

The Cannabinoid CB1 Receptor Antagonist Rimonabant Stimulates 2-Deoxyglucose Uptake in Skeletal Muscle Cells by Regulating the Expression of Phosphatidylinositol-3-kinase

Iolanda Esposito, Maria Chiara Proto, Patrizia Gazerro, Chiara Laezza, Claudia Miele, Anna Teresa Alberobello, Vittoria D'Esposito, Francesco Beguinot, Pietro Formisano, and Maurizio Bifulco

Dipartimento di Biologia e Patologia Cellulare e Molecolare & Istituto di Endocrinologia ed Oncologia Sperimentale del Consiglio Nazionale delle Ricerche, Università degli Studi di Napoli "Federico II", Napoli, Italy (I.E., C.L., C.M., A.T.A., V. D.E., F.B., P.F.); and Endocannabinoid Research Group, Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, Fisciano (SA), Italy (M.C.P., P.G., M.B.)

Received May 26, 2008; accepted September 18, 2008

ABSTRACT

The endocannabinoid system regulates food intake, energy, and glucose metabolism at both central and peripheral levels. 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 small interfering RNA. 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, phosphoinositide-

dependent kinase-1, Akt/protein kinase B, and protein kinase C ζ activities were rapidly induced after SR141716 treatment of L6 cells in a PI3K-dependent manner. The stimulatory effect of SR141716 on PI3K expression and activity was largely prevented by *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline (H-89), an inhibitor of the cAMP-dependent protein kinase. Moreover, SR141716-stimulated 2-DG uptake was blunted by the incubation either with H-89 or with the PI3K inhibitor 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride (LY294002), both in L6 cells and in mouse primary myocytes. Thus, modulation of CB1 regulates glucose uptake at the level of the PI3K signaling system in skeletal muscle cells. Interfering with CB1 signaling may therefore ameliorate glucoregulatory functions in peripheral tissues.

Type 2 diabetes (T2D) is a genetically determined disorder, affecting more than 150 million people worldwide. T2D is char-

acterized by several metabolic defects, among which β -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 believed 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 patients with T2D, suggesting that common molecular events may contribute to the onset and/or the progression of both disorders.

This study was supported in part by the European Community's FP6 EUGENE2 (LSHM-CT-2004-512013) and FP7 PREPOBEDIA (number 201-681), grants from the Associazione Italiana per la Ricerca sul Cancro (to F.B. and P.F.), the Ministero dell'Università e della Ricerca Scientifica (to F.B. and P.F. and FIRB Idea Progettuale RBIP0689BS), the Ministero della Salute, the European Federation for the Study of Diabetes, and by the Associazione Educazione e Ricerca Medica Salernitana (ERMES). The financial support of Telethon-Italy is also gratefully acknowledged. I.E. was awarded a travel grant from the Università di Napoli "Federico II"–International Exchange Program.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.108.049205.

ABBREVIATIONS: T2D, type 2 diabetes; 2-DG, 2-deoxyglucose; PKB, protein kinase B; CB1, cannabinoid receptor type 1; CHX, cycloheximide; CREB, cAMP response element-binding protein; DMEM, Dulbecco's modified Eagle's medium; ECS, endocannabinoid system; GLUT, glucose transporter; IRS, insulin receptor substrate; IRTX, iodo-resinatoxin; PeSt, penicillin-streptomycin; PKC ζ , 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; SR141716, rimonabant; H-89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

These defects may affect feeding behavior, 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 and 2-arachidonoyl-glycerol) have been found in obese individuals (Engeli et al., 2005; Osei-Hyiaman et al., 2005, 2006) 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 and Taylor, 2001; Williams and Kirkham, 2002; Cota et al., 2003a). Mice carrying the ablation of the CB1 gene are lean and resistant to diet-induced obesity (Ravinet Trillou et al., 2004). It has been shown recently 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 favorable modifications in serum biochemical and lipid profiles (Poirier et al., 2005; Pi-Sunyer et al., 2006).

Long-term 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 or are 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 Ca^{2+} 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 (Osei-Hyiaman et al., 2005). In addition, modulation of CB-1 activity in isolated mouse adipocytes increases the activity of the lipogenic enzyme lipoprotein lipase (Cota et al., 2003b) and adiponectin expression (Bensaid et al., 2003). Very little information is available, however, concerning cannabinoid receptor function in the skeletal muscle. Recently, CB1 expression has been detected in soleus muscle (Pagotto et al., 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 (Cavuto 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 signaling and a consequent regulation of glucose uptake in the skeletal muscle cells.

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 extracellular signal-regulated kinase antibodies were purchased from

Cell Signaling Technology (Danvers, MA). Actin antibody was from Sigma (St. Louis, MO). Antibodies directed against CB1, GLUT1, GLUT4, extracellular signal-regulated kinases, phospho-Thr410 PKC ζ , PKC ζ , p110, PTEN, PKB, CREB, and phospho-Ser133 CREB were from Santa Cruz Biotechnology (Santa Cruz, CA). PDK1, IRS-1, IRS-2, 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 enhanced chemiluminescence reagents were from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Other reagents were from Sigma.

Cell Culture. The L6 skeletal muscle cells were plated (6×10^3 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 was achieved as described previously (Caruso et al., 1997).

