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The cannabinoid CB1 receptor antagonist Rimonabant stimulates 2-deoxyglucose uptake in skeletal muscle cells by regulating the expression of phosphatidylinositol-3-kinase.

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Running Title: Effect of Rimonabant in skeletal muscle cells.

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ABBREVIATIONS: 2-AG, 2-arachidonoyl-glycerol; 2-DG, 2-deoxyglucose; AEA, anandamide (*N*-arachidonoylethanolamine); Akt/PKB, Protein Kinase B; BSA, bovine serum albumin; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; CHX, cycloheximide ; CREB, cAMP response element-binding; DMEM, Dulbecco's modified Eagle's medium; ECL, Enhanced ChemiLuminescence; ECS, Endocannabinoid system; ERKs, Extracellular signal regulated kinases; GLUT, glucose transporter; IRS, insulin receptor substrate; IRTX, iodoresinatoxin; PBS, Phosphate buffered saline; PeSt, Penicillin-Streptomycin; PKCζ, Protein kinase C ζ; PDK-1, Phosphoinositide-dependent kinase-1; PI3K, phosphatidylinositol-3-kinase; PKA, Protein kinase A; PTEN, phosphatase and tensin homolog; siRNA, small interfering RNA; SREBP-1c , sterol regulatory element binding protein 1c; T2D, Type 2 Diabetes; TBS, Tris-buffered saline; THC, Δ^9-tetrahydrocannabinol; TRPV1, Transient receptor potential vanilloid 1.

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ABSTRACT

The endocannabinoid system regulates food intake, energy and glucose metabolism at both central and peripheral level. We have investigated the mechanism by which it may control glucose uptake in skeletal muscle cells. Detectable levels of the cannabinoid receptor type 1 (CB1) were revealed in L6 cells. Exposure of differentiated L6 myotubes to the CB1 antagonist Rimonabant (SR141716) selectively increased 2-deoxyglucose uptake (2-DG) in a time- and dose-dependent manner. A similar effect was induced by genetic silencing of CB1 by siRNA. Protein expression profiling revealed that both the regulatory p85 and the catalytic p110 subunits of the phosphatidylinositol-3-kinase (PI3K) were increased by SR141716. No significant change in the cellular content of other known molecules regulating PI3K was observed. However, PDK-1, Akt/PKB and PKCζ activities were rapidly induced following SR141716 treatment of L6 cells in a PI3K-dependent manner. The stimulatory effect of SR141716 on PI3K expression and activity was largely prevented by H-89, an inhibitor of the cAMP-dependent protein-kinase. Moreover, SR141716-stimulated 2-DG uptake was blunted by the co-incubation either with H-89 or with the PI3K inhibitor LY294002, both in L6 cells and in mouse primary myocytes. Thus, modulation of CB1 regulates glucose uptake at the level of the PI3K signalling system in skeletal muscle cells. Interfering with CB1 signalling may therefore ameliorate gluco-regulatory functions in peripheral tissues.

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INTRODUCTION

Type 2 diabetes (T2D) is a genetically determined disorder, affecting over 150 million people worldwide. T2D is characterized by several metabolic defects, among which beta-cell secretory dysfunction and peripheral insulin resistance are considered as hallmarks of the disease in humans (Kahn, 2003). Common forms of T2D arise because of the progressive failure of endocrine pancreas to adequately cope with the increased insulin demand in insulin-resistant states (Lazar, 2005). In particular, obesity is thought to play a central role as a causative factor of insulin-resistance (Lazar, 2005). Moreover, genetic and functional abnormalities found in obese individuals show a certain degree of overlap with those detected in T2D patients, suggesting that common molecular events may contribute to the onset and/or the progression of both disorders. These defects may affect feeding behaviour, as well as energy expenditure and nutrient metabolism.

The endocannabinoid system, for instance, is an important regulator for all of those functions (Boyd 2006; Pagotto et al., 2006). Elevated levels of the endogenous cannabinoids (anandamide - AEA - and 2-arachidonoyl-glycerol - 2AG) have been found in obese individuals (Engeli et al. 2005; Osei-Hyiaman et al., 2005a and 2005b) and correlate with intra-abdominal adiposity (Cote et al, 2007). Both exogenous cannabinoids and endocannabinoids increase food intake and promote weight gain by activating the specific CB1 receptor (Jamshidi et al., 2001; Williams et al., 2002; Cota et al., 2003). Mice carrying the ablation of the CB1 gene are lean and resistant to diet-induced obesity (Ravinet-Trillou et al., 2004). It has recently been shown that rimonabant (SR141716), a CB1-receptor inverse agonist (Bouaboula et al., 1997), produces a marked and sustained decrease in body weight which is associated with favourable modifications in serum biochemical and lipid profiles (Poirier et al., 2005; Pi-Sunyer et al., 2006).

Chronic blockade of the CB1 receptor is also accompanied by reduced blood pressure and fasting glucose and insulin levels (Ravinet-Trillou et al., 2003; Poirier et al., 2005; Van Gaal et al., 2005). Nevertheless, whether these additional beneficial effects are merely the consequence of weight loss

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or also the result of peripheral actions of the CB1 targeted molecules is still unknown. It has been shown that CB1 receptors are expressed in several mammalian tissues relevant to insulin action (Engeli et al., 2005; Juan-Pico et al., 2006). For instance, pancreatic islets express functional cannabinoid receptors, which may regulate Ca2+ signals and insulin secretion (Juan-Pico et al., 2006). In the liver, the activation of CB1 increases de novo synthesis of fatty acids by activating the transcription factor sterol regulatory element binding protein 1c (SREBP-1c) (Osei-Hyiaman et al., 2005). Also, modulation of CB-1 activity in isolated mouse adipocytes increases the activity of the lipogenic enzyme lipoprotein lipase (Cota et al., 2003) and adiponectin expression (Bensaid et al., 2003). Very little information, however, is available concerning cannabinoid receptor function in the skeletal muscle. Recently, CB1 expression has been detected in soleus muscle (Pagotto 2006) and SR141716 has been shown to affect glucose uptake in the isolated soleus of genetically obese mice (Liu et al., 2005) and the expression of genes involved in oxidative metabolism (Cavuoto et al., 2007). However, the molecular mechanisms of CB1 action in skeletal muscle cells remain largely undefined.

