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“Molecular mechanisms of insulin resistance: role of endocannabinoid system in the regulation of glucose metabolism and insulin action”

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“Molecular mechanism of insulin resistance: role of endocannabinoid system in the regulation of glucose metabolism and insulin action”

TABLE OF CONTENTS

LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

1. Fiory F, Oriente F, Miele C, Romano C, Trencia A, Alberobello AT, **Esposito I**, Valentino R, Beguinot F, Formisano P. Protein kinase C-zeta and protein kinase B regulate distinct steps of insulin endocytosis and intracellular sorting. J Biol Chem 2004;279:11137-11145.

2. Fiory F, Alberobello AT, Miele C, Oriente F, **Esposito I**, Corbo V, Ruvo M, Tizzano B, Rasmussen TE, Gammeltoft S, Formisano P, Beguinot F. Tyrosine phosphorylation of phosphoinositide-dependent kinase 1 by the insulin receptor is necessary for insulin metabolic signaling. Mol Cell Biol 2005;25(24):10803-14.

3. Perfetti A, Oriente F, Iovino S, Alberobello AT, Barbagallo APM, **Esposito I**, Fiory F, Teperino R, Ungaro P, Miele C, Formisano P, Begiunot F. Phorbol esters induce intracellular accumalation of the antiapoptotic protein PED/PEA-15 by preventing ubiquitinylation and proteasomal degradation. J Biol Chem 2007;282(12):8648-57.

ABSTRACT

Molecular mechanisms of insulin resistance: role of endocannabinoid system in the regulation of glucose metabolism and insulin action

Hypothalamic endocannabinoids are considered to belong to the growing family of orexigenic mediators. The endocannabinoid system participates in the regulation of food intake and energy metabolism at both central and peripheral level. It has been shown that the endocannabinoids, anandamide and 2-arachydonoyl glycerol, stimulate feeding via activation of the cannabinoid CB1 receptors. SR141716 is a potent and selective central cannabinoid receptor antagonist, which also may act as an inverse agonist. It antagonises the hyperphagia induced by anandamide and 2-arachidonoyl glycerol. In addition to its effect on food intake, SR141716 decreases body weight and adiposity in diet-induced obese mice by increasing energy expenditure. In this work, I have investigated whether it may control glucose metabolism in skeletal muscle cells. Detectable levels of the endocannabinoid CB1 receptor were revealed in L6 cell model. However, differentiated myotubes displayed 35 and 45% reduction, respectively, of CB1 mRNA and protein levels, compared to undifferentiated myoblasts. Exposure of L6 myotubes to the metabolically stable endocannabinoid analogue 2-methyl-2'-F-anandamide (Met-F-AEA) inhibited 2-deoxyglucose (2-DG) uptake in a dose-dependent manner. At variance, the treatment with SR141716 increased 2-DG uptake. Protein expression profiling revealed that both the regulatory p85 and the catalytic p110 subunits of the phosphatidilinositol-3-kinase (PI3K) were increased by SR141716. No significant change of expression of other known PI3K downstream molecules and lipid phosphatases was observed. However, PDK-1, Akt/PKB and PKCζ activities were acutely induced following SR141716 treatment of L6 cells, and the effect was detectable up to 72 h. These stimulatory effects on PI3K expression and activity were largely prevented by H-89, an inhibitor of the cAMP-dependent protein-kinase A (PKA). Moreover, SR141716-stimulated 2-DG uptake was blunted by the co-incubation either with H-89 or with the PI3K inhibitor LY294002. Thus, pharmacological 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.

BACKGROUND

1. Insulin sensitivity and insulin-resistance

Over the course of evolution, organisms have evolved complex mechanisms to regulate fuel metabolism in response to food availability (Taylor 1999). Insulin is the principal hormone that coordinates these processes. When food is eaten, pancreatic β cells secrete insulin, which directs the body to store fuel. Insulin promotes the deposition of glycogen in liver and triglyceride in adipose tissue, and also activates glucose transport and glycogen synthesis in muscle.

1.1 Insulin action and glucose metabolism

Insulin stimulates glucose uptake into skeletal muscle tissue and into adipose tissue mainly through GLUT4 translocation from intracellular pools to the plasma membrane (Klip et al., 1993; Bryant et al., 2002; Saltiel and Pessin, 2002). Following insulin binding, insulin receptor tyrosine kinase undergoes autophosphorylation and catalyzes the phosphorylation of several intracellular protein substrates, such as members of IRS family (Insulin Receptor Substrate) and Shc (Src-Homology Collagen) proteins. These initial events generate multiple signalling cascades that mediate the final cellular responses to insulin (Pessin and Saltiel 2000; Van Obberghen et al. 2001). In particular, tyrosine phosphorylation of IRS-1 by insulin activates phosphatidylinositol 3-kinase (PI3-K) and induces activation of downstream signal molecules, such as protein kinase B (PKB/Akt) (Kohn et al., 1996; Tanti et al., 1997; Hill et al., 1999; Wang et al., 1999) and atypical PKCs (aPKCs) ζ and λ/ι (Bandyopadhyay et al.1999; Standaert et al., 1997; Kotani et al., 1998). It is plausible that PKCζ may be implicated in actin remodelling for the translocation of the insulin-sensitive glucose transporter GLUT4 in muscle cells (Liu et al. 2006) (Figure 1).