Mouse Primary Fibroskeletal Muscle Cell Culture. Skeletal muscle biopsies were obtained after C57 BL6 strain ($n = 9$) mice were sacrificed by pentobarbitone overdose, as described previously (Krämer et al., 2007). The biopsies were collected in cold phosphate-buffered saline supplemented with 1% PeSt (100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin), dissected, finely minced, and transferred to a digestion solution (0.015 g Collagenase IV, 8% 10 \times trypsin, 0.015 g of bovine serum albumin, 1% PeSt, in DMEM supplemented with 10% fetal calf serum, 10,000 U/ml penicillin, 10 mg/ml streptomycin, and 2% L-glutamine) and incubated with gentle agitation at 37°C for 15 to 20 min. Thereafter, undigested tissue was allowed to settle, and the supernatant was collected and mixed with DMEM supplemented with 20% fetal calf serum and 1% PeSt. The remaining tissue was digested for a further 15 to 20 min at 37°C with fresh digestion solution. The resultant supernatant was then pooled with the previous cells and centrifuged for 10 min at 350g. The cell pellet was resuspended in DMEM supplemented with 20% fetal calf serum and 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 and 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 of 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, 0.1, 0.3, 1, and 10 μM) were simultaneously added to the media, as described in the figure legend. Likewise, 0.1 μM SR144528 and 0.3 μM iodo-resinatoxin (IRTX) were added to the serum-free media for the indicated time. For PI3K inhibition studies, cells were pretreated 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 pretreated with 10 μM LY294002 for 30 min, followed by further incubation with LY294002 and 0.1 μM SR141716 for an additional 30 min. For PKA inhibition studies, cells were pretreated with 15 μM H-89 for 30 min followed by combined treatment with H-89 and 0.1 μM SR141716 for an additional 30 min or 24 h as indicated. To study the SR141716 effect on protein synthesis, the cells were incubated with 40 $\mu\text{g}/\text{ml}$ 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'-CCCAAGUGAC-GAAAAACAUU-dTdT-3'. The cells were transfected using FuGENE (Roche, Indianapolis, IN) and 100 nM siRNA for each transfection, in accordance with the manufacturer's instruction. An equal concentration of Silencer Negative Control-1 siRNA (Ambion, Austin, TX) 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. 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 preadipocytes and L6 cells by using the RNeasy Kit (QIAGEN Sciences, Valencia, CA) according to the manufacturer's instruction. For real-time RT-PCR analysis, 1 μ g of cell RNA was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). PCR was analyzed using SYBR Green mix (Invitrogen). Reactions were performed using Platinum SYBR Green Quantitative PCR SuperUDG using an iCycler IQ multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA). All reactions were performed in triplicate, and β -actin was used as an internal standard. Primer sequences used were as follows: CB1R sense, 5'-CTA CTG GTG CTG TGT GTC ATC-3'; antisense, 5'-GCT GTC TTT ACG GTG GAA TAC-3'; and β -actin: forward, 5'-GCGTGACATCAAAGA-GAAG-3'; 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 $\text{Na}_2\text{P}_2\text{O}_7$, 2 mM sodium orthovanadate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, pH 7.4, and 1% (v/v) Triton X-100. The lysates were clarified by centrifugation at 12,000g for 20 min at 4°C. Proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted on Immobilon-P membranes (Millipore, Billerica, MA). Membranes were blocked for 1 h in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, and 140 mM NaCl) containing 4% (w/v) bovine serum albumin and then incubated with the indicated antibodies. Detection of blotted proteins was performed by enhanced chemiluminescence according to the manufacturer's instruction. Immunoprecipitation experiments were performed as described previously (Formisano et al., 1998). Densitometric analysis was performed using a Scion Image Analyzer. All of the data were expressed as mean \pm S.D. Significance was assessed by Student's *t* test for comparison between two means. Data were analyzed with Statview software (Abacus Concepts, Piscataway, NJ) by one-factor analysis of variance. *P* values of less than 0.05 were considered statistically significant.

2-Deoxy-D-glucose Uptake. The measurement of 2-deoxy-D-[^{14}C]glucose uptake was taken as a measure of glucose uptake by muscle cells, as described previously (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 μ Ci/assay) for 15 min in HEPES buffer. The reaction was terminated by the addition of 10 μ M cytochalasin B, and the cells were washed three times with ice-cold isotonic saline solution before 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 5×10^5 cells/well. Cells were treated with 1 μ M forskolin for 30 min and with 0.1 μ M SR141716 for 24 h. After treatment, cells were washed twice with cold phosphate-buffered saline, and intracellular cAMP was extracted by the addition of 500 μ l of 0.1 N HCl with 0.1% Triton, followed by three cycles of freezing and thawing. cAMP concentration was measured by radioimmunoassay (PerkinElmer Life and Analytical Sciences, Waltham, MA) (Nikodemova et al., 2003).

Results

Effect of CB1 Modulation on Glucose Uptake. To investigate whether the endocannabinoid system may operate in a skeletal muscle cell model, we 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 used 3T3 L1 cells, in which CB1 expression has already been described previously (Yan et al., 2007). In L6 myotubes, the CB1 mRNA (Fig. 1A) and protein (Fig. 1, B and C) were expressed at levels similar to those detected in 3T3 L1 cells.

Next, 2-deoxy-glucose (2-DG) uptake was measured in the myotubes in the absence or presence of increasing concentrations of the CB1 receptor inverse agonist SR141716 (Fig. 2A). At variance, preincubation of L6 myotubes with 0.1 and 0.3 μ M 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 μ M SR141716 (Fig. 2A). Increasing SR141716 concentrations to 1 and 10 μ M led to a progressive reduction of 2-DG uptake, however, consistent with a partial agonist effect. In addition, no significant effect was achieved after treatment of L6 cells with 0.1 μ M SR144528 (a selective CB2 receptor antagonist) and 0.3 μ M IRTX, a potent transient receptor potential vanilloid 1 receptor antagonist (Fig. 2A). Time course analysis revealed that the effect of 0.1 μ M 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).

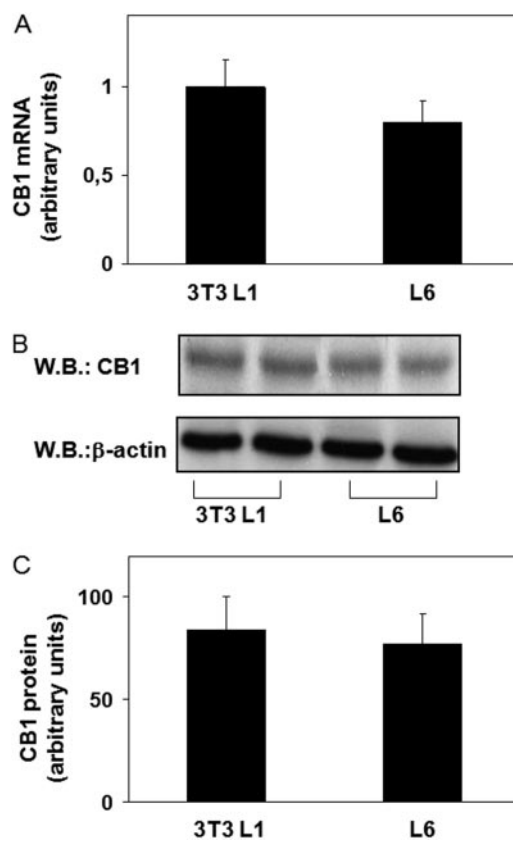


Fig. 1. CB1 expression in myoblasts and myotubes. A, the abundance of mRNA for CB1 was determined by real-time PCR analysis of total RNA isolated from 3T3 L1 cells and L6 myotubes. The results have been analyzed as described under *Materials and Methods*. Bars represent the mRNA levels in L6 myotubes and are relative to those in 3T3 L1 cells. Data are expressed as means \pm S.D. 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 immunoblotted with CB1 antibodies. The autoradiograph shown is representative of five independent experiments in duplicate. C, filters obtained in B have been analyzed by laser densitometry as described under *Materials and Methods*.

