Inhibition of cannabinoid CB1 receptor upregulates *Slc2a4* expression via nuclear factor-κB 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 (*Slc2a4* gene) expression by CB1 receptor. For this, 3T3-L1 adipocytes were incubated in the presence of a highly selective CB1 receptor agonist (1 μM arachidonyl-2′-chloroethylamide) and/or a CB1 receptor antagonist/inverse agonist (0·1, 0·5, or 1 μM 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) *Slc2a4*, *Cnr1* (CB1 receptor-encoded gene), and *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)-κB and sterol regulatory element-binding protein (SREBP)-1 specifically in the promoter of *Slc2a4* gene (electrophoretic mobility shift assay). Results revealed that both acute and chronic CB1 receptor antagonism greatly increased (∼2·5-fold) *Slc2a4* mRNA and protein content. Additionally, CB1-induced upregulation of *Slc2a4* was accompanied by decreased binding activity of NF-κB 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 *Slc2a4*/GLUT4 expression in adipocytes, a feature that involves NF-κB 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), cannabinoid 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.* 2005, Matias *et al.* 2006, Vettor & Pagano 2009). In this condition, insulin resistance is currently associated, and the pharmacological treatment of obesity with SR141716 (*N*-(piperidin-1-yl)-5-(4-chloro-phenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazo le-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 glycemia, and insulinemia (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 *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,

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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 S*lc2a4*/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′-chloroethyl-amide) 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 mmol/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 (ImageScanner 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 DNaseI (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-TGCCATCTTGATGACCGTG-3′ forward (fw) and 5′-GTTGGAGAAACCAGCGACAGC-3′ reverse (rv). Primers for mouse *Srebf1* type a (fw: 5′-TAGTCCGAAG-CCGGGTGGGCGCCGGCGCCAT-3′, rv: 5′-GATGTCG-TTCAAAACCGCTGTGTGTCCAGTTC-3′), *Srebf1* type c (fw: 5′-ATCGGCGCGG-AAGCTGTCGGGGTAGCGTC-3′, rv: 5′-ACTGTCTT-GGTTGTTGATGAGCTGGAGCAT-3′),

and mouse *Cnr1* (fw: 5′-TGGTTCTGATCCTGGTGGTG-TTG-3′, *rv*: 5′-TTCAGCAGGGAGAGCATACTACA-3′) were previously described (Shimomura *et al.* 1997, Bellocchio *et al.* 2008). As housekeeping genes, the following primer pairs were used: mouse *36b4* (*Rplp0*) (fw: 5′-GAGGAATCAGATGAGGATATGGGA-3′, rv: 5′-AA-GCAGGCTGACTTGGTTGC-3′) (Dalen *et al.* 2003) and mouse *Gapdh* (fw: 5′-GAAGGTCGGTGTGAACGGATT-3′, rv: 5′-AAGACACCAGTAGACTCCACGA-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), GTGAA**GGG**CG**T**GT**CC**TATGGCG, corresponding to the −83 to −62 bp κB site of *Slc2a4* gene 5′-flanking sequence, *Slc2a4*–NF-κB (−134), GGGTGG**GGG**CG**T**GG**CC**TTTTGG corresponding to −134 to −113 bp κB site of *Slc2a4* gene 5′-flanking sequence, and *Slc2a4*-sterol regulatory element-binding protein (SREBP1), GGCCTTTTGGGGTGTGCGGG, 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 polydeoxyinosinicdeoxycytidylic 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.

Statistical analysis

All data are reported as mean ± S.E.M. of three to five different experiments, each one assayed in duplicate. The data were first analyzed by the non-parametric one-way ANOVA using the Kruskal–Wallis test. Subsequently, the means of the groups were compared by ANOVA, Student–Newman–Keuls as a *post hoc* test. When applicable, Student's *t*-test was used. Pearson's correlation was used to confirm AM251 and *Slc2a4*/GLUT4 expression dose–response effect.