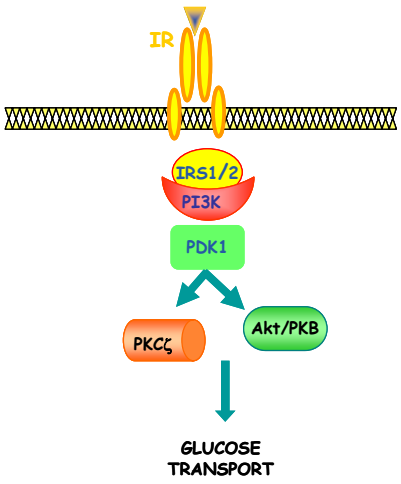


Figure 1: Molecular mechanism of insulin-stimulated glucose transport.

1.2 Insulin-resistance and type 2 diabetes

Insulin resistance is a rather complex condition, which is contributed to by genetic, nutritional and other environmental factors. Insulin resistance is a key factor in the pathogenesis of type 2 diabetes (T2D) and a co-factor in the development of dyslipidaemia, hypertension and atherosclerosis. Type 2 diabetes 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; Rizza et al. 1990; Weyer et al. 1999). It is generally acknowledged that the disease arises because of the progressive failure of endocrine pancreas to adequately cope with the increased insulin demand in insulin resistance states (Lazar 2005)

1.3 Molecular mechanisms of insulin-resistance

Resistance to insulin action is a common abnormality present in major human diseases such as diabetes mellitus and obesity. Insulin resistance in diabetes is genetically determined, but its incidence is also affected by environmental conditions and by factors secondary to diabetes itself (Kahn and Flier 2000). These acquired and secondary factors further impair insulin action in the diabetic individual. For instance, chronic hyperglycemia *per se* promotes insulin resistance (Hager et al. 1991; Davidson et al. 1994). A number of mechanisms have been proposed to explain hyperglycemia-induced insulin resistance. These include abnormalities in the PKC signalling system (Idris et al. 2002) and activation of the NF-κB transcription factors by chronically elevated glucose concentrations (Yerneni et al. 1999, Nishikawa et al. 2000). However, the molecular mechanism(s) through which hyperglycemia exacerbates insulin resistance in diabetes have only partially been elucidated. This occurs, at least in part, because of the increased production of advanced glycation end products (AGEs) (Vlassara 1997). We have previously shown that AGEs derange insulin signals, activating PKCα (Miele et al. 2003). During first two years of my doctorate programme, I have studied the molecular mechanism through which AGEs interfere with insulin action in L6 skeletal muscle cells. AGEs, binding their transmembrane receptor (RAGE, Receptor for AGEs), activate intracellular tyrosine kinase Src and cause an increase of Src-dependent diacylglycerol levels. These events concur to activate PKCα, which interacts with RAGE, IRS1 and Src (Submitted for publication). AGEs activated PKCα reduces IRS1 and IRS2 tyrosine phosphorylation and impairs insulin activation of glucose transport and of glycogen synthase (Miele et al. 2003). These novel findings indicate that AGEs induce formation of a multimolecular complex which includes RAGE, Src activated PKCα and IRS1. This complex may lead to the AGE-dependent inhibition of insulin action in L6 skeletal muscle cells. (Figure 2)

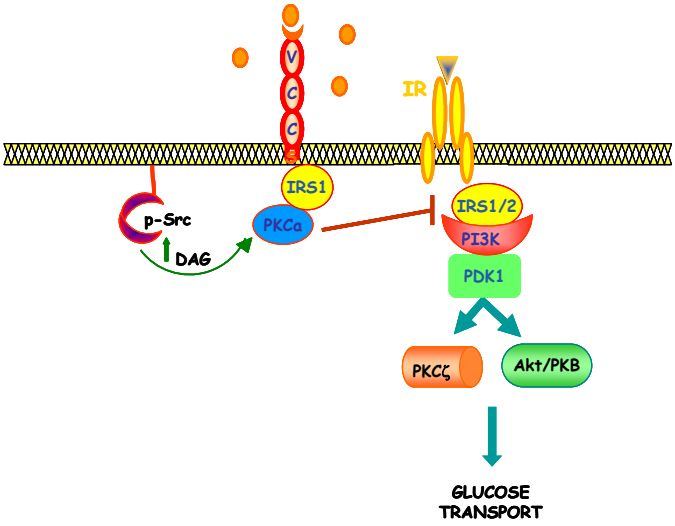


Figure 2: Proposed model for AGEs effect on insulin signalling.

Furthermore, obesity is thought to play a central role as a causative factor of insulin-resistance (Kahn and Flier 2000). Moreover, genetic and functional abnormalities found in obese individuals show a certain degree of overlap with those detected in type 2 diabetes patients, suggesting that common molecular events may contribute to the onset and/or progression of both disorders. Several factors have been identified through which obesity is thought to cause insulin resistance: free fatty acids (FFAs) (Randle et al. 1963; Boden 1997), tumour necrosis factor-α (TNF-α) (Hube and Hauner 1999; Sigal and Warram 1996) and resistin (Steppan et al. 2001). These defects may affect feeding behaviour, as well as energy expenditure and nutrients metabolism. Human obesity has been moreover associated with dysregulation of the peripheral and adipose tissue endocannabinoid system (Murdolo et al. 2007).

2. The endocannabinoid system

Recent studies have provided evidence that the endocannabinoid system has significant effects on energy balance and metabolism through the central control of appetite and by affecting peripheral metabolism (Despres 2007). The endocannabinoid system comprises two cannabinoid receptor subtypes, CB1 and CB2, their endogenous ligands [the endocannabinoids, N-arachidonyl ethanolamine, or AEA, named anandamide, and 2-arachidonoylglucerol (2-AG)], and enzymes for ligands biosynthesis and degradation, i.e. the *sn*-1-selective diacylglycerol-α (DAGLα), the monoacylglycerol lipase (MAGL), the *N*-arachidonoyl-phosphatidylethanolamine phospholipase D (NAPE-PLD) and the fatty acid amide hydrolase (FAAH) (Piomelli et al 1999, De Petrocellis et al.