Protein Expression Profile upon CB1 Modulation. To address the mechanism by which CB1 may regulate glucose uptake in the L6 cells, protein lysates were obtained after-treatment with 0.1 μ M SR141716 for 16 h. Protein expression profiling was achieved by immunoblot with specific antibodies (Table 1). The intracellular content of the regulatory (p85 α) and the catalytic (p110 α) subunits of class I PI3K was increased by 2.0- and 1.7-fold, respectively, upon SR141716 exposure (Table 1 and Fig. 3). No significant change, instead, was detected for the insulin receptor, 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 1). The expression levels of protein kinase B α /Akt1 were also increased by SR141716, although differences did not reach statistically significant values (Table 1). 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 increased up to 24 h and remained stable for 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).

To assess whether CB1-mediated regulation of p85 expres-

TABLE 1

Effect of SR141716 on proteins involved in glucose uptake

The cells were incubated or not with 0.1 μ M SR141716 for 16 h. Filters were immunoblotted with specific antibodies as indicated and analyzed by laser densitometry, as described under *Materials and Methods*. The values have been normalized to the actin levels and represent means \pm S.D. of three independent experiments in duplicate.

Protein	SR141716
	% of control
Insulin receptor	108 \pm 8.4
IRS1	107 \pm 7.0
IRS2	100 \pm 5.4
p85	196 \pm 8.8***
p110	168 \pm 9.3*
PDK1	99 \pm 9.2
PKC ζ	112 \pm 5.6
Akt/PKB	123 \pm 16.9
PTEN	107 \pm 10.2
GLUT1	100 \pm 11.2
GLUT4	108 \pm 7.8

* $P < 0.05$; *** $P < 0.001$ (statistically significant difference of the samples obtained from treated versus untreated cells).

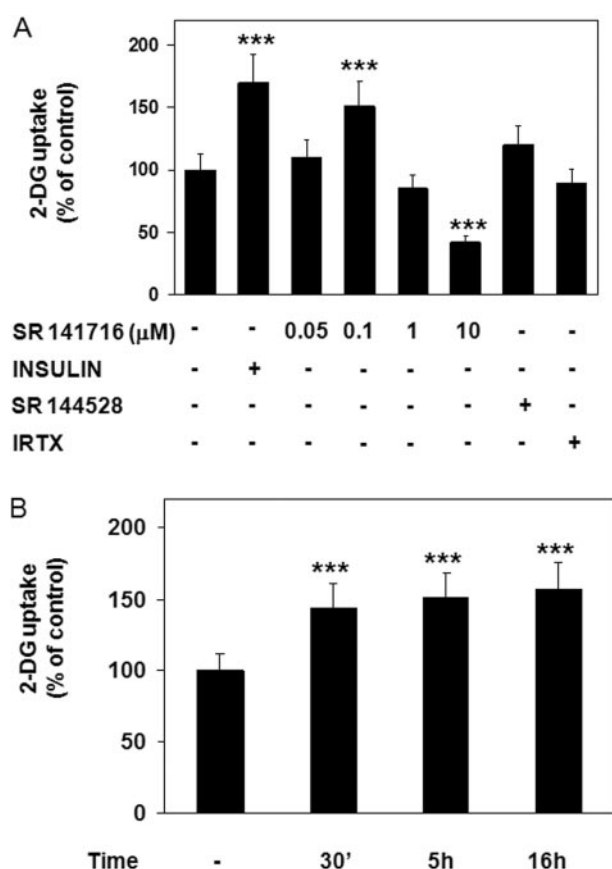


Fig. 2. 2-DG uptake upon SR141716 incubation. A, L6 myotubes were incubated in serum-free media for 16 h before exposure to 100 nM insulin for 30 min as indicated. On the other hand, increasing concentrations (0.05, 0.1, 0.3, 1, and 10 μ M) of SR141716, or 0.1 μ M SR144528 or 0.3 μ M IRTX was simultaneously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

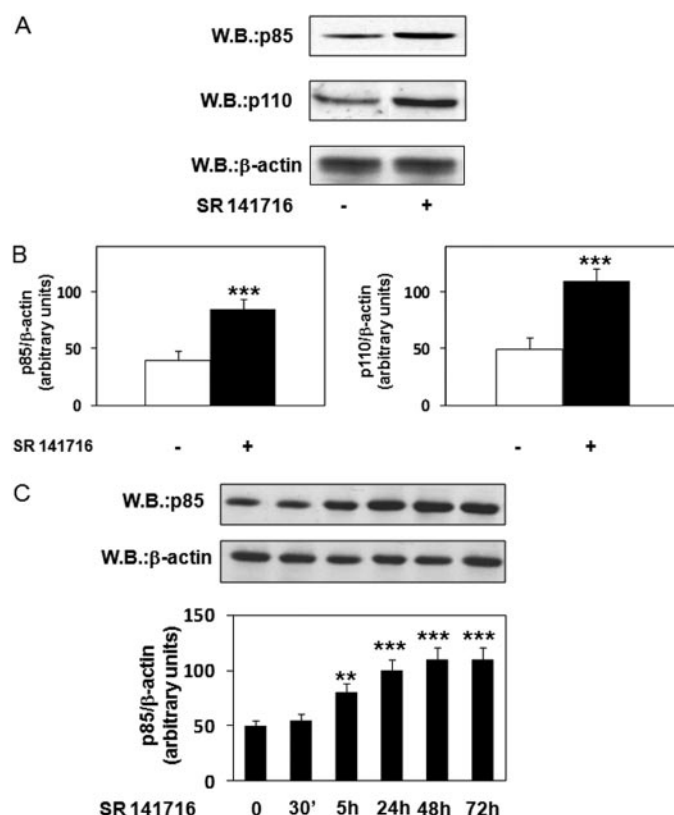


Fig. 3. p85 and p110 regulation by SR141716. A, L6 myotubes were serum-starved and incubated with 0.1 μ M SR141716 for 16 h, as described in the legend to Fig. 2 and under *Materials and Methods*. Cell lysates were separated on SDS-PAGE and analyzed by p85, p110, and β -actin immunoblot. The autoradiographs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