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 $r=0{\cdot}9569$, $P<0{\cdot}05$) and GLUT4 protein (Pearson $r=0{\cdot}9606$, $P<0{\cdot}05$) content. Furthermore, the results (Fig. 2B) show that ACEA exerted an inhibitor effect on the GLUT4 protein expression ($P<0{\cdot}001$, C vs Ac1 and $P<0{\cdot}05$, 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 ($P<0{\cdot}05$ vs control and ACEA) (Fig. 3A). However, chronic CB1 agonist

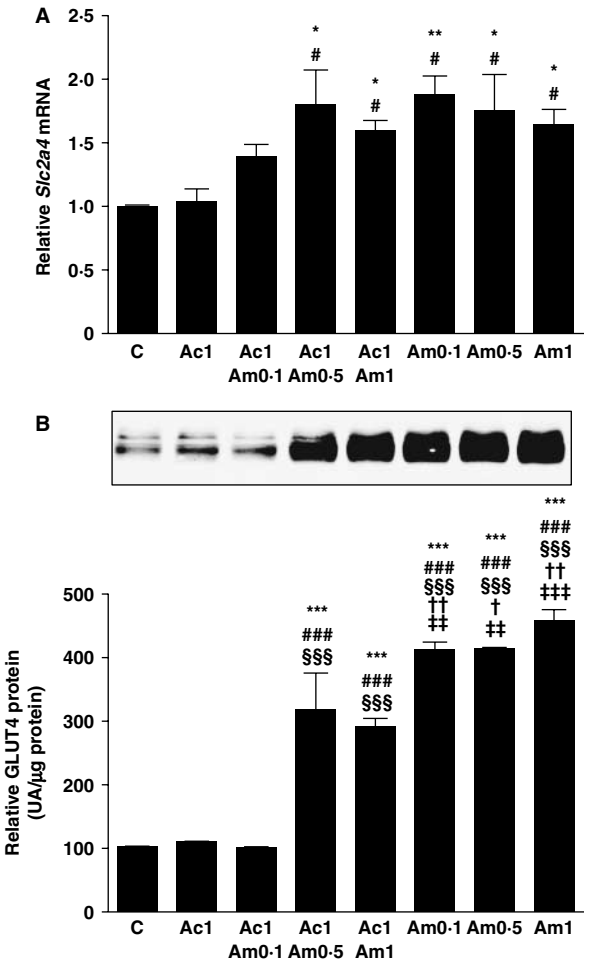

Figure 1 Effect of acute ACEA and/or AM251 treatment on *Slc2a4* mRNA and GLUT4 protein content. 3T3-L1 adipocytes were treated with vehicle (control, C) or 1 μmol/l ACEA (Ac1) and/or 0·1, 0·5, and 1 μmol/l AM251 (Am0·1, Am0·5, and Am1 respectively) for either 2 h (for mRNA analysis) or 4 h (for protein 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) $P=0{\cdot}0063$ and (B) $P=0{\cdot}0025$, (B) Kruskal–Wallis. $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$, $^{***}P<0{\cdot}001$ vs C; $^{\#}P<0{\cdot}01$, $^{\#\#\#}P<0{\cdot}001$ vs Ac1; $^{\S\S\S}P<0{\cdot}001$ vs Ac1/Am0·1, $^{\dagger}P<0{\cdot}05$, $^{\dagger\dagger}P<0{\cdot}01$ vs Ac1/Am0·5, $^{\ddagger\ddagger}P<0{\cdot}01$, $^{\ddagger\ddagger\ddagger}P<0{\cdot}001$ vs Ac1/Am1, ANOVA (Student–Newman–Keuls).

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

Chronic and acute CB1 antagonism decreases NF-κB 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-κB binding to the *Slc2a4* promoter, two oligonucleotides containing specific kB sites in the mouse *Slc2a4* promoter region were used: the −83 to −62 bp sequence, named *Slc2a4*–NF-κB (−83); and the −113 to −134 sequence, named *Slc2a4*–NF-κB (−134). Both *Slc2a4*–NF-B (−83) and *Slc2a4*–NF-B (−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