2004, Pagotto et al. 2006). Elevated levels of the endogenus cannabinoids (anandamide –AEA- and 2-arachidonoyl-glycerol -2AG-) have been found in obese individuals (Engeli et al. 2005; Osei-Hyiaman et al. 2005) and correlate with intra-abdominal adiposity (Cote at al. 2007).

2.1 Endocannabinoid receptors

The endocannabinoid system controls food intake via both central and peripheral mechanisms, and it may also stimulate lipogenesis and fat accumulation. Exogenous cannabinoids and endocannabinoids increase food intake and promote weight gain by activating specific receptors. (Jamshidi and Taylor 2001; Williams and Kirkham 2002; Williams and Kirkham 1999; Cota et al. 2003). Two cannabinoid receptors have been identified and molecularly characterized so far, namely the seven transmembrane G protein-coupled cannabinoid receptor type 1 (CB1 receptor) and type 2 (CB2 receptor). CB1 receptor was originally described as the 'brain type' cannabinoid receptor, because its levels of expression were high in the brain (Herkenham et al. 1990). However, recent studies attribute new sites of action of endocannabinoids to many peripheral organs through CB1 receptor activation. In fact, CB1 receptor expression was found in pituitary, adrenal glands, reproductive organs, adipocytes, muscle and liver. The generalization for CB1 receptor being the eminent 'brain type' receptor is therefore no longer appropriate. CB2 receptor expression and action seem to be restricted to keratinocytes and immune and blood cells, where they may participate in regulating immune responses (Howlett et al. 2002; Ibrahim et al. 2005). Juan-Pico and coworkers have observed that 2-AG through CB2 receptors regulates $[Ca^{2+}]_i$ signals in β-cells and, as a consequence, it decreases insulin secretion (Juan-Pico et al 2006). Recently, Nakata and Yada have observed that CB1 receptor, but not CB2 receptor, was expressed in mouse pancreatic islets. Moreover, it seems that anandamide and CB1 agonists inhibit glucose-induced insulin secretion from mouse pancreatic islets. Both anandamide and CB1 agonist inhibit glucose-induced increases in $[Ca^{2+}]_i$ in mouse pancreatic β-cells (Nakata and Yada 2007).

The signal transduction of cannabinoids receptors has been extensively described. CB1 receptor activation might lead to the stimulation of different intracellular pathways, depending on the cell type involved and the experimental conditions. Intracellular effects of CB1 receptor stimulation include the regulation of the cAMP cascade, modulation of ion channels, stimulation of kinase pathways, and induction of immediate early genes. It has been described that activation of CB1 leads to the stimulation of $G_{i/0}$ proteins, that, in turn, inhibits the adenylate cyclase-mediated conversion of ATP to cAMP. cAMP molecules can bind the regulatory subunits of protein kinase A (PKA) and cause the liberation of the catalytic subunits. Activated PKA can phosphorylate A-type potassium (K^+_A) channels. $G_{i/0}$ activated by CB1 can also directly inhibit N- or P/Q-type channels and activate inwardly rectifying potassium (K_{ir}) channels. These last two effects are controlled by protein kinase C (PKC), which, after

activation, can phosphorylate CB1 in the third cytoplasmatic loop and uncouple the receptor from ion channels. Activation of CB1 can also stimulate several intracellular kinases, such as focal adhesion kinase (FAK), phosphatidyl inositol-3-kinase (PI3K) and its downstream effector protein kinase B (PKB/AKT), ERKs, c-Jun N-terminal kinase (c-JNK) and p38 MAPK (p38). Stimulation of cytoplasmatic kinases could also mediate the CB1-induced expression of the immediate early genes (IEG), such as the transcriptor factors c-fos, c-jun, and the brain-derived neurotrophic factor (BDNF) (Figure 3). However, CB1 receptor, which normally inhibits adenylate cyclase, can also stimulate the cAMP pathway in particular conditions (Glass and Felder 1997; Kearn et al. 2005). Moreover, recent results suggest the possibility of functional interactions of CB1 receptor with other receptors, for istance, with type 1 orexin receptor (Hilairet et al. 2003), $5HT_2$ serotonin receptors (Devlin and Christopoulos 2002), and dopamine receptor type 2 (D2) (Kearn et al. 2005). The possibility that such interactions depend on heterooligomerization processes might represent a very interesting novel aspect (Kearn et al. 2005), which will expand the view of the pharmacology and physiology of endocannabinoid system. Similar to CB1, CB2 receptors can modulate adenylyl cyclase and MAP kinase activity, through their ability to couple to $G_{i/0}$ proteins (Felder et al. 1995; Kobayashi et al. 2001). However, in contrast to CB1, CB2 receptor stimulation is believed not to modulate ion channel function (Felder et al. 1995), and CB2 does not couple to G_s (Glass and Felder 1997; Calandra et al 1999).