We now show that, in a skeletal muscle cultured cell model, the L6 cells, pharmacological regulation of the endocannabinoid system controls glucose uptake at the level of the phosphatidylinositol-3-kinase (PI3K). In particular, the CB1 inverse agonist SR141716 selectively increases PI3K expression and activity in a protein-kinase A (PKA) -dependent manner. This, in turn, leads to changes of the PI3K downstream signalling and a consequent regulation of glucose uptake in the skeletal muscle cells.

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MATERIALS AND METHODS

General-Media, sera and antibiotics for cell cultures were from Invitrogen Ltd. (Paisley, United Kingdom). Phospho-Ser241 PDK1, phospho-Ser473 PKB, phospho-serine and phospho-Thr202/Tyr204 ERKs antibodies were purchased from Cell Signaling Technology (Beverly MA). Actin antibody was from Sigma (St. Louis, MO). Antibodies directed against CB1, GLUT1, GLUT4, ERKs, phospho-Thr410 PKCζ, PKCζ, p110, PTEN, PKB, CREB and phospho-Ser133 CREB were from Santa Cruz Biotechnology (Santa Cruz, Calif.). PDK1, IRS1, IRS2 and p85 antibodies were from Upstate Cell Signaling Technology (Lake Placid, NY). Electrophoresis and Western blot reagents were from Bio-Rad (Richmond, Va.). 2-deoxy-[^{14}C]-glucose and ECL reagents were from Amersham Biosciences (Arlington Heights, Ill.). Other reagents were from Sigma.

Cell Culture-The L6 skeletal muscle cells were plated (6x 10^3 cells/cm^2) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 2 mM glutamine. Cultures were maintained at 37 C, in a humidified atmosphere containing 5% (v/v) CO_2. L6 differentiation has been achieved as previously described (Caruso et al., 1997).

Mouse primary fibro-skeletal muscle cell culture-Skeletal muscle biopsies were obtained after C57 BL6 strain (n=9) mice were sacrificed by pentobarbitone overdose, as previously described (Kramer et al., 2007). The biopsies were collected in cold-buffered saline (PBS) supplemented with 1% PeSt (100 U/mL Penicillin, 100 µg/mL Streptomycin), dissected finely minced and transferred to a digestion solution (0.015 g Collagenase IV, 8% 10X trypsin, 0.015 g BSA, 1% PeSt, in DMEM supplemented with 10% fetal calf serum, 10000 U/mL Penicillin, 10 mg/mL Streptomycin and 2% L-Glutamine) and incubated with gentle agitation at 37 C for 15-20 min. Thereafter, undigested tissue was allowed to settle and the supernatant was collected and mixed with DMEM supplemented with 20% fetal calf serum, 1% PeSt. The remaining tissue was digested for a further 15-20 min at 37°C with fresh digestion solution. The resultant supernatant was then pooled with the

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previous cells and centrifuged for 10 min at 350 x g. The cell pellet was resuspended in DMEM supplemented with 20% fetal calf serum, 1% PeSt and was then seeded and grown in culture flask. After this, medium was again changed to DMEM supplemented with 10% fetal calf serum, 1% PeSt. Before any experiment, cells were incubated in serum-free media for 16 h, and stimulated with insulin or with SR141716 as indicated.

Cell Treatment-For all the experiments L6 cells and myocytes were incubated in serum-free media supplemented with 0.25% bovine serum albumin. Unless specified, different concentrations of SR141716 (0.05 μM, 0.1 μM, 0.3 μM, 1 μM, 10 μM) were simultaneously added to the media, as described in the figure legend. Similarly, 0.1 μM SR144528 and 0.3 μM iodoresinatoxin (IRTX) were added to the serum-free media for the indicated time. For PI3K inhibition studies, cells were pre-treated with 10 μM LY294002 for 30 min followed by further incubation with LY294002 and 0.1 μM SR141716 for additional 24 h. For early times, the cells were serum-starved for 16 h and then pre-treated with 10 μM LY294002 for 30 min, followed by further incubation with LY294002 and 0.1 μM SR141716 for additional 30 min. For PKA inhibition studies, cells were pre-treated with 15 μM H-89 for 30 min followed by combined treatment with H-89 and 0.1 μM SR141716 for additional 30 min or 24 h as indicated. To study SR141716 effect on protein synthesis, the cells were incubated with 40 μg/ml cycloheximide in presence of 0.1 μM SR141716 for 24 h.

Transient transfection-For knocking down CB1 expression, a 21-nucleotide small interfering RNA duplex (Dharmacon Research, Lafayette, CO) was used, designed for specific silencing of CB1 (siRNA-CB1), covering the sequence sense 5'-CCCAAGUGACGAAAAACAUU – dTdT – 3'. The cells were transfected using FuGENE (Roche) and 100 nM siRNA for each transfection, in accordance with the manufacturer's instruction. An equal concentration of Silencer Negative Control-1 siRNA (Ambion) was used as negative control. Transfected L6 cells were serum starved and incubated with 0.1 μM SR141716 and after 16 h assayed for 2-deoxy-D-glucose uptake.

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Specific silencing of CB1 gene was confirmed by RT-PCR and Western blot analysis for CB1 receptor.