sion occurred at the level of protein synthesis, L6 cells were treated with 40 $\mu\text{g/ml}$ cycloheximide (CHX). At the baseline, CHX treatment reduced p85 cellular abundance (Fig. 4, A and B). The treatment with SR141716, however, was still able to increase p85 immunodetection, indicating that regulation occurred at a 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 shown previously that CB1 receptor may regulate PKA activity (Bidaut-Russell et al., 1990). To investigate whether SR141716 may regulate PKA activity in the L6 cells, we evaluated the phosphorylation of its substrate, CREB. Immunoblot with specific antibodies revealed that SR141716 induced the phosphorylation of CREB on Ser133 (Fig. 5, A and B). We have therefore tested whether PKA

inhibition was able to revert the SR141716 effect on p85. To this end, L6 cells were treated with H-89 (15 μM), a PKA inhibitor, in the absence or presence of SR141716. It is interesting that no increase of p85 cellular abundance was detected in cells treated with H-89 (Fig. 5, A and B). To further clarify the hypothesis that SR141716 may regulate PKA activity, we 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 ($p < 0.001$), approximately 50% lower than that observed upon short-term stimulation (30 min) with 1 μM forskolin, a well known adenylyl cyclase stimulator (Fig. 5C). Moreover, we have investigated whether SR141716 stimulates p85 phosphorylation via PKA. To this aim, L6 cells were pretreated with H-89 for 30 min and stimulated with SR141716 for additional 30 min. SR141716 treatment increased p85

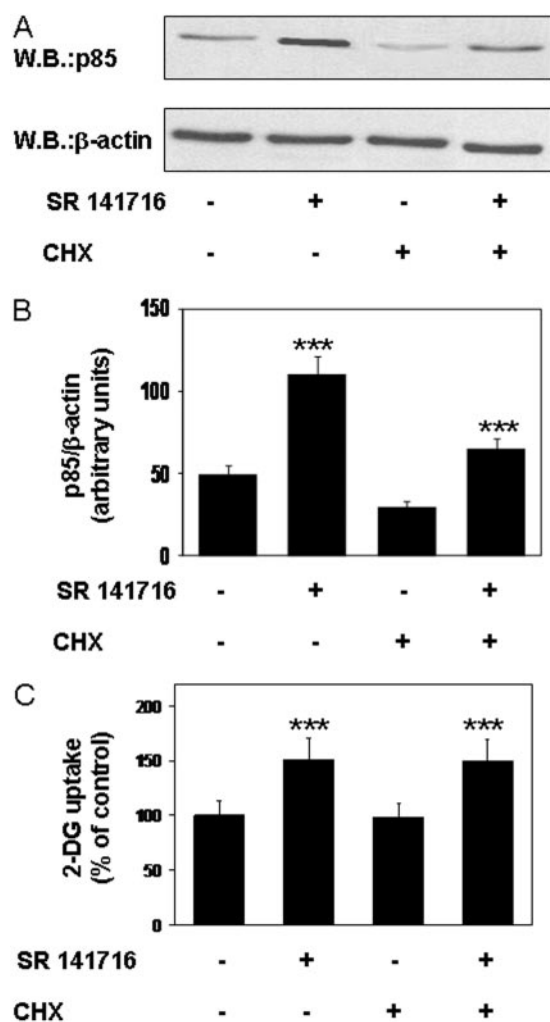


Fig. 4. Effect of CHX on p85 and 2-deoxyglucose uptake. A, cells were serum-starved and incubated with 40 $\mu\text{g/ml}$ cycloheximide in the absence or in the presence of SR141716 for 24 h, as described in the legend to Fig. 1 and under *Materials and Methods*. Cell lysates were then analyzed by immunoblot with p85 and β -actin antibodies. The autoradiographs shown are representative of five independent experiments. B, filters obtained in A were analyzed by laser densitometry as described under *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 under *Materials and Methods*. Bars represent mean \pm S.D. of three different experiments in triplicate. Asterisks indicate statistically significant differences versus untreated cells (***, $p < 0.001$).

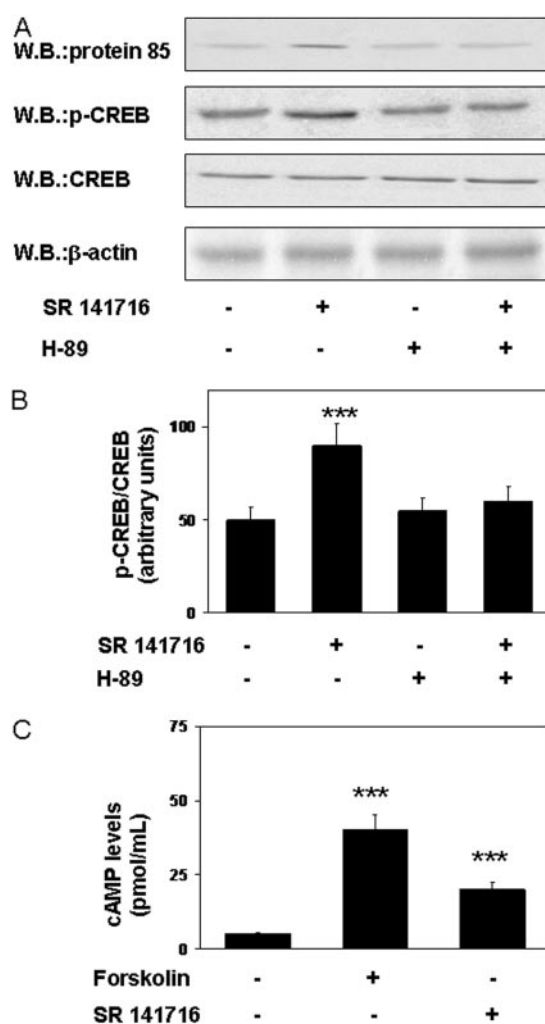


Fig. 5. SR141716 regulates p85 levels via PKA. A, cells were pretreated with 15 μM H-89 and incubated with 0.1 μM SR141716 for 24 h, as described in the legend to Fig. 2 and under *Materials and Methods*. Cell lysates were then analyzed by immunoblot with p85, CREB, phospho-CREB, and β -actin antibodies. The autoradiographs shown are representative of five independent experiments. B, filters obtained in A were analyzed by laser densitometry as described under *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 \pm S.D. of three different experiments in triplicate. Asterisks indicate statistically significant differences versus untreated cells (***, $p < 0.001$).

serine phosphorylation, and this effect is prevented by H-89 pretreatment (Fig. 6).