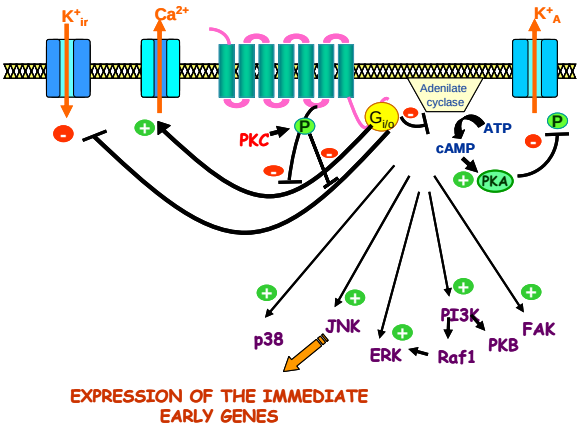


Figure 3: Signal transduction of CB1 receptor

2.2 Endocannabinoids and exogenous cannabinoids

Cannabinoid receptors are able to bind exogenous cannabinoids, such as Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive constituent of marijuana, and their endogenous ligands: the endocannabinoids (Mechoulam et al. 1995;

Matsuda et al. 1990; Munro et al. 1993; Devane et al. 1992; Sugiura et al. 1995). In 1992, the first endogenous cannabinoid, N-arachidonyl ethanolamine, or AEA, also called anandamide, was identified (Devane et al. 1992). Subsequently, a second endocannabinoid, 2-arachidonoyl glycerol (2-AG), was discovered (Mechoulam et al. 1995; Sugiura et al. 1995) (Figure 4). Both these compounds are derivatives of arachidonic acid and are able to bind to CB1 and CB2 receptors, although with difference in affinities and activation efficacies (Howlett 2002). During the last few years, several other bioactive lipid mediators have been described; they appear to act, at least in part, through CB1 and/or CB2 receptors and confer specific pharmacological effects *in vivo* (Bradshaw et al. 2005). Specifically, these compounds are 2-arachidonoyl-glyceryl-ether (noladin ether) (Hanus et al. 2001), O-arachidonoyl-ethanolamine (virodhamine) (Porter et al. 2002), N-arachidonoyl-dopamine (Huang et al. 2002), and possibly oleamide (Leggett et al. 2004). However, the endogenous function in physiological processes for all these latter compounds have not yet been established in detail and need further investigation (De Petrocellis et al. 2004).

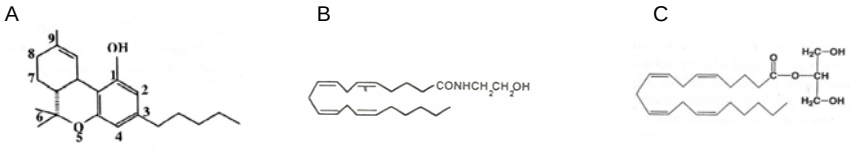


Figure 4: A. Δ^9-tetrahydrocannabinol (Δ^9-THC); B. Anandamide (AEA); C. 2-arachidonoil glycerol (2-AG)

Anandamide and 2-AG are synthesized via phospholipid-dependent pathway. The enzymes that catalyze the formation of anandamide and 2-AG from arachidonic acid are N-acylphosphatidylethanolamine-selective phospholipase D (NAPE-PLD) and diacylglycerol (DAG) lipase, respectively. Endocannabinoids are very lipofilic and thus cannot be stored in vesicles like other neurotrasmitters. Consequently, the regulation of endocannabinoid signalling is tightly controlled by their synthesis, release, uptake and degradation (Piomelli 2003). Several different stimuli, including membrane depolarization and increased intracellular Ca^{2+} and/or receptor stimulation, can activate complex enzymatic machineries, which lead to the cleavage of membrane phospholipids and eventually to the synthesis of endocannbainoids. Importantly, different enzymes are involved in the synthesis of distinct endocannabinoids, indicating an independent involvement of endocannabinoids in different conditions. After synthesis, endocannabinoids can activate cannabinoid receptors, either after previous release into the extracellular space or directly moving within the cell membrane. Endocannabinoid signalling is limited by very efficient degradation processes, involving facilitated uptake from the extracellular space into the cell and enzymatic catabolism mediated by specific intracellular enzymes. The enzymes

able to degrade endocannabinoids are quite well characterized. They are fatty acid amide hydrolase (FAAH) for anandamide and related compounds (Giang et al. 1997) and monoglycerol lipase for 2-AG (Dinh et al. 2002). An interesting aspect of endocannabinoids activity is the rapid induction of their synthesis, receptor activation and degradation (Piomelli 2003; Di Marzo 2004). The endocannabinoid system has thus been suggested to act on demand, with a tightly regulated spatial and temporal selectivity. The system exerts its modulatory actions only when and where it is needed. This fact poses an important distinction between the physiological functions of the endocannabinoid system (selective in time and space) and the pharmacological actions of exogenous cannabinoid agonists, which lack such selectivity. Concerning degradation of endocannabinoids, which represents an important regulatory aspect of the activity of the endocannabinoid system, it should also be mentioned that a recent study investigated whether endocytic processes are involved in the uptake of endocannabinoids and found that about half of the AEA uptake occurs via a caveola/lipid raft-related process (McFarland et al. 2004).

Several mechanisms underlying endocannabinoid-mediated signalling have been reported. 1) In the central nervous system (CNS), endocannabinoids can act as neurotrasmitters transferring information from one neuron to the next. Here, postsynaptic site where they activate CB1 receptors. They thus mediate a retrograde signal (Freund et al. 2003; Wilson and Nicoll 2002; Alger 2002). The overall effect is a decrease in the release of neurotrasmitters such as glutamate and GABA. This phenomenon is present in synaptic connections of many brain regions, thus representing an important modulatory mechanism of neuronal transmission. 2) Endocannabinoids may act in a paracrine or autocrine manner, not involving synaptic transmission. This is presumably for glial cells (Stella 2004) and in non-neuronal cells such as the adipocytes and the hepatocytes. 4) Because endocannabinoids and CB1 receptor are also present within the cell, it cannot be excluded that endocannabinoids may act as intracellular signalling molecules. Endocannabinoids appear to be very versatile signalling mediators, involved in a broad spectrum of physiological regulatory processes.