Real-time PCR-Total RNA was isolated from 3T3 L1 pre-adipocytes and L6 cells by using the Rneasy Kit (Qiagen Sciences) according to the manufacturer's instruction. For real-time RT-PCR analysis, 1 μg cell RNA was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, Calif.). PCR were analyzed using SYBR Green mix (Invitrogen). Reactions were performed using Platinum SYBR Green Quantitative PCR Super-UDG using an iCycler IQ multicolour Real-Time PCR Detection System (Biorad Hercules, CA). All reactions were performed in triplicate and β-actin was used as an internal standards. Primer sequences used were as follows: CB1R sense primer 5'-CTA CTG GTG CTG TGT GTC ATC-3', antisense primer 5'-GCT GTC TTT ACG GTG GAA TAC-3' and β-actin forward 5'-GCGTGACATCAAAGAGAAG-3', β-actin reverse 5'-ACTGTGTTGGCATAGAGG-3'

Immunoblot Analysis and Immunoprecipitation Procedure-Cells were solubilized for 20 min at 4 C with lysis buffer containing 50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM sodium orthovanadate, 50 mM NaF, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.4, and 1% (v/v) Triton X-100 (TAT buffer). The lysates were clarified by centrifugation at 12,000 x g for 20 min at 4 C. Proteins were separated by SDS poly-acrylamide gel electrophoresis and blotted on Immobilon-P membranes (Millipore Corp., Bedford, MA). Membranes were blocked for 1h in TBS (10 mM Tris-HCl, pH 7.4, 140 mM NaCl), containing 4% (w/v) bovine serum albumin and then incubated with indicated antibodies. Detection of blotted proteins was performed by ECL according to the manufacturer's instruction. Immunoprecipitation experiments were performed as previously described (Formisano et al., 1998). Densitometric analysis was performed using a Scion Image Analyzer. All the data were expressed as mean ± SD. Significance was assessed by Student's t test for comparison between two means. Data were analyzed with Statview software (Abacusconcepts) by one-factor analysis of variance. P values of less than 0.05 were considered statistically significant.

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2-Deoxy-D-glucose Uptake-The measurement of 2-deoxy-D-[^{14}C] glucose uptake was taken as a readout of glucose uptake by muscle cells, as previously described (Klip et al., 1982). Cells were incubated in serum free Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.2% (w/v) bovine serum albumin for 18 h in the presence or absence of SR141716 at different concentrations. Cells were incubated in glucose-free 20 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $MgSO_4$, 5 mM KCl, 1 mM $CaCl_2$ (HEPES buffer) and exposed or not to 100 nM insulin for 30 min. Glucose uptake was measured by incubating cells with 0.15 mM 2-deoxy-D-[^{14}C]glucose (0.5 μCi/assay) for 15 min in HEPES buffer. The reaction was terminated by the addition of 10 μM cytochalasin B, and the cells were washed three times with ice-cold isotonic saline solution prior to lysis in 1 M NaOH. Incorporated radioactivity was measured in a liquid scintillation counter.

Measurement of intracellular cAMP-Cells were plated in 12-well plates at a density of $5x10^5$ cells per well. Cells were treated with 1 μM forskolin for 30', with 0.1 μM SR141716 for 24 h. After treatment, cells were washed twice with cold PBS, and intracellular cAMP was extracted by addition of 500 μl of 0.1 N HCl with 0.1% Triton, followed by three cycles of freezing and thawing. cAMP concentration was measured by radioimmunoassay (Perkin Elmer, Norwalk, CT) (Nikodemova et al., 2003).

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RESULTS

Effect of CB1 modulation on glucose uptake. In order to investigate whether the endocannabinoid system may operate in a skeletal muscle cell model we have measured the expression levels of the CB1 receptor by real-time RT-PCR and immunoblot experiments in the L6 myotubes (Fig.1). As control, we have used 3T3 L1 cells, in which CB1 expression has already been described (Yan et al., 2007). In L6 myotubes the CB1 mRNA (Fig. 1A) and protein (Fig. 1B, C) were expressed at similar levels to those detected in 3T3 L1 cells.

Next, 2-deoxy-glucose (2-DG) uptake was measured in the myotubes in the absence or in the presence of increasing concentrations of the CB1 receptor inverse agonist SR141716 (Fig. 2A). At variance, pre-incubation of L6 myotubes with 0.1 and 0.3 μM SR141716, respectively, led to 50% and 45% increases of 2-DG uptake ($p<0.001$), only slightly lower than that observed upon acute insulin stimulation (100 nM for 30 min). No effect was observed with 0.05 μM SR141716 (Fig. 2A). Raising SR141716 concentrations to 1 μM and 10 μM led to a progressive reduction of 2-DG uptake, however, consistent with a partial agonist effect. In addition, no significant effect was achieved following treatment of L6 cells with 0.1 μM SR144528 (a selective CB2 receptor antagonist) and 0.3 μM iodoresinatoxin (IRTX), a potent TRPV1 receptor antagonist (Fig.2A).

Time-course analysis revealed that the effect of 0.1 μM SR141716 was rapidly induced upon 30 min treatment and persisted up to 16 h (Fig. 2B) and longer (up to 72 h, data not shown).

Protein expression profile upon CB1 modulation. In order to address the mechanism by which CB1 may regulate glucose uptake in the L6 cells, protein lysates were obtained following treatment with 0.1 μM SR141716 for 16 h. Protein expression profiling was achieved by immunoblot with specific antibodies (Table I). The intracellular content of the regulatory (p85α) and the catalytic (p110α) subunits of class I PI3K was increased by 2.0 and 1.7-fold, respectively, upon SR141716 exposure (Table I and Fig. 3). No significant change, instead, was detected for the insulin receptor,

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IRS-1 and -2, phosphoinositide-dependent-kinase 1 and protein kinase C-ζ, as well as for the lipid phosphatase PTEN and glucose transporters GLUT-1 and -4 (Table I). The expression levels of protein kinase Bα/Akt1 were also increased by SR141716, although differences did not reach statistically significant values (Table I). We then evaluated the timing of PI3K regulation by CB1. The treatment of L6 cells with SR141716 for 5 h led to a slight increase of p85 expression, which raised up to 24 h and remained stable up to 72 h (Fig. 3C). No detectable change was observed upon 30 min exposure to SR141716. Very similar results were obtained with p110 (data not shown).

In order to assess whether CB1-mediated regulation of p85 expression occurred at the level of protein synthesis, L6 cells were treated with 40 μg/ml cycloheximide (CHX). At the baseline, CHX treatment reduced p85 cellular abundance (Fig. 4A and 4B). The treatment with SR141716 however was still able to increase p85 immuno-detection, indicating that regulation occurred at post-translational level. Similar results were obtained with p110 (data not shown). Consistent with this hypothesis CHX did not affect SR141716-induced 2-DG uptake (Fig. 4C).