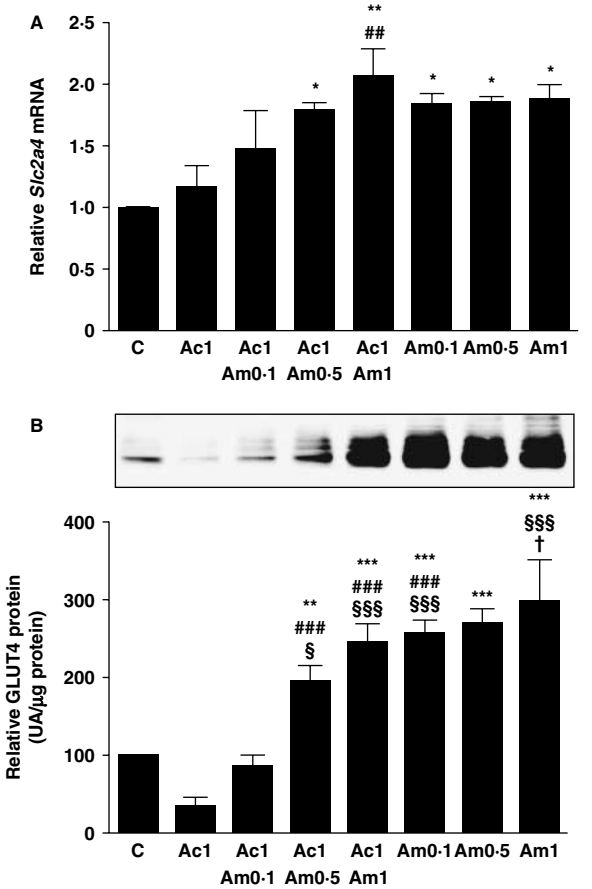

Figure 2 Effect of chronic ACEA and/or AM251 treatment on *Slc2a4* mRNA and GLUT4 protein content. 3T3-L1 adipocytes were treated with vehicle (control, C) or 1 μmol/l ACEA (Ac1) and/or 0·1, 0·5, and 1 μmol/l AM251 (Am0·1, Am0·5, and Am1 respectively) for 24 h. (A) Relative values of *Slc2a4* mRNA. *36b4* 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) $P=0{\cdot}0453$ and (B) $P=0{\cdot}008$, Kruskal–Wallis. $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$, $^{***}P<0{\cdot}001$ vs C; $^{\#\#}P<0{\cdot}01$, $^{\#\#\#}P<0{\cdot}001$ vs Ac1; $^{\S}P<0{\cdot}01$, $^{\S\S\S}P<0{\cdot}001$ vs Ac1/Am0·1, $^{\dagger}P<0{\cdot}05$ one-way ANOVA (Student–Newman–Keuls).

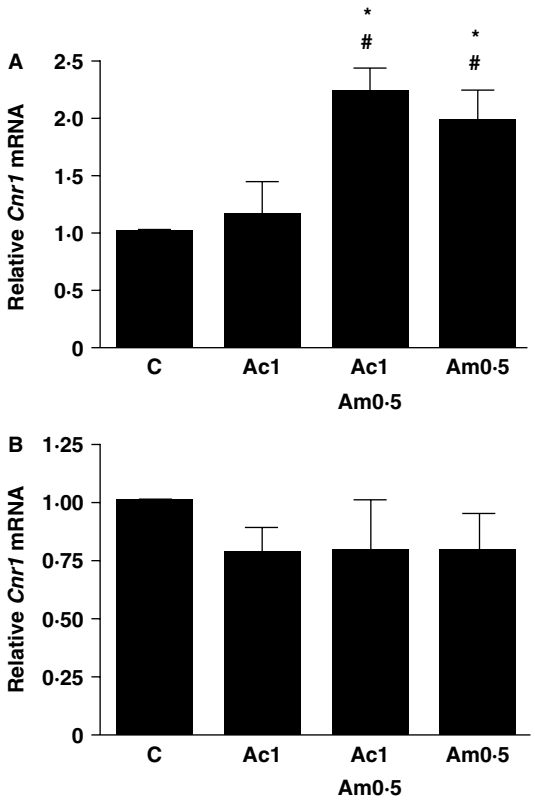

Figure 3 Effect of acute and chronic ACEA and/or AM251 treatment on *Cnr1* (CB1 receptor) mRNA content. 3T3-L1 adipocytes were treated with vehicle (control, C) or 1 μmol/l ACEA (Ac1) and/or 0·5 μmol/l AM251 (Am0·5) for either (A) 2 h or (B) 24 h and the relative values of *Cnr1* mRNA content were calculated. *36b4* gene was used for normalization. Results represent (A) five and (B) four independent experiments. (A) $P=0{\cdot}0119$, Kruskal–Wallis. $^{*}P<0{\cdot}05$ vs C, $^{\#}P<0{\cdot}05$ vs Ac1, one-way ANOVA (Student–Newman–Keuls).

the formation of the A1, A2, and B complexes for both oligonucleotides (Fig. 4, lane 3 for *Slc2a4*–NF-B (−83) and lane 5 for *Slc2a4*–NF-B (−134)).