Pharmacological investigations have placed emphasis on the generation of substances acting as specific antagonists of cannabinoid receptors. Among the increasing number of compounds sharing CB1 receptor antagonistic properties (Lange and Kruse 2004; Gatley et al. 1996), the compounds most characterized are SR141716 (Figure 5) (Rinaldi-Carmona et al. 1994), SR14778 (Rinaldi-Carmona et al. 2004), AM251 (Gatley et al. 1996), AM281 (Lan et al. 1999), LY320135 (Felder et al. 1998), and SLV319 (Lange et al. 2004). The CB1 receptor antagonists known so far are diarylpyrazoles, or aminoalkylindoles, or triazole derivatives. Diarylpyrazoles include SR141716, which is the first selective CB1 receptor antagonist reported. It was discovered approximately a decade ago, and it has been the compound most studied so far. Pharmacologically, SR141716 shows a K_i value of binding to rat brain synaptosome of 1.98 ± 0.36 nM (Rinaldi-Carmona et al. 2004). Few data on the metabolism and pharmacokinetics of SR141716 are available in humans (Huestis et al. 2001). The dose of SR141716 that produced a 50% antagonism of agonist

effect in the mouse was 0.23 mg/Kg, and a dose of 3 mg/kg produces a long lasting (18 hrs) blockade of the effect of the agonistic agent WIN-55212-3 (Dutta et al. 1994). There are different possible mechanisms by which CB1 receptor antagonists produce their effects on the CB1 receptor (Pertwee 2005). The ligands can be competitive antagonists of CB1 receptor activation by endogenously released endocannabinoids, or they can act as inverse agonists and modulating constitutive CB1 receptor activity by shifting it from an active "on" to an inactive "off" state (Bouaboula et al. 1997). They may also act by CB1 receptor independent mechanisms (Pertwee 2005). These mechanisms are not mutually exclusive.

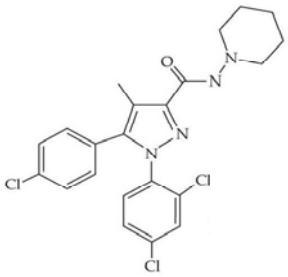


Figure 5: Chemical structure of SR141716 or rimonabant.

2.3 The functions of endocannabinoid system

The endocannabinoid system is involved in a plethora of physiological functions (Piomelli 2003; De Petrocellis et al. 2004). During the last few years, an overwhelming amount of data has been acquired to understand the biological roles of this system in more detail. However, many questions are still open, and promising new discoveries await us in the near future. In general, the endocannabinoid system is involved in many different physiological functions, many of which relate to stress-recovery systems and to maintenance of homeostatic balance (Di Marzo et al. 1998). Among other functions, the endocannabinoid system is involved in neuroprotection (Panikashvili et al. 2001; Marsicano et al. 2003; Panikashvili et al. 2005), modulation of nociception (Cravatt et al.2004), regulation of motor activity (van der Stelt and Di Marzo 2003), and the control of certain phases of memory processing (Wotjak 2005; Marsicano et al. 2002; Varvel and Lichtman 2002). In addition, the endocannabinoid system is involved in modulating the immune and inflammatory responses (Walter et al. 2003; Klein et al. 2003, Massa et al. 2004). It also influences the cardiovascular and respiratory systems by controlling heart rate, blood pressure, and bronchial functions (Mendizabal et al. 2003). Yet importantly, endocannabinoids are known to exert important antiproliferative actions in tumor cells (Bifulco and Di Marzo 2002). It has been known for a long time that exogenous cannabinoids are able to affect secretion of pituitary

hormones, thus having a strong effect on peripheral target organ functions. The hypothalamus is generally considered as the main site of cannabinoid action on neuroendocrine functions. Endocannabinoids act as retrograde messengers activating CB1 receptors expressed at presynaptic glutamatergic terminals in the hypothalamus (Di et al. 2003). The subsequent activation of the CB1 receptor signalling cascade leads to the inhibition of the release of the excitatory neurotransmitter glutamate onto the neuroendocrine cells of the PVN and the supraoptic nucleus (Di et al. 2003). This leads to a general suppressive effect on neuroendocrine cells and a final inhibitory effect on neuroendocrine function (Figure 6).

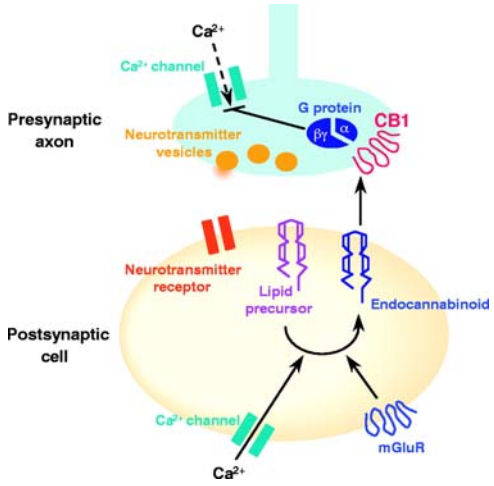


Figure 6: Endocannabinoids act at presynaptic CB1 receptors to inhibit transmitter release.