It has been previously shown that CB1 receptor may regulate protein kinase A (PKA) activity (Bidaut-Russell et al., 1990). In order to investigate whether SR141716 may regulate PKA activity in the L6 cells, we have evaluated the phosphorylation of its substrate CREB. Immunoblot with specific antibodies revealed that SR141716 induced the phosphorylation of CREB on Ser133 (Fig. 5A and 5B). We have therefore tested whether PKA inhibition was able to revert SR141716 effect on p85. To this end, L6 cells were treated with H-89 (15 μM), a PKA inhibitor, in the absence or in the presence of SR141716. Interestingly, no increase of p85 cellular abundance was detected in cells treated with H-89 (Fig. 5A and 5B). To further clarify the hypothesis that SR141716 may regulate PKA activity, we have measured intracellular cAMP levels. Incubation of L6 myotubes with 0.1 μM SR141716 for 24 h led to a significant 5-fold increase of intracellular cAMP production ($p<0.001$), about 50% lower than that observed upon acute stimulation (30 min) with 1μM forskolin, a well known adenylyl cyclase stimulator (Fig. 5C). Moreover, we have investigated

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whether SR141716 stimulates p85 phosporylation via PKA. To this aim, L6 cells were pre-treated with H89 for 30 min and stimulated with SR141716 for additional 30 min. SR141716 treatment increased p85 serine phosphorylation and this effect is prevented by H89 pre-treatment (Fig. 6).

Regulation of PI3K signalling by CB1. Next, we investigated whether CB1 modulation affects signalling downstream PI3K. To this end, L6 cells have been treated with 0.1 μM SR141716. Immuno-detection of the phosphorylated forms of PDK1, Akt/PKB and PKCζ was taken as readout for PI3K activity (Fig. 7A and 7B). Indeed, these kinases represent downstream targets of PI3K (Hirsch et al., 2007). Treatment with SR141716 increased the phosphorylation of all these proteins. The positive effect of SR141716 on PDK1, Akt/PKB and PKCζ phosphorylation was already evident upon 30 min treatment and remained stable up to 24 h (Fig. 7A and 7B). L6 myotubes were therefore pre-treated with 10 μM LY294002, in order to block PI3K activity, or with 15 μM H89, in order to block PKA activity, and stimulated with 0.1 μM SR141716 for 30 min or for 24 h. In both conditions, SR141716 failed to induce phosphorylation of PDK1, Akt/PKB and PKCζ (Fig.8A and 8B). Also, the effect of SR141716 on 2-DG uptake was blunted following LY294002 and H89 pre-treatment (Fig. 9A). Moreover, we investigated the SR141716 effect on 2-DG uptake in primary myocytes. Treatment with 0.1 μM SR141716 for 30 min or for 24 h stimulated 2-DG uptake ($p<0.01$) by about 50%, at similar level to that observed upon acute insulin stimulation (100 nM for 30 min) (Fig.9B). Also, the effect of SR141716 on 2-DG uptake was reverted following LY294002 and H89 pre-treatment (Fig. 9B).

Effect of CB1 silencing on glucose uptake. Finally, we investigated whether CB1 silencing affects glucose uptake induced by SR141716. To this aim, L6 cells have been transfected with 100 nM siRNA-CB1, which specifically inhibited CB1 expression by about 80% (Fig. 10A). In untrasfected L6 cells SR141716 increased 2-DG uptake. Interestingly, the transfection of siRNA increased glucose uptake but abolished further increases induced by SR141716 (Fig.10B). Moreover, the

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transfection of silencer negative control siRNA did not modify 2-DG uptake induced by SR141716 treatment (Fig. 10B).

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DISCUSSION

The Endocannabinoid system (ECS) is a crucial regulator of several physiologic processes, including the control of energy balance (Pagotto, 2006; Osey-Hyiaman et al., 2006; Bifulco et al., 2007). Studies in genetically engineered murine models have, indeed, proven that removal of the CB1 receptor produces lean animals, with grossly modified feeding behaviour and increased energy consumption (Ravinet-Trillou et al., 2004). More recent evidence in humans has indicated that pharmacologic blockade of CB1 is accompanied by significant reduction of body weight and of plasma levels of cholesterol and triglycerides (Despres et al., 2005; Van Gaal et al., 2005). It also appears that ECS targeting reduces blood glucose levels (Hollander, 2007) and may directly regulate glucose metabolism in peripheral tissues. We have investigated the molecular mechanism by which CB1 exerts its modulatory action in the L6 cells, a well characterized model of differentiating skeletal muscle cells (Klip et al., 1982). Exposure of the myotubes to SR141716 significantly increased glucose uptake. No effect was elicited, instead, by SR144528 and IRTX, which are antagonists of CB2 and TRPV1, respectively. Genetic silencing of CB1 by siRNA, instead, elicited similar effects as SR141716. This is also in agreement with the recent observation that treatment of leptin-deficient obese mice with CB1 antagonists enhances glucose uptake by skeletal muscle (Liu et al., 2005). Accordingly, Cavuoto and co-workers have shown that CB1 agonists and antagonists modify the expression of genes regulating skeletal muscle oxidative pathways (Cavuoto et al., 2007). Altogether, these observations indicate that, in addition to its effect in the central nervous system (Matias et al., 2006), and similar as in liver cells and adipocytes (Teixeira-Clerc et al., 2006; Gasperi et al., 2007), ECS may directly modulate nutrient metabolism in the skeletal muscle.

Elevated levels of endocannabinoids, which have been found in obese animal models (Di Marzo et al., 2001) and humans (Cote et al., 2007), may enhance CB1 activity and interfere with glucose metabolism in muscle cells. Thus, it is conceivable that activation of CB1 may down-regulate

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glucose uptake. Consistently, either genetic silencing or pharmacologic interference up-regulates this function. However, whether SR141716 works as an antagonist, by inhibiting constitutive CB1 activity in the L6 cells, or as an inverse agonist, as largely recognized in other cell types (Bouaboula et al., 1997, Xie et al., 2007), is currently under investigation in our laboratories. It should be pointed out that SR141716 effect occurred in the absence of exogenous anandamide and 2-AG, suggesting an inverse agonist effect. However, autonomous cellular production of endocannabinoids can not be excluded, also raising the possibility of an antagonist effect of the compound. Intriguingly, higher concentrations of the compound produced a paradoxical decrease of 2-DG uptake. The latter effect is possibly due to the partial agonist activity of SR141716 (De Vry 2004; Krylatov 2005). One alternative explanation could be found in the up-regulation of CB1 occurring at micromolar concentrations of SR141716 in the L6 cells and in the primary myocytes (data not shown). However, this effect is most probably a feature of cultured cell systems, and it may not occur "*in vivo*", because of the drug turnover, mainly operated by liver metabolism (Padwal and Majumdar 2007).