Regulation of PI3K Signaling by CB1. Next, we investigated whether CB1 modulation affects signaling downstream PI3K. To this end, L6 cells have been treated with 0.1 μ M SR141716. Immunodetection of the phosphorylated forms of PDK1, Akt/PKB, and PKC ζ was taken as functional marker for PI3K activity (Fig. 7, A and B). 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 PKC ζ phosphorylation was already evident upon 30-min treatment and remained stable up to 24 h (Fig. 7, A and B). L6 myotubes were therefore pretreated with 10 μ M LY294002, to block PI3K activity, or with 15 μ M H-89, to block PKA activity, and stimulated with 0.1 μ M SR141716 for 30 min or for 24 h. In both conditions, SR141716 failed to induce phosphorylation of PDK1, Akt/PKB, and PKC ζ (Fig. 8, A and B). In addition, the effect of SR141716 on 2-DG uptake was blunted after LY294002 and H-89 pretreatment (Fig. 9A). Moreover, we investigated the SR141716 effect on 2-DG uptake in primary myocytes. Treatment with 0.1 μ M SR141716 for 30 min or for 24 h stimulated 2-DG uptake ($p < 0.01$) by approximately 50%, a level similar to that observed upon short-term insulin stimulation (100 nM for 30 min) (Fig. 9B). In addition, the effect of SR141716 on 2-DG uptake was reverted after LY294002 and H-89 pretreatment (Fig. 9B).

Effect of CB1 Silencing on Glucose Uptake. Finally, we investigated whether CB1 silencing affects glucose up-

take induced by SR141716. To this end, L6 cells were transfected with 100 nM siRNA-CB1, which specifically inhibited CB1 expression by approximately 80% (Fig. 10A). In untransfected L6 cells, SR141716 increased 2-DG uptake. It is interesting that the transfection of siRNA increased glucose uptake but abolished further increases induced by SR141716 (Fig. 10B). Moreover, the transfection of silencer negative control siRNA did not modify 2-DG uptake induced by SR141716 treatment (Fig. 10B).

Discussion

The endocannabinoid system (ECS) is a crucial regulator of several physiological processes, including the control of energy balance (Osei-Hyiaman et al., 2006; Pagotto 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 behavior and increased energy consumption (Ravinet Trillou et al., 2004). More recent evidence in humans has indicated that pharmacological blockade of CB1 is accompanied by significant reduction of body weight and of plasma levels of cholesterol and triglycerides (Despres et al., 2005;

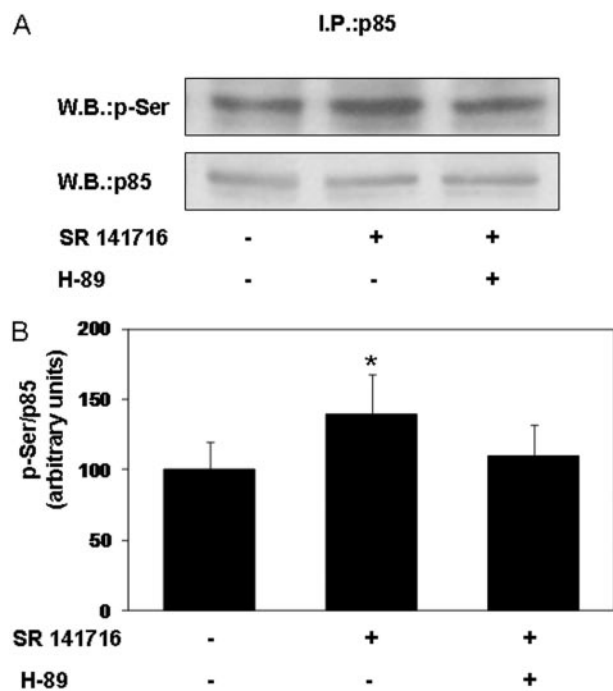


Fig. 6. SR141716 increases p85 phosphorylation via PKA. A, L6 myotubes were pretreated with 15 μ M H-89 for 30 min and incubated with 0.1 μ M SR141716 for additional 30 min, as described in the legend to Fig. 2 and under *Materials and Methods*. Cells were then solubilized, precipitated with anti-p85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

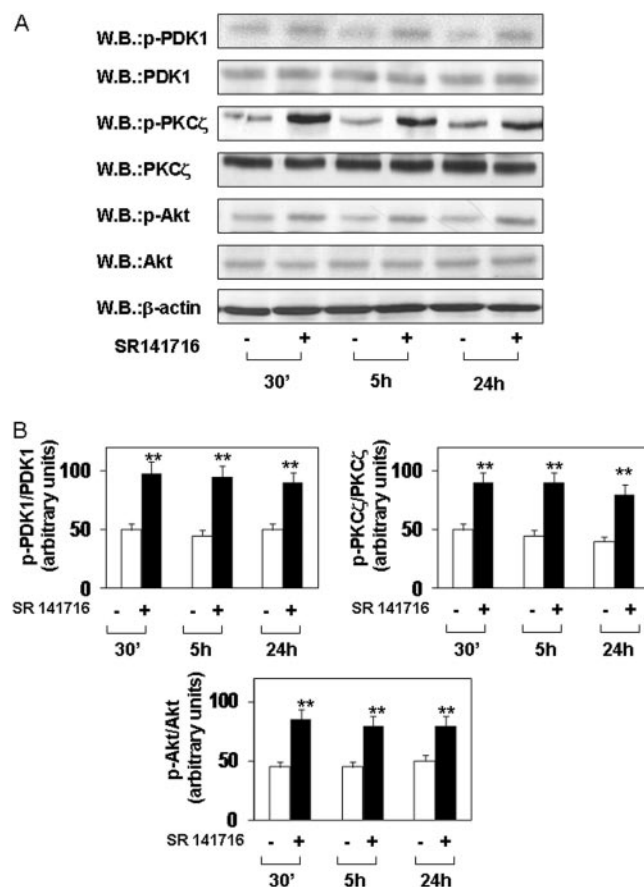


Fig. 7. Effect of CB1 modulation on PI3K signaling. A, L6 myotubes were incubated with 0.1 μ M SR141716 for different times, as described in the legend to Fig. 2 and under *Materials and Methods*. Cells were then solubilized and phosphorylation and expression of PDK1, PKC ζ , and Akt were analyzed by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