3. The role of endocannabinoid system in the modulation of energy balance

Several studies have been performed to prove the stimulating effect of cannabis on hunger in humans (Abel 1975). Greenberg et al. (Greenberg et al. 1976) were the first to systematically asses, under rigorous experimental conditions, the effect of a well-defined amount of Δ^9-THC in terms of changes in feeding behaviour and in body weight in humans. Both parameters increased after the first few days of the experiment. However, after this period, body weight continued to rise, whereas a stabilization of energy intake was observed. This pioneer study already suggested that the ability of cannabinoids to stimulate hunger may vanish with time, whereas a possible metabolic effect of the drug may remain active longer (Greenberg et al. 1976). Nonetheless, later studies did not investigate the metabolic idea further, preferring to concentrate interest on the ability of cannabis to stimulate hyperphagia and overconsumption of high palatable food at the central level. This hypothesis was substantiated by other studies later, demonstrating that increased total food intake particularly related to consumption of palatable food (sweet solid snack) was a main effect of smoked

marijuana (Foltin et al. 1987). The stimulating effect of cannabinoids on appetite observed in healthy subjects promoted assessment of the efficacy of a cannabinoid treatment for clinical syndromes featuring loss of appetite or weight, such as cancer or AIDS associated anorexia (Cat and Coleman 1994; Beal et al. 1997; Gorter 1999), or as adjuvant with most chemotherapeutic drugs (Sallan et al. 1975). The U.S. Food and Drug Administration officially approved the used of Δ^9-THC (commercially name Dronabinol) for the treatment of chemotherapy-induced nausea and vomiting refractory to other drugs, and for the treatment of patients with HIV-induced wasting syndrome.

3.1 Endocannabinoid functions at mesolimbic level

After the finding of the hyperphagic effect of Δ^9-THC mediated by CB1 receptor activation, Williams and Kirkham (Williams and Kirkham 1999) reported that endocannbainoids were also able to stimulate hunger in a dose-dependent manner. Importantly, the effect of AEA was completely blocked by pretreating the animals with SR141716, confirming the pivotal role of CB1 receptor activation in the hyperphagic effect of endocannbainoids (Williams and Kirkham 2002; Kirkham and Williams 2001). With the advent of CB1 receptor-specific antagonists, it became clear that, even when injected alone, these compounds are able to modify ingestive behaviour. The high expression of CB1 receptor in areas involved in reward, such as hypothalamus area, constitutes a strong indication that the endocannabinoid system is directly involved in various physiological functions controlled in these brain regions, including feeding (Breivogel and Childers 1998). In the hypothalamus endocannabinoid system interacts with the other reward pathways. The most relevant reward pathway is represented by the mesolimbic dopaminergic system. Both CB1 receptor and endocannabinoids are found in the rat limbic forebrain (Bisogno et al.1999), in which colocalization with dopamine D1 and D2 and CB1 receptor were described (Hermann et al. 2002). A relevant interplay also exists between the endocannabinoid system and the endogenous opioid peptides (Corchero et al. 2004). Both systems are linked to central reward processes, and there is increasing evidence supporting an important functional cross-talk between the two systems, in relation to a wide range of physiological processes, including appetite The existence of cross-talk between the endocannabinoid and opioid systems in controlling food intake was also confirmed by several studies in which naloxone and SR141716 synergistically depress food intake at doses that do not alter food intake on their own (Kirkham and Williams 2001; Rowland et al. 2001). According to the involvement of serotonin in the control of feeding behaviour (Vickers et al. 2001), the interaction of the endocannabinoid system with the serotoninergic system has also been investigated. However, the administration of a CB1 receptor antagonist in rats combined with dexflenfluramine, an anorectic drug stimulating the release of serotonin, led to additional but not synergistic effects on reducing food intake, which is consistent with the hypothesis that the two pathways work via independent mechanisms of

action (Rowland et al. 2001). This notion is important, because it makes it possible to exclude a synergistic effect in a possible future combination of antiobesity drugs, such as those inhibiting serotonin reuptake, like subutramine (Vettor et al. 2005) and CB1 receptor antagonists.

3.2 Endocannabinoid functions at hypothalamic level

A complex and redundant neuronal hypothalamic network provides high levels of adaptability of feeding behaviour to various central and peripheral stimuli (Flier 2004). Redundancy in appetite-stimulating signalling is conceivable in view of the vital importance of feeding for survival (Flier 2004). Signals coming from various peripheral organs, such as the liver, gastrointestinal tract, and adipose tissue, are conveyed mainly at the hypothalamic level to constantly inform the brain about the state of nutrition (Flier 2004; Wynne et al. 2005). An example of such peripheral control is the adipocyte-derived hormone leptin, which acts on receptors located in the hypothalamus (Flier 2004). A milestone in the identification of the endocannabinoid system as a new player in the regulation of food intake at hypothalamic level was the finding that leptin is a strong modulator of hypothalamic endocannabinoid levels (Di Marzo et al. 2001). Di Marzo et al. showed that acute leptin treatment reduced AEA and 2-AG not only in the hypothalami of normal mice but also in mice lacking leptin signalling. They also described the defect in leptin signalling as being constitutively associated with elevated hypothalamic levels of endocannabinoids. In these animals, SR141716 was able to reduce food intake, confirming the anorectic properties of the compound (Di Marzo et al. 2001). These findings suggest that, at least in genetically modified animal models, obesity is associated with a chronic hypothalamic overactivation of the endocannabinoid system, which may in turn explain the hyperphagic behaviour of the animals having leptin signal impairment.