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

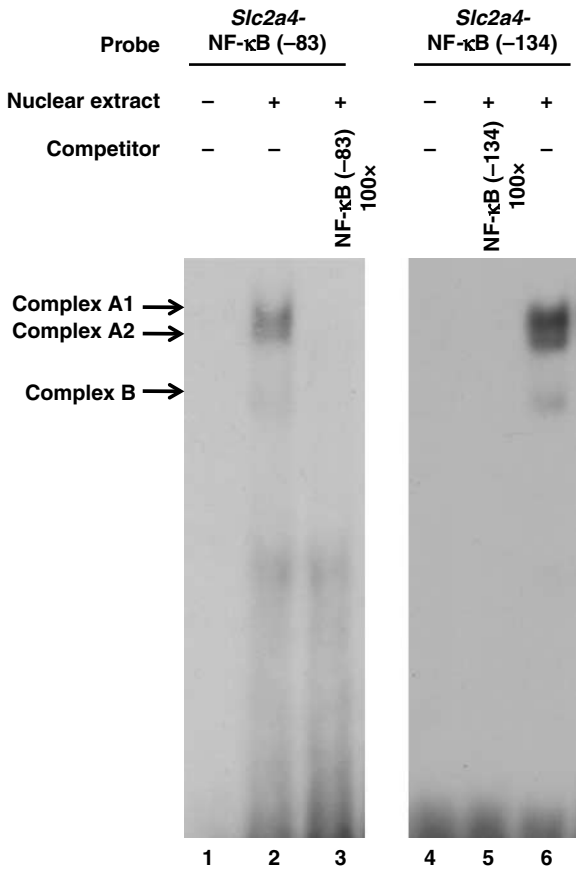

Figure 4 Identification of nuclear proteins interacting with the NF-κB site of the mouse *Slc2a4* promoter in 3T3-L1 cells. EMSA was performed as described in Materials and methods. Nuclear extracts of 3T3-L1 adipocytes (lanes 2, 3, 5, and 6) were incubated with the following radiolabeled probes: *Slc2a4*–NF-κB (−83), in lanes 1, 2, and 3; and *Slc2a4*–NF-κB (−134), in lanes 4, 5, and 6, in the absence (−) (lanes 2 and 6) or the presence of an excess amount (100-fold) of nonlabeled competitors.

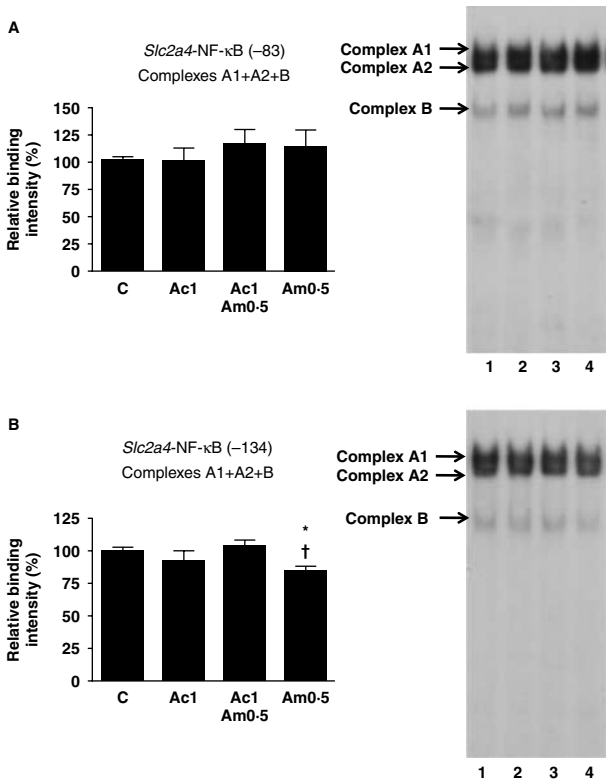

Figure 5 Effect of acute ACEA and/or AM251 treatment on NF-κB binding activity to the *Slc2a4* promoter. 3T3-L1 adipocytes were treated with vehicle (control, C) or 1 μmol/l ACEA (Ac1) and/or 0·5 μmol/l AM251 (Am0·5) for 2 h. EMSA was performed as described in Materials and methods. Nuclear extracts 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) $P=0{\cdot}0391$, Kruskal–Wallis. $^{*}P<0{\cdot}05$ vs C, $^{\dagger}P<0{\cdot}05$ 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).