Van Gaal et al., 2005). It also seems 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 transient receptor potential vanilloid 1, 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). Therefore, Cavuoto et al. (2007) have shown that CB1 agonists and antagonists modify the expression of genes regulating skeletal muscle oxidative pathways. 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 glucose uptake. Consistently, either genetic silencing or pharmacological 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 the SR141716 effect occurred in the absence of exogenous anandamide and 2-arachidonoyl-glycerol, suggesting an inverse agonist effect. However, autonomous cellular production of endocannabinoids cannot be excluded, also raising the possibility of an antagonist effect of the compound. It is intriguing that 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 and Jentzsch, 2004; Krylatov et al., 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

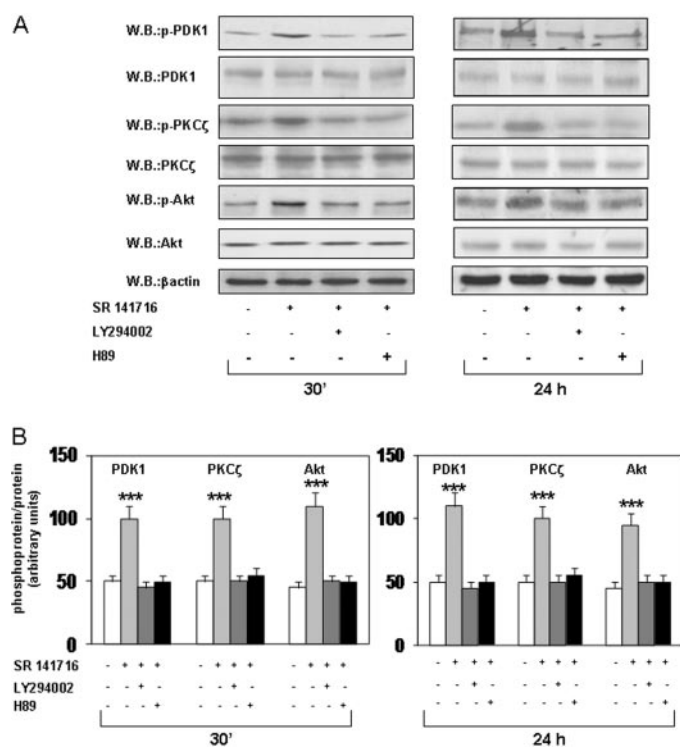


Fig. 8. SR141716 increases PI3K signaling via PKA. A, cells were serum-starved and incubated with LY294002 or with H-89, in the presence of 0.1 μ M SR141716 for 30 min or for 24 h, as described in the legend to Fig. 2 and under *Materials and Methods*. Then cell extracts were subjected to SDS-PAGE followed 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 were analyzed by laser densitometry as described under *Materials and Methods*. Asterisks indicate statistically significant differences (**, $p < 0.01$).

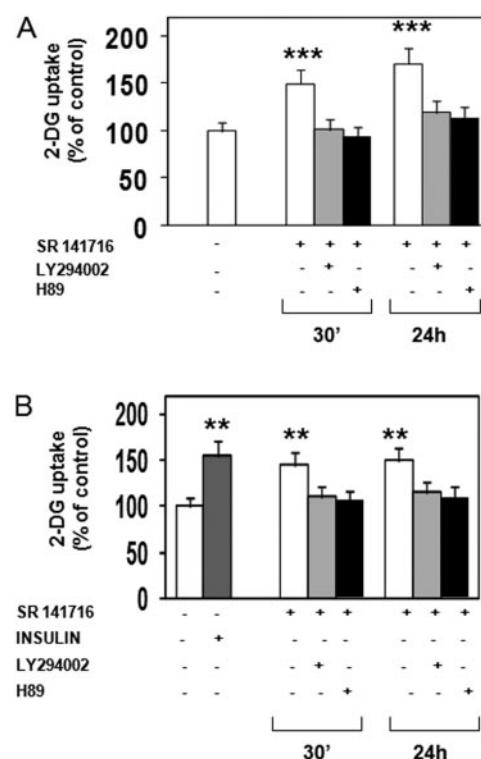


Fig. 9. SR141716 increases glucose uptake via PI3K and PKA. A, L6 myotubes were serum-starved and incubated with LY294002 or with H-89 in the presence of 0.1 μ M SR141716 for 30 min or for 24 h. Then the cells were assayed for 2-DG uptake as described under *Materials and Methods*. Bars represent mean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

unmodified upon CB1 pharmacological targeting. Several lines of evidence indicate that CB1 regulation of glucose uptake occurs through PI3K signaling. First, dose- and time-dependent increases of both the regulatory (p85) and the catalytic (p110) subunits were observed after treatment with low concentrations of SR141716. Second, these effects were paralleled by increased activity of several PI3K downstream molecules (PDK1, PKC ζ , and Akt/PKB). Third, the inhibition of PI3K activity counteracted the effect of SR141716 on glucose uptake.

It is noteworthy that the SR141716 effect occurred with 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.

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 in intracellular cAMP levels and CREB phosphorylation at a PKA consensus site. H-89, a pharmacological PKA blocker, inhibits both CREB phosphorylation and PI3K signaling 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 $_{i/o}$ 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 increase 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 cannot 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 described recently 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: 1) a short-term mechanism, which directly stimulates PI3K phosphorylation and activation, and 2) 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 this hypothesis. In conclusion, beside antiobesity and antineoplastic effects (Van Gaal et al., 2005; Pi-Sunyer et al., 2006; Sarnataro et al., 2006; Bifulco et al., 2007), SR141716 may potentially possess a glucoregulatory function, which is exerted at least in part by direct regulation of glucose metabolism in skeletal muscle cells.