3.3 The peripheral effect of the endocannabinoid system in the modulation of metabolic functions

Several lines of evidence are currently converging, indicating that the effects of CB1 receptor blockade on food intake and body weight are not limited to a central mode of action. In the last two years, the use of $CB1^{-/-}$ mice has represented an important tool to substantiate further the hypothesis of an additional effect of endocannabinoids in peripheral organs. Indeed, the lack of CB1 receptor in mutant mice causes hypophagia and body fat reduction. Importantly, pair-feeding experiments showed that in young $CB1^{-/-}$ mice, the lean phenotype is predominantly caused by decreased caloric intake, whereas in adult $CB1^{-/-}$ mice metabolic factors appear to be the major cause of the lean phenotype. These experiments therefore suggested that the endocannabinoid system might regulate central food intake related mechanisms at young ages, but that this

function diminishes with age (Cota et al. 2003). These observations converge on the idea that additional peripheral food intake-independent metabolic functions may participate, or even predominate, in the control of energy balance exerted by the endocannabinoid system (Cota et al. 2003). Even more prominent differences in terms of body weight regulation are obtained when a high-fat diet is administered to adult $CB1^{-/-}$ mice and wild-type littermates. In contrast to wild-type littermates, $CB^{-/-}$ mice do not display hyperphagia or reduction of their relative energy intake and were resistant to diet-induced obesity (DIO) (Ravinet-Trillou et al. 2004). Importantly, the obesity-prone diet induced a significant increase of fasting glycemia in the two genotypes, but the sensitivity to insulin remained unchanged in $CB1^{-/-}$ mice, whereas it was significantly reduced in the wild-type animals (Ravinet-Trillou et al. 2004). The expression of CB1 receptor in adipocytes and the ability of SR141716 to block lipogenesis stimulated by cannabinoids represent a first important step forward in understanding the peripheral mechanisms of action of the endocannabinoid system in regulating metabolic processes (Cota et al. 2003). Importantly, a recent study shed further light on the mechanisms of action of the endocannabinoid system on adipose tissue. By using SR141716 in DIO mice, Jbilo *et al.* (Jbilo et al. 2005) were able to reverse the phenotype of obese adipocytes at both macroscopic and genomic levels. They showed that a major restoration of white adipocyte morphology similar to lean animals occurred in adipocytes derived from obese animals after CB1 antagonist treatment. More importantly, they found that the major alterations in gene expression levels induced by obesity in white adipose tissue were mostly reversed in SR141716-treated obese mice. Importantly, the transcriptional patterns of treated obese mice were similar to those obtained in the $CB1^{-/-}$ mice fed with a high-fat diet, supporting a CB1 receptor-mediated process. Functional analysis of these modulations indicated that the reduction of adipose mass by the drug was due to enhanced lipolysis through the induction of enzymes of the β-oxidation and tricarboxylic acid cycle; increased energy expenditure, mainly through futile cycling (calcium and substrate); and a tight regulation of glucose homeostasis. In particular, in this last context the SR141716-induced increased expression of glucose transporter 4 (GLUT4), the insulin-responsive glucose transporter, appears very important (Jbilo et al. 2005). This finding makes it possible to hypothesize that cannabinoid antagonists may also be attractive drugs in fighting diabetes. Altogether, these data confirmed that the endocannabinoid system has a major role in the regulation of energy metabolism in adipocytes. Importantly, CB1 receptor expression has been found to be higher in adipocytes derived from obese animals compared with lean controls (Bensaid et al. 2003). Similar to the finding of higher levels of endocannabinoids in the hypothalamus derived from obese animals, the overexpression of CB1 receptor in adipocytes of obese rats seems to confirm the notion that hyperactivity of the endocannabinoid system is associated with the obesity state. Finally, the increase in levels of adiponectin in Zucker obese rats chronically treated with SR141716 *in vivo* (Bensaid et a. 2003) points to a close relationship between CB1 receptor blockade and the production of this antiatherogenic and antidiabetic adipocyte-derived protein (Chandran et al. 2003). The quick and strong improvement of

hyperinsulinemia detected after a very short-term treatment with SR141716 (4 d) in obese Zucker rats was also attributed to an increase in adiponectin (Bensaid et al. 2003). However, the well-known reduction in food intake and the consequent body weight loss displayed at the beginning of SR141716 treatment may be the most obvious explanation for the changes in adiponectin levels. The ability of long-term treatment with SR141716 to enhance the circulating levels of adiponectin was further confirmed in DIO mice (Poirier et al. 2005). In the last few years, several studies using different CB1 receptor antagonists confirmed the hypothesis that a potential peripheral mode of action of pharmacological CB1 receptor blockade may play a relevant role in the final weight loss effect. Very recently, Poirier et al. (Poirier et al. 2005) monitored weight and metabolic marker changes in three groups of mice after establishing a condition of obesity by a 5-month high-fat diet. Two groups of animals were maintained on a high-fat diet, but one was treated for 10-weeks with 10 mg/kg SR141716 and the other one with a vehicle. A third group received a dietary switch to standard food after the 5 months on a high-fat diet. SR141716 induced a weight loss of approximately of 78% in comparison to the weight of the animals receiving the vehicle. More importantly, the antiobesity effect of the drug was equivalent (both in terms of time course and maximum effect) to that achieved by switching obese mice to a normal diet (Poirier et al. 2005). Again, the authors demonstrated that the anorectic effect of the CB1 receptor antagonist vanished with time because the energy intake in the SR141716-treated animals was equivalent to animals on a high-fat diet during the last 6 weeks of the experiment and significantly greater than in the group receiving standard diet. Consistent with a previous report (Ravinet-Trillou et al. 2003), the SR141716-induced weight loss was accompanied by normalization of leptin, insulin, and glucose levels (Poirier et al. 2005). Notably, SR141716 also normalized triglycerides and low-density lipoprotein-cholesterol. Moreover, the high-density lipoprotein (HDL)-cholesterol/low-density lipoprotein-cholesterol ratio after SR141716 treatment was significantly higher than in the other two groups (Poirier et al. 2005). Whether this effect on lipid metabolism is indirectly related to an elevation of adiponectin is still a matter of debate.