As shown by protein expression profiling, the regulatory effect of CB1 on glucose uptake is not due to changes in cellular abundance of the main glucose transporters GLUT1 and GLUT4. The expression of major proteins involved in the early events of insulin action on glucose uptake was also unmodified upon CB1 pharmacological targeting. Several lines of evidence indicate that CB1 regulation of glucose uptake occurs through PI3K signalling. First, dose- and time-dependent increases of both the regulatory (p85) and the catalytic (p110) subunits were observed following treatment with low concentrations of SR141716. Second, these effects were paralleled by increased activity of several PI3K downstream molecules (PDK1, PKCζ and Akt/PKB). Third, the inhibition of PI3K activity counteracted the effect of SR141716 on glucose uptake.

Noteworthy, SR141716 effect occurred upon both short (30 min) and long term incubation. This is only partially consistent with the timing of up-regulation of the PI3K subunits by the CB1 antagonist compound.

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The molecular events involved in both short and long term CB1 regulation of PI3K, require PKA activity. Indeed, in parallel with p85 and p110 expression, SR141716 induces increases intracellular cAMP levels and CREB phosphorylation at a PKA consensus site. H89, a pharmacological PKA blocker, inhibits both CREB phosphorylation and PI3K signalling as well as SR141716-induced glucose uptake. It is currently unknown whether CREB transcriptional activity is involved in CB1-mediated regulation of glucose uptake. It has been reported that CB1 is coupled to $G_{i/0}$ proteins (Howlett et al., 1986). Then, engagement of CB1 by endogenous ligands causes inhibition of adenylate cyclase and reduction of cellular cAMP levels (Bidaut-Russell et al., 1990). SR141716 may uncouple CB1 from the inhibitory proteins and raise cAMP levels, with a consequent activation of PKA. PKA, in turn, regulates the expression of both p85 and p110, at least in part, at the post-translational level as indicated by the experiments in the presence of protein synthesis inhibitors.

However, the rapid stimulatory effect of SR141716 on PI3K activity and glucose uptake can not be accounted for by changes in the content of the PI3K subunits. CB1 modulation therefore may also either directly activate PI3K, independent of its expression, or regulate the activity of its downstream targets (i.e. Akt). This is consistent with the potential role of PKA to regulate PI3K activity in other cellular systems. It has been recently described that PKA phosphorylates p85 on Ser83 (Cosentino et al., 2007; De Gregorio et al., 2007). We have now shown that SR141716 increases serine phosphorylation of p85 in a PKA dependent manner. Therefore, it could be inferred that CB1 modulates PI3K by a dual mechanism: i) a short term mechanism, which directly stimulates PI3K phosphorylation and activation, and ii) a long term mechanism, mediated by the enhanced PI3K expression. Both effects are largely mediated by PKA activation.

Thus, at least in cultured cellular models, CB1 receptor exerts an inhibitory action on glucose uptake, which could be augmented by endocannabinoid stimulation. SR141716 removes the inhibitory constraint maintained by CB1 tonic activity and induces glucose uptake by cAMP/PKA- and PI3K-mediated pathways. Genetic silencing of CB1 in skeletal muscle cells further supports

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this hypothesis. In conclusion, beside anti-obesity and anti-neoplastic effects (Van Gaal et al., 2005; Pi-Sunyer et al., 2006; Sarnataro et al., 2006; Bifulco et al. 2007), SR141716 may potentially possess a gluco-regulatory function, which is exerted at least in part by direct regulation of glucose metabolism in skeletal muscle cells.

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LEGENDS FOR FIGURES

Figure 1: **CB1 expression in myoblasts and myotubes**. A. The abundance of mRNA for CB1 was determined by real-time PCR analysis of total RNA isolated from 3T3 L1 cells and L6 myotubes. The results have been analysed as described in Materials and Methods. Bars represent the mRNA levels in L6 myotubes and are relative to those in 3T3 L1 cells. Data are expressed as means ± SD of triplicate reactions for total RNAs from each cell type in five independent experiments. B. 3T3 L1 and L6 were solubilized and cell lysates were separated on SDS-PAGE and immuno-blotted with CB1 Ab. The autoradiograph shown is representative of five independent experiments in duplicate. C. Filters obtained in B have been analyzed by laser densitometry as described in Materials and Methods.

Figure 2: **2-deoxyglucose (2-DG) uptake upon SR141716 incubation.** A. L6 myotubes were incubated in serum-free media for 16 h before exposure to 100 nM insulin for 30 min, as indicated. Alternatively, raising concentrations (0.05, 0.1, 0.3, 1 and 10 μM) of SR141716, or 0.1 μM SR144528 or 0.3 μM IRTX were simultaneously added to serum-free media for 16 h (in the absence of insulin). Then the cells were assayed for 2-DG uptake as described in Materials and Methods. Bars represent mean ± S.D. of three different experiments in triplicate. Asterisks indicate statistically significant differences vs untreated cells (***, $p<0.001$). B. The cells were treated with 0.1 μM SR141716 for different times (as indicated). 2-DG uptake assay was performed as described in Materials and Methods. Bars represent mean ± S.D. of three different experiments in triplicate. Asterisks indicate statistically significant differences vs untreated cells (***, $p<0.001$).

