and because of this we quantified the total activity (complex A plus complex B). Interestingly, the results reveal that only the binding to Slc2a4–NF-κB (−134) site is being regulated by CB1 activity. This fact can be a consequence of different nucleotides in the core of the Slc2a4–NF-κB domains (T for Slc2a4–NF-κB (−83) or G for Slc2a4–NF-κB (−134)) or different nucleotides that comprise the flanking sequences, resulting in impairment of the binding activity.

From another standpoint, it was found that 24 h blockade of the CB1 receptor increases the binding activity of SREBP-1, a positive regulator of Slc2a4 gene expression (Im et al. 2006), to the Slc2a4 promoter; thus, this finding contributes to the increased Slc2a4 mRNA expression observed in this condition.

The nature of Srebf1 is not well established yet. One study reported that SREBP-1 protein in 3T3-L1 cells is exclusively of SREBP-1a type (Shimomura et al. 1997). Another study demonstrated that in this same cell line SREBP-1 protein is mostly of SREBP-1c type (Kim et al. 1998). It is known that Srebf1 type a and Srebf1 type c mRNAs are transcribed from the same gene (Srebf1), differing in the first exon that is spliced to a second common exon. Here, Srebf1 type c expression was undetectable in 3T3-L1 cells, agreeing with a previous report (Shimomura et al. 1997), whereas Srebf1 type a expression was clearly increased in cells treated for 24 h with AM251 alone. In this manner, this finding suggests that SREBP-1a may have an important participation in CB1-mediated Slc2a4 upregulation.

Considering that NF-κB activity is increased in obesity, a known inflammatory condition (Cancelli et al. 2005, Furuya et al. 2010), and that CB1 antagonism reduces body weight and adipocyte size (Jbilo et al. 2005, Pi-Sunyer et al. 2006, van Gaal et al. 2008), inflammatory activity should decrease via the CB1 receptor. Then, it is expected that NF-κB expression should decrease by the action of the CB1 receptor. As a matter of fact, the treatment of obese mice with the CB1 antagonist, SR141716, was reported to induce loss of body weight with reduction in adipocyte size and expression of some inflammatory markers (Jbilo et al. 2005). Therefore, both the expected decreased gene expression of NF-κB and its decreased transcriptional activity described here contribute to the reduction of Slc2a4 gene expression.
In summary, the present research contributes importantly to unravel the CBI role in the modulation of Slc2a4 gene expression in adipocytes. CBI antagonism/inverse agonism upregulates Slc2a4 gene expression and this occurs by decreasing NF-kB and increasing SREBP-1 control upon Slc2a4 transcriptional activity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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