References

- Bensaid M, Gary-Bobo M, Esclançon A, Maffrand JP, Le Fur G, Oury-Donat F, and Soubrié P (2003) The cannabinoid CB1 receptor antagonist SR141716 increases Acip30 mRNA expression in adipose tissue of obese fa/fa rats and in cultured adipocyte cells. *Mol Pharmacol* **63**:908–914.
- Bidaut-Russell M, Devane WA, and Howlett AC (1990) Cannabinoid receptors and modulation of cyclic AMP accumulation in the rat brain. *J Neurochem* **55**:21–26.
- Bifulco M, Grimaldi C, Gazzero P, Pisanti S, and Santoro A (2007) Rimonabant: just an antiobesity drug? Current evidence on its pleiotropic effects. *Mol Pharmacol* **71**:1445–1456.
- Bouaboula M, Perrachon S, Milligan L, Canat X, Rinaldi-Carmona M, Portier M, Barth F, Calandra B, Pecceu F, Lupker J, et al. (1997) A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. Evidence for a new model of receptor/ligand interactions. *J Biol Chem* **272**:22330–22339.
- Boyd ST (2006) The endocannabinoid system. *Pharmacotherapy* **26**:218S–221S.
- Caruso M, Miele C, Formisano P, Condorelli G, Bifulco G, Oliva A, Auricchio R, Riccardi G, Capaldo B, and Beguinot F (1997) In skeletal muscle, glucose storage and oxidation are differentially impaired by the IR1152 mutant receptor. *J Biol Chem* **272**:7290–7297.
- Cavuto P, McAinch AJ, Hatzinikolas G, Cameron-Smith D, and Wittert GA (2007) Effects of cannabinoid receptors on skeletal muscle oxidative pathways. *Mol Cell Endocrinol* **267**:63–69.
- Cosentino C, Di Domenico M, Porcellini A, Cuozzo C, De Gregorio G, Santillo MR, Agnese S, Di Stasio R, Feliciello A, Migliaccio A, et al. (2007) p85 regulatory subunit of PI3K mediates cAMP-PKA and estrogens biological effects on growth and survival. *Oncogene* **26**:2095–2103.
- Cota D, Marsicano G, Lutz B, Vicennati V, Stalla GK, Pasquali R, and Pagotto U (2003a) Endogenous cannabinoid system as a modulator of food intake. *Int J Obes Relat Metab Disord* **27**:289–301.
- Cota D, Marsicano G, Tschöp M, Gröbler Y, Flachskamm C, Schubert M, Auer D, Yassouridis A, Thöne-Reineke C, Ortmann S, et al. (2003b) The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. *J Clin Invest* **112**:423–431.
- Cote M, Matias I, Lemieux I, Petrosino S, Almeras N, Despres JP, and Di Marzo V (2007) Circulating endocannabinoid levels, abdominal adiposity and related cardiometabolic risk factors in obese men. *Int J Obes (Lond)* **31**:692–699.
- De Gregorio G, Coppa A, Cosentino C, Ucci S, Messina S, Nicolussi A, D'Inzeo S, Di Pardo A, Avvedimento EV, and Porcellini A (2007) The p85 regulatory subunit of PI3K mediates TSH-cAMP-PKA growth and survival signals. *Oncogene* **26**:2039–2047.
- De Vry J and Jentzsch KR (2004) Partial agonist-like profile of the cannabinoid receptor antagonist SR141716A in a food-reinforced operant paradigm. *Behav Pharmacol* **15**:13–20.
- Després JP, Golay A, and Sjöström L (2005) Rimonabant in Obesity-Lipids Study Group. Effects of rimonabant on metabolic risk factors in overweight patients with dyslipidemia. *N Engl J Med* **353**:2121–2134.
- Di Marzo V, Goparaju SK, Wang L, Liu J, Bátkai S, Jári Z, Fezza F, Miura GI,

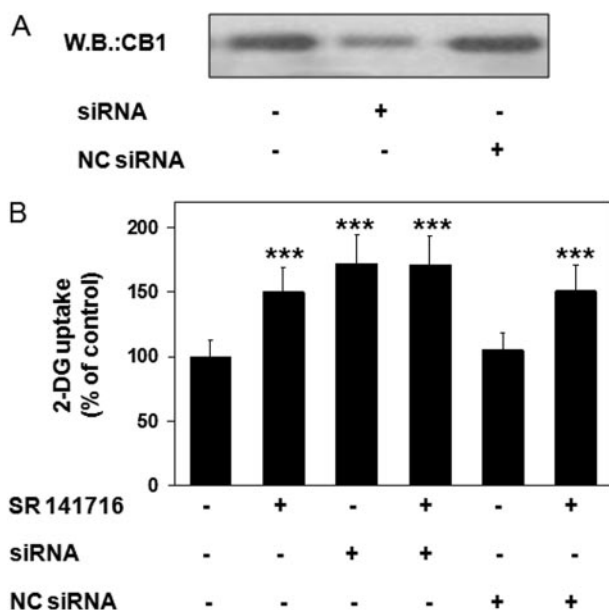


Fig. 10. Effect of CB1 silencing on glucose uptake. A, cells were transfected with siRNA-CB1 or with silencer negative control-1 siRNA. After 6 h, cells were serum-starved. Cell lysates were then analyzed by CB1 immunoblot as indicated. The autoradiographs shown are representative of three independent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