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Figure 3: **p85 and p110 regulation by SR141716.** A. L6 myotubes were serum-starved and incubated with 0.1 μM SR141716 for 16 h, as described in the legend to Fig.2 and in Materials and Methods. Cell lysates were separated on SDS-PAGE and analyzed by p85, p110 and β-actin immunoblot. The autoradiographs shown are representative of five independent experiments. B. Filters obtained in A have been analyzed by laser densitometry as described in Materials and Methods. Asterisks indicate statistically significant differences (***, $p<0.001$). C. Cells were treated with 0.1 μM SR141716 for different times as indicated. Cell lysates were then analyzed by p85 and β-actin immunoblot as indicated. The autoradiographs shown are representative of five independent experiments. Then, filters obtained in C have been analyzed by laser densitometry as described in Materials and Methods. Asterisks indicate statistically significant differences (***, $p<0.001$, **, $p<0.01$)

Figure 4: **Effect of CHX on p85 and 2-deoxyglucose uptake.** A. Cells were serum-starved and incubated with 40 μg/ml cycloheximide in the absence or in the presence of SR141716 for 24 h, as described in the legend to Fig.1 and in Materials and Methods. Cell lysates were then analyzed by immunoblot with p85 and β-actin antibodies. The autoradiographs shown are representative of five independent experiments. B. Filters obtained in A have been analyzed by laser densitometry as described in Materials and Methods. Asterisks indicate statistically significant differences (***, $p<0.001$). C. Cells were treated as described above and assayed for 2-DG uptake as described in Materials and Methods. Bars represent mean ± S.D. of three different experiments in triplicate. Asterisks indicate statistically significant differences vs untreated cells (***, $p<0.001$).

Figure 5: **SR141716 regulates p85 levels via PKA.** A. Cells were pre-treated with 15 μM H-89 and incubated with 0.1 μM SR141716 for 24 h, as described in the legend to Fig.2 and in Materials and Methods. Cell lysates were then analyzed by immunoblot with p85, CREB, phospho-CREB and

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β-actin antibodies. The autoradiographs shown are representative of five independent experiments. B. Filters obtained in A have been analyzed by laser densitometry as described in Materials and Methods. Asterisks indicate statistically significant differences (***, $p<0.001$). C. Myotubes were exposed to 1 μM forskolin for 30 min and to 0.1 μM SR141716 for 24 h. Then the cells were assayed for cAMP production. Bars represent mean ± S.D. of three different experiments in triplicate. Asterisks indicate statistically significant differences vs untreated cells (***, $p<0.001$).

Figure 6: **SR141716 increases p85 phosphorylation via PKA.** A. L6 myotubes were pre-treated with 15 μM H-89 for 30 min and incubated with 0.1 μM SR141716 for additional 30 min, as described in the legend to Fig.2 and in Materials and Methods. Cells were then solubilized, precipitated with anti-p85 antibodies, and immunoblotted with anti phospho-serine antibodies. The autoradiographs shown are representative of three independent experiments. B. Filters obtained in A have been analyzed by laser densitometry as described in Materials and Methods. Asterisks indicate statistically significant differences (*, $p<0.05$).

Figure 7: **Effect of CB1 modulation on PI3K signalling.** A. L6 myotubes were incubated with 0.1 μM SR141716 for different times, as described in the legend to Fig.2 and in Materials and Methods. Cells were then solubilized and phosphorylation and expression of PDK1, PKCζ and Akt were analyzed by Western blot with specific antibodies (phospho-PDK1/PDK1, phospho-PKCζ/ PKCζ, phospho-Akt/Akt), as indicated. The autoradiographs shown are representative of three independent experiments. B. Filters obtained in A have been analyzed by laser densitometry as described in Materials and Methods. Asterisks indicate statistically significant differences (**, $p<0.01$).

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Figure 8: **SR141716 increases PI3K signalling via PKA.** A. Cells were serum-starved and incubated with LY294002 or with H-89, in presence of 0.1 μM SR141716 for 30 min or for 24 h, as described in the legend to Fig.2 and in Materials and Methods. Then, cell extracts were subjected to SDS-PAGE followed by immunoblotting with specific anti phospho- or control antibodies, as indicated. The autoradiographs shown are representative of three independent experiments. B. Filters obtained in A have been analyzed by laser densitometry as described in Materials and Methods. Asterisks indicate statistically significant differences (**, $p<0.01$).

Figure 9: **SR141716 increases glucose uptake via PI3K and PKA.** A. L6 myotubes were serum-starved and incubated with LY294002 or with H-89, in presence of 0.1 μM SR141716 for 30 min or for 24 h. Then the cells were assayed for 2-DG uptake as described in Materials and Methods. Bars represent mean ± S.D. of three different experiments in triplicate (***, $p<0.001$). B. Prymary myocytes were serum-starved for 24 h before exposure to 100 nM insulin for 30 min, as indicated. Alternatively, myocytes were incubated with LY294002 or with H-89, in presence of 0.1 μM SR141716 for 30 min or for 24 h. Then the cells were assayed for 2-DG uptake as described in Materials and Methods. Bars represent mean ± S.D. of three different experiments in triplicate (**, $p<0.01$).

Figure 10: **Effect of CB1 silencing on glucose uptake.** A. Cells were transfected with siRNA-CB1 or with silencer negative control-1 siRNA. After 6 h, cells were serum-starved. Cell lysates were then analyzed by CB1 immunoblot as indicated. The autoradiographs shown are representative of three independent experiments. B. Cells were treated as described above and incubated with 0.1 μM SR141716 for 16 h. Then they were assayed for 2-DG uptake as described in Materials and Methods.

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Table 1. **Effect of SR141716 on proteins involved in glucose uptake.**

Protein	SR141716 (% of control)
Insulin Receptor	108±8.4
IRS1	107±7.0
IRS2	100±5.4
p85	196***±8.8
p110	168*±9.3
PDK1	99±9.2
PKCζ	112±5.6
Akt/PKB	123±16.9
PTEN	107±10.2
GLUT1	100±11.2
GLUT4	108±7.8

The cells were incubated or not with 0.1 μM SR141716 for 16 h. Filters were immuno-blotted with specific antibodies as indicated and analyzed by laser densitometry, as described in Materials and Methods. The values have been normalized on the actin levels and represent means ± S.D. of three independent experiments in duplicate. Asterisks denote statistically significant difference of the samples obtained from treated vs. untreated cells (* $p < 0.05$; *** $p < 0.001$).