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,

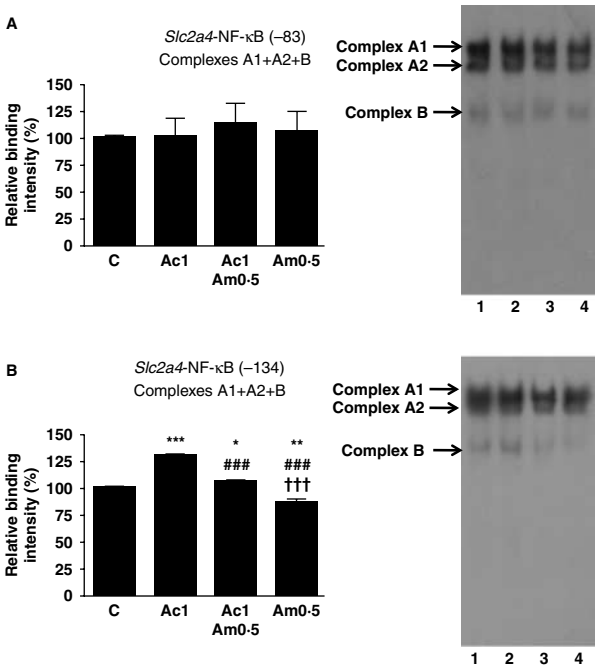

Figure 6 Effect of chronic ACEA and/or AM251 treatment on NF-κB binding to the *Slc2a4* promoter. 3T3-L1 adipocytes were treated with vehicle (control, C) or 1 μmol/l ACEA (Ac1) and/or 0·5 μmol/l AM251 (Am0·5) for 24 h. EMSA was performed as described in Materials and methods. Nuclear extracts of 3T3-L1 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) $P=0{\cdot}0151$, Kruskal–Wallis. $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$, $^{***}P<0{\cdot}001$ vs C, $^{\#\#\#}P<0{\cdot}001$ vs Ac1, $^{\dagger\dagger\dagger}P<0{\cdot}001$ vs Ac1/Am0·5, one-way ANOVA (Student–Newman–Keuls).

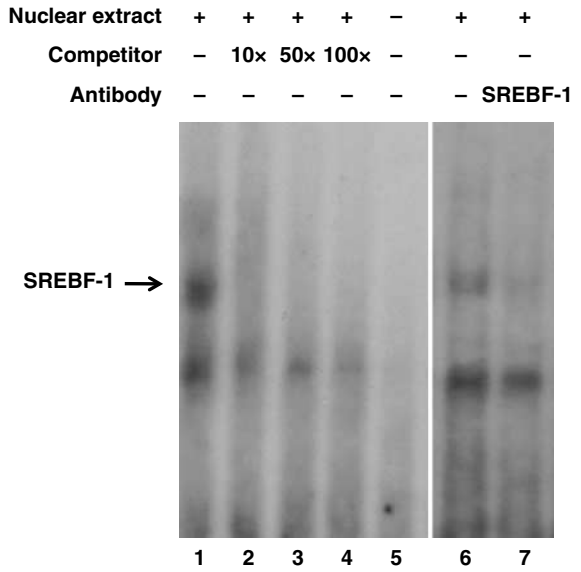

Figure 7 Identification of nuclear proteins interaction with SRE site of *Slc2a4* promoter. EMSA was performed as described in Materials and methods. SRE radiolabeled probe was incubated in the absence (−), in lane 5, or in the presence, in other lanes, of 3T3-L1 nuclear extracts. For competition analysis, 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).

Pagano *et al.* 2007, Eckardt *et al.* 2009), and we tested concentrations from 0·1 to 1 μmol/l. Since clear effects on *Slc2a4*/GLUT4 expression were observed with the intermediary concentration of the antagonist AM251 (0·5 μmol/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 *Slc2a4* mRNA and GLUT4 protein by AM251-induced CB1 receptor antagonism/inverse agonism. Similarly, a chronic *in vivo* effect of CB1 antagonism on *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 *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 μmol/l) in the presence of the agonist ACEA (1 μmol/l) showed a progressive dose–response enhancing effect on *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 *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) and GLUT4 protein expression (Jbilo *et al.* 2005), which can be explained by the mechanisms we are reporting here.