- Palmiter RD, Sugiura T, et al. (2001) Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature* **410**:822–825.
- Engeli S, Böhnke J, Feldpausch M, Gorzelniak K, Janke J, Bätke S, Pacher P, Harvey-White J, Luft FC, Sharma AM, et al. (2005) Activation of the peripheral endocannabinoid system in human obesity. *Diabetes* **54**:2838–2843.
- Formisano P, Oriente F, Miele C, Caruso M, Auricchio R, Vigliotta G, Condorelli G, and Beguinot F (1998) In NIH-3T3 fibroblasts, insulin receptor interaction with specific protein kinase C isoforms controls receptor intracellular routing. *J Biol Chem* **273**:13197–13202.
- Gasperi V, Fezza F, Pasquariello N, Bari M, Oddi S, Agrò AF, and Maccarrone M (2007) Endocannabinoids in adipocytes during differentiation and their role in glucose uptake. *Cell Mol Life Sci* **64**:219–229.
- Hirsch E, Costa C, and Ciralo E (2007) Phosphoinositide 3-kinases as a common platform for multi-hormone signaling. *J Endocrinol* **194**:243–256.
- Hollander P (2007) Endocannabinoid blockade for improving glycemic control and lipids in patients with type 2 diabetes mellitus. *Am J Med* **120** (2 Suppl 1):S18–S32.
- Howlett AC, Qualy JM, and Khachatrian LL (1986) Involvement of Gi in the inhibition of adenylate cyclase by cannabimimetic drugs. *Mol Pharmacol* **29**:307–313.
- Jamshidi N and Taylor DA (2001) Anandamide administration into the ventromedial hypothalamus stimulates appetite in rats. *Br J Pharmacol* **134**:1151–1154.
- Juan-Picó P, Fuentes E, Bermúdez-Silva FJ, Javier Diaz-Molina F, Ripoll C, Rodríguez de Fonseca F, and Nadal A (2006) Cannabinoid receptors regulate Ca^{2+} signals and insulin secretion in pancreatic beta-cell. *Cell Calcium* **39**:155–162.
- Kahn SE (2003) The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. *Diabetologia* **46**:3–19.
- Klip A, Logan WJ, and Li G (1982) Hexose transport in L6 muscle cells. Kinetic properties and the number of [3H]cytochalasin B binding sites. *Biochim Biophys Acta* **687**:265–280.
- Krämer DK, Al-Khalili L, Guigas B, Leng Y, Garcia-Roves PM, and Krook A (2007) Role of AMP kinase and PPAR δ in the regulation of lipid and glucose metabolism in human skeletal muscle. *J Biol Chem* **282**:19313–19320.
- Krylatov AV, Maslov LN, Lasukova OV, and Pertwee RG (2005) Cannabinoid receptor antagonists SR141716 and SR144528 exhibit properties of partial agonists in experiments on isolated perfused rat heart. *Bull Exp Biol Med* **139**:558–561.
- Lazar MA (2005) How obesity causes diabetes: not a tall tale. *Science* **307**:373–375.
- Liu YL, Connoley IP, Wilson CA, and Stock MJ (2005) Effects of the cannabinoid CB1 receptor antagonist SR141716 on oxygen consumption and soleus muscle glucose uptake in Lep(ob)/Lep(ob) mice. *Int J Obes (Lond)* **29**:183–187.
- Matias I, Bisogno T, and Di Marzo V (2006) Endogenous cannabinoids in the brain and peripheral tissues: regulation of their levels and control of food intake. *Int J Obes (Lond)* **30** (Suppl 1):S7–S12.
- Nikodemova M, Kasckow J, Liu H, Manganiello V, and Aguilera G (2003) Cyclic adenosine 3',5'-monophosphate regulation of corticotropin-releasing hormone promoter activity in AtT-20 cells and in a transformed hypothalamic cell line. *Endocrinology* **144**:1292–1300.
- Osei-Hyiaman D, DePetrillo M, Pacher P, Liu J, Radaeva S, Bätke S, Harvey-White J, Mackie K, Offertaler L, Wang L, et al. (2005) Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. *J Clin Invest* **115**:1298–1305.
- Osei-Hyiaman D, Harvey-White J, Bätke S, and Kunos G (2006) The role of the endocannabinoid system in the control of energy homeostasis. *Int J Obes (Lond)* **30** (Suppl 1):S33–S38.
- Padwal RS and Majumdar SR (2007) Drug treatments for obesity: orlistat, sibutramine, and rimonabant. *Lancet* **369**:71–77.
- Pagotto U, Marsicano G, Cota D, Lutz B, and Pasquali R (2006) The emerging role of the endocannabinoid system in endocrine regulation and energy balance. *Endocr Rev* **27**:73–100.
- Pi-Sunyer FX, Aronne LJ, Heshmati HM, Devin J, and Rosenstock J; RIO-North America Study Group (2006) Effect of rimonabant, a cannabinoid-1 receptor blocker, on weight and cardiometabolic risk factors in overweight or obese patients: RIO-North America: a randomized controlled trial [published erratum appears in *JAMA* **295**:1252]. *JAMA* **295**:761–775.
- Poirier B, Bidouard JP, Cadrouvele C, Marniquet X, Staels B, O'Connor SE, Janiak P, and Herbert JM (2005) The anti-obesity effect of rimonabant is associated with an improved serum lipid profile. *Diabetes Obes Metab* **7**:65–72.
- Ravinet Trillou C, Arnone M, Delgorge C, Gonalons N, Keane P, Maffrand JP, and Soubrie P (2003) Anti-obesity effect of SR141716, a CB1 receptor antagonist, in diet-induced obese mice. *Am J Physiol Regul Integr Comp Physiol* **284**:R345–R353.
- Ravinet Trillou C, Delgorge C, Menet C, Arnone M, and Soubrie P (2004) CB1 cannabinoid receptor knockout in mice leads to leanness, resistance to diet-induced obesity and enhanced leptin sensitivity. *Int J Obes Relat Metab Disord* **28**:640–648.
- Sarnataro D, Pisanti S, Santoro A, Gazzerò P, Malfitano AM, Laezza C, and Bifulco M (2006) The cannabinoid CB1 receptor antagonist rimonabant (SR141716) inhibits human breast cancer cell proliferation through a lipid raft-mediated mechanism. *Mol Pharmacol* **70**:1298–1306.
- Teixeira-Clerc F, Julien B, Grenard P, Tran Van Nhieu J, Deveaux V, Li L, Serriere-Lanneau V, Ledent C, Mallat A, and Lotersztajn S (2006) CB1 cannabinoid receptor antagonism: a new strategy for the treatment of liver fibrosis. *Nat Med* **12**:671–676.
- Van Gaal LF, Rissanen AM, Scheen AJ, Ziegler O, and Rössner S; RIO-Europe Study Group (2005) Effects of the cannabinoid-1 receptor blocker rimonabant on weight reduction and cardiovascular risk factors in overweight patients: 1-year experience from the RIO-Europe study [published erratum appears in *Lancet* **366**:370, 2005]. *Lancet* **365**:1389–1397.
- Williams CM and Kirkham TC (2002) Observational analysis of feeding induced by Delta9-THC and anandamide. *Physiol Behav* **76**:241–250.
- Xie S, Furjanic MA, Ferrara JJ, McAndrew NR, Ardino EL, Ngondara A, Bernstein Y, Thomas KJ, Kim E, Walker JM, et al. (2007) The endocannabinoid system and rimonabant: a new drug with a novel mechanism of action involving cannabinoid CB1 receptor antagonism—or inverse agonism—as potential obesity treatment and other therapeutic use. *J Clin Pharm Ther* **32**:209–231.
- Yan ZC, Liu DY, Zhang LL, Shen CY, Ma QL, Cao TB, Wang LJ, Nie H, Zidek W, Tepel M, et al. (2007) Exercise reduces adipose tissue via cannabinoid receptor type 1 which is regulated by peroxisome proliferator-activated receptor-delta. *Biochem Biophys Res Commun* **354**:427–433.

Address correspondence to: Dr. Pietro Formisano, Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università degli Studi di Napoli “Federico II”, Via Pansini, 5-80131 Napoli, Italy. E-mail: pfietro@unina.it