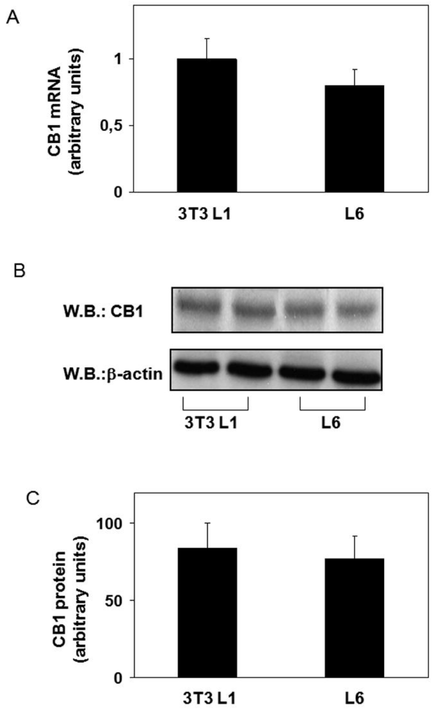

A
CB1 mRNA
(arbitrary units)
1
0,5
0
3T3 L1
L6
B
W.B.: CB1
W.B.:β-actin
3T3 L1
L6
C
CB1 protein
(arbitrary units)
100
50
0
3T3 L1
L6

Fig.1

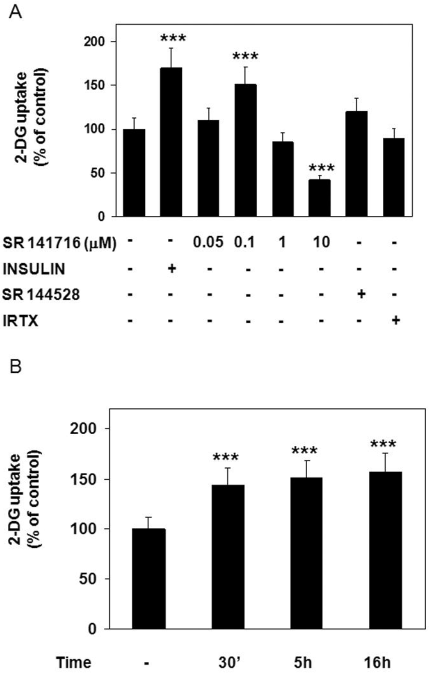

A
2-DG uptake
(% of control)
200
150
100
50
0
***
***
***
SR 141716 (μM) - - 0.05 0.1 1 10 - -
INSULIN - + - - - - - -
SR 144528 - - - - - - + -
IRTX - - - - - - - +
B
2-DG uptake
(% of control)
200
150
100
50
0
***
***
***
Time - 30' 5h 16h

Fig.2

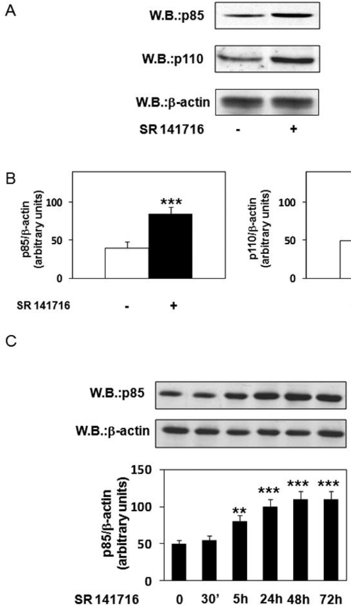

A
W.B.:p85
W.B.:p110
W.B.:β-actin
SR 141716 - +
B
p85/β-actin (arbitrary units)
0
50
100
***
SR 141716 - +
p110/β-actin (arbitrary units)
0
50
100
***
- +
C
W.B.:p85
W.B.:β-actin
p85/β-actin (arbitrary units)
0
50
100
150
**
***
***
***
SR 141716 0 30' 5h 24h 48h 72h

Fig.3

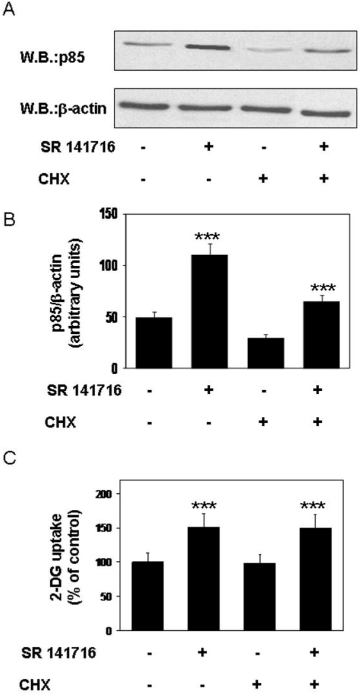

A
W.B.:p85
W.B.:β-actin
SR 141716 - + - +
CHX - - + +
B
150
100
50
0
p85/β-actin
(arbitrary units)
***
***
SR 141716 - + - +
CHX - - + +
C
200
150
100
50
0
2-DG uptake
(% of control)
***
***
SR 141716 - + - +
CHX - - + +

Fig.4

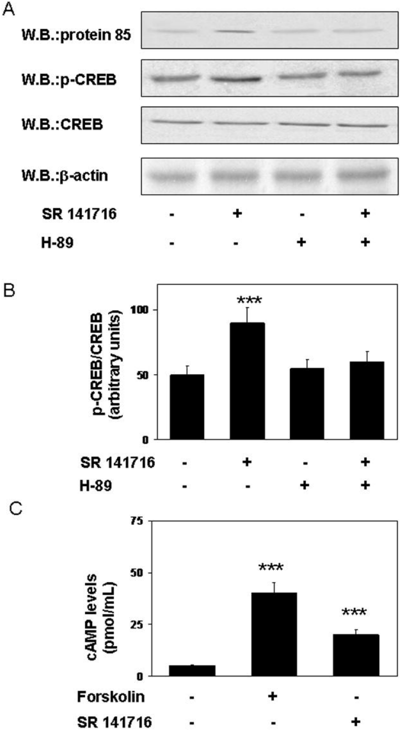

A
W.B.:protein 85
W.B.:p-CREB
W.B.:CREB
W.B.:β-actin
SR 141716 - + - +
H-89 - - + +
B
p-CREB/CREB (arbitrary units)
***
100
50
0
SR 141716 - + - +
H-89 - - + +
C
cAMP levels (pmol/mL)
***
***
75
50
25
0
Forskolin - + -
SR 141716 - - +