Although 0·5 μmol/l AM251 acutely increased *Cnr1* mRNA expression, both the agonist and antagonist studied had no effect on *Cnr1* expression after 24-h incubation, pointing out that the 24-h effects observed in *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

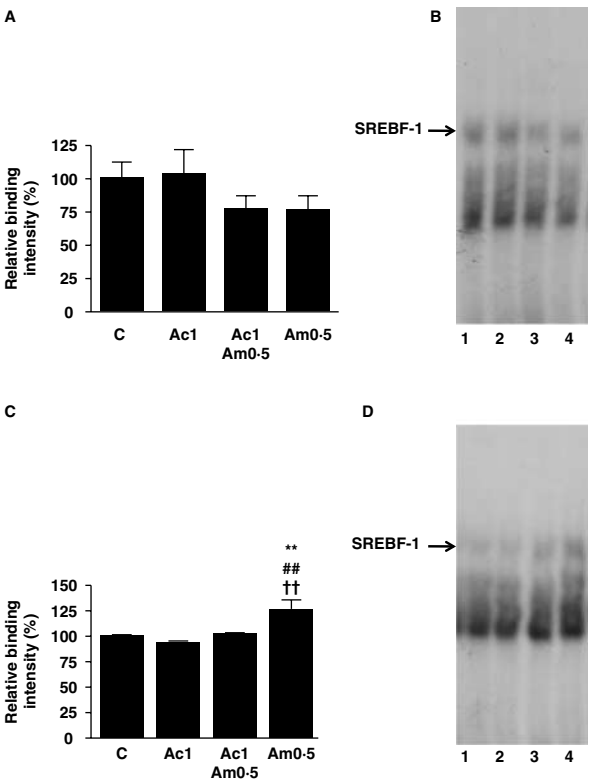

Figure 8 Effect of acute and chronic ACEA and/or AM251 treatment on SREBP-1 binding activity to the *Slc2a4* gene promoter. 3T3-L1 adipocytes were treated with vehicle (control, C) or 1 μmol/l ACEA (Ac1) and/or 0·5 μmol/l AM251 (Am0·5) for either (A and B) 2 h or (C and D) 24 h. EMSA was performed as described in Materials and methods. Left: relative binding intensity of SREBP-1 to the *Slc2a4* promoter; and right: image of a typical experiment. Results represent three independent experiments. (C) $P=0{\cdot}0257$, Kruskal–Wallis. $^{**}P<0{\cdot}01$ vs C, $^{\#\#}P<0{\cdot}01$ vs Ac1, $^{\dagger\dagger}P<0{\cdot}01$ vs Ac1/Am0·5, one-way ANOVA (Student–Newman–Keuls).

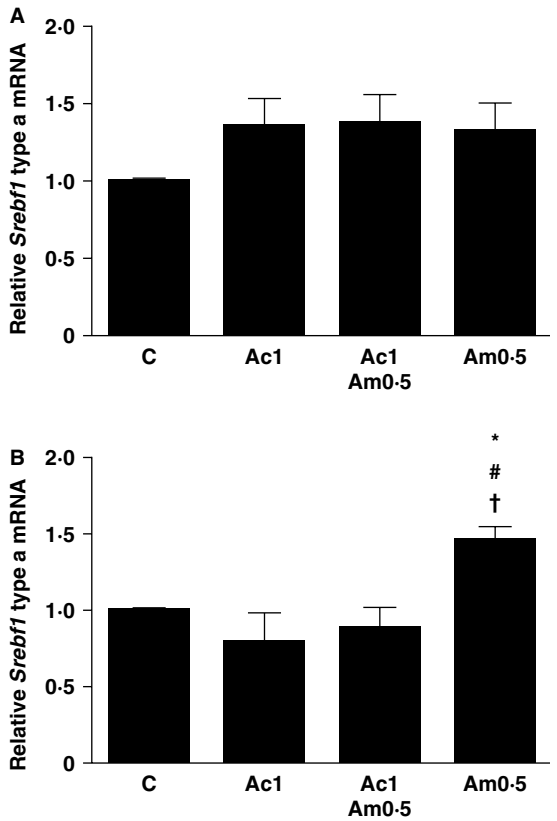

Figure 9 Effect of acute and chronic ACEA and/or AM251 treatment on *Srebf1* type a mRNA content. 3T3-L1 adipocytes were treated with vehicle (control, C) or 1 μmol/l ACEA (Ac1) and/or 0·5 μmol/l AM251 (Am0·5) for either (A) 2 h or (B) 24 h and the relative values of *Srebf1* type a mRNA were calculated. *36B4* gene was used for normalization. Results represent four independent experiments. (B) $P=0{\cdot}0323$, Kruskal–Wallis. $^{*}P<0{\cdot}05$ vs $^{\#}P<0{\cdot}05$ vs Ac1, $^{\dagger}P<0{\cdot}05$ 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 (Cancello *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 CB1 role in the modulation of *Slc2a4* gene expression in adipocytes. CB1 antagonism/inverse agonism upregulates *Slc2a4* gene expression and this occurs by decreasing NF-κB 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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