Fig.5

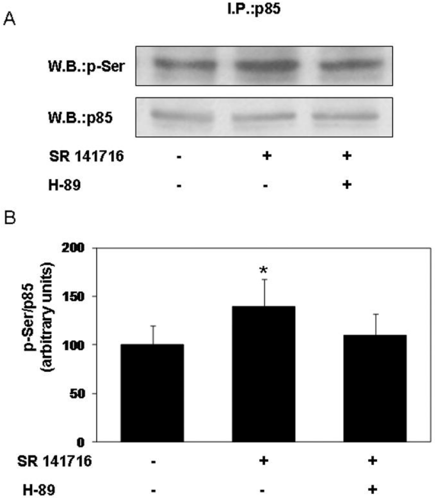

A
I.P.:p85
W.B.:p-Ser
W.B.:p85
SR 141716
-
+
+
H-89
-
-
+
B
200
150
100
50
0
p-Ser/p85
(arbitrary units)
*
SR 141716
-
+
+
H-89
-
-
+

Fig.6

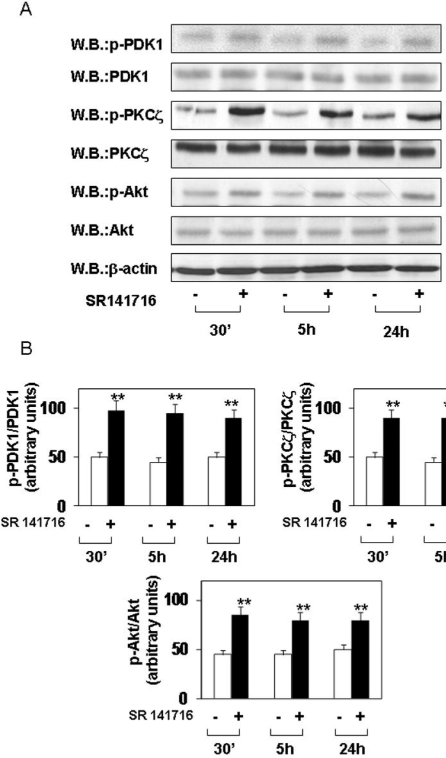

A
W.B.:p-PDK1
W.B.:PDK1
W.B.:p-PKCζ
W.B.:PKCζ
W.B.:p-Akt
W.B.:Akt
W.B.:β-actin
SR141716
- + - + - +
30'
5h
24h
B
p-PDK1/PDK1 (arbitrary units)
p-PKCζ/PKCζ (arbitrary units)
p-Akt/Akt (arbitrary units)
100
50
0
**
SR 141716
- + - + - +
30'
5h
24h

Fig.7

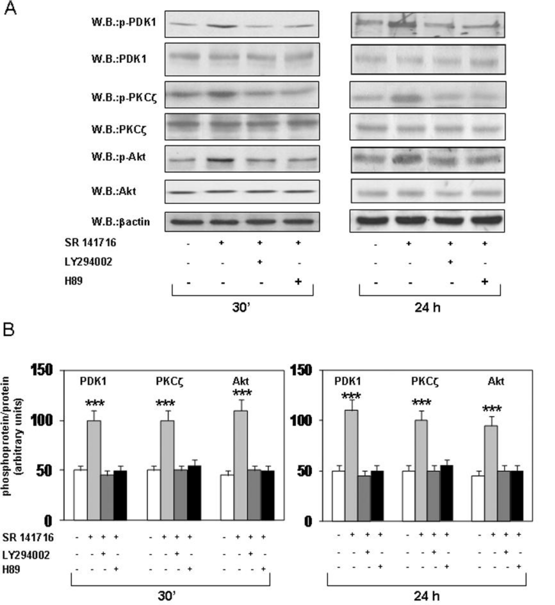
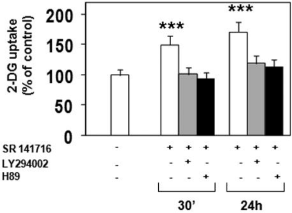

A
W.B.:p-PDK1
W.B.:PDK1
W.B.:p-PKCζ
W.B.:PKCζ
W.B.:p-Akt
W.B.:Akt
W.B.:βactin
SR 141716
LY294002
H89
30'
24 h
B
phosphoprotein/protein (arbitrary units)
150
100
50
0
PDK1
PKCζ
Akt
***
SR 141716
LY294002
H89
30'
24 h

Fig.8

A


2-DG uptake
(% of control)
200
150
100
50
0
***
***
SR 141716
LY294002
H89
30'
24h

B

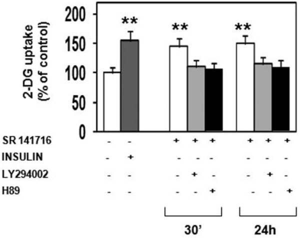

2-DG uptake
(% of control)
200
150
100
50
0
**
**
**
SR 141716
INSULIN
LY294002
H89
30'
24h

Fig.9

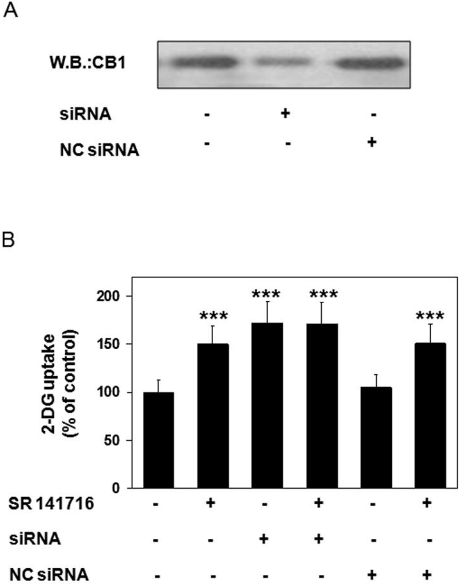

A
W.B.:CB1
siRNA - + -
NC siRNA - - +
B
2-DG uptake
(% of control)
0
50
100
150
200
***
***
***
***
SR 141716 - + - + - +
siRNA - - + + - -
NC siRNA - - - - + +

Fig.10