Moreover, Liu et al. (Liu et al. 2005) found that a 7-days treatment with SR141716 induces an increase in basal oxygen consumption compared with the vehicle in ob/ob mice. The authors were not able to identify the mechanism by which SR141716 treatment is able to affect energy expenditure. A start on clarifying the molecular mechanism by which treatment with SR141716 may favor thermogenesis has been made with the microarray experiment performed by Jbilo et al. (Jbilo et al. 2005). These data suggest that the cannabinoid antagonist treatment is able to stimulate the expression of genes favoring energy dissipation through mitochondrial heat production in brown adipose tissue (Jbilo et al. 2005). Liu et al. (Liu et al. 2005) also showed that a 7-days treatment of SR141716 induces a significant increase in glucose uptake in isolated soleus muscle. This activity might contribute to the improved hyperglycemia seen after SR141716 treatment in previous studies.

Hepatocytes, key players in the metabolic processes, were not considered as a target of endocannabinoid action for a long period of time. Very recently, Osei-Hyiaman et al. (Osei-Hyiaman et al. 2005) strongly substantiated this hypothesis by a series of experiments in which they identified the liver as a primary site for endocannabinoid-mediated modulation of lipogenesis. In fact, probably via inhibition of adenylate cyclase, the cannabinoid agonist HU210 stimulates the expression of several genes involved in the *de novo* synthesis of fatty acids, such as lipogenic transcription factor SREBP-1c and its targets acetyl-CoA carboxylase-1 and fatty acid synthase. The inhibition of this lipogenic response by SR141716 and its absence in $CB1^{-/-}$ mice confirms the lipogenic role of CB1 receptors localized in hepatocytes. High-fat diet also induces an increase in the number of CB1 receptors and in hepatic levels of AEA, strongly suggesting that the blockade of the endocannabinoid system plays an important protection against the pathological consequences of a fat diet in the liver (Osei-Hyiaman et al. 2005). These data pave the way to hypothesize the clinical use of CB1 antagonists in preventing or reversing the development of fatty liver.

4. Cannabinoid receptor antagonist: SR141716 or Rimonabant

The whole body of data mentioned above highlights the role of the endocannabinoid system in feeding and energy balance regulation. Indeed, it was reasonable to hypothesize a therapeutic role for cannabinoid antagonists in the treatment of obesity. SR141716, also named rimonabant (commercialized as Acomplia), underwent multicenter randomized, double-blind phase III trials to assess the effects on weight loss in obese patients with or without comorbidities with dyslipidemia and with type 2 diabetes (Fernandez and Allison 2004).

The CB1 receptor antagonist rimonabant was initially tested in humans not as an antiobesity drug but for its potential ability to reduce subjective intoxication and tachycardia in healthy subjects with a history of marijuana use or as an antipsychotic agent in schizophrenic patients.

The most promising data seem to derive from rimonabant as a treatment for obesity. Concerning studies in humans, a very recent report (Sipe et al. 2005) confirms, on a genetic basis, the possible association between the chronic pathological overactivation of the endocannabinoid system and the development of obesity. In fact, in a large cohort of Caucasian and black subjects, overweight and obesity have been found to be associated with a polymorphism in FAAH. This genetic variant predicts a substitution of threonine for a highly conserved proline residue (P129T). It has been observed that patients carrying this polymorphism may have approximately half the enzymatic activity of FAAH. This may lead to a reduced inactivation of AEA and, eventually, to an inappropriate chronic increase of endocannabinoid tone (Sipe et al. 2005). In such a context, a recent work (Engeli et al. 2005) showed increased circulating levels of AEA and 2-AG in obese women when compared with a lean control group. Moreover, in the same study, a marked downregulation of FAAH gene expression in adipose tissue of

obese women has been found, suggesting that the increased endocannabinoid levels may be secondary to decreased enzymatic degradation (Engeli et al. 2005).

A large phase III trial named as RIO (rimonabant in obesity) was initiated in August 2001 including more than 6600 overweight or obese patients (Fernandez et al. 2004). Rimonabant treatment was well tolerated, and the most common adverse events experienced with 20 mg rimonabant were gastrointestinal symptoms such as nausea and diarrhea and mood disorders such as anxiety and depression. However, the effects were found to be mild, and the discontinuation rate due to these events was similar between patients taking 20 mg rimonabant or placebo. The genesis of these adverse events might be explained by bearing in mind that CB1 receptor plays a role in gastrointestinal motility and in hypothalamic-pituitary-adrenal axis activation. Nausea and diarrhea on the one hand and anxiety and depression on the other hand might be due to CB1 receptor pharmacological blockade. Nevertheless, it has recently been observed that patients taking the weight loss pill Acomplia reported twice as many psychiatric side effects, including depression, anxiety and sleep problems, than those who received a placebo. So, the drug has not been approved by Food and Drug Administration (FDA) in the United States. All studies have already been concluded, and some of them are already reported in the literature (Cleland et al. 2004; Van Gaal et al. 2005). Two of these studies, named RIO-North America and RIO-Europe, recruited obese and overweight patients with or without comorbidities who were treated for 2 years with 5 or 20 mg rimonabant *vs.* placebo. RIO-Lipids and RIO-Diabetes are the other two clinical trials with rimonabant aimed at investigating the amelioration, after treatment with the CB1 receptor antagonist, of specific comorbidity factors associated with obesity or overweight such as hyperlipidemia and diabetes. Most patients who completed treatment with 20 mg/ml rimonabant achieved from 5% to 10% weight loss. The pattern of weight loss appeared to be sustained for up to 36-40 weeks. Rimonabant was associated with a significant improvement of lipid and glycemic profile an important and significant reduction in waist circumference, tryglicerides, and C reactive protein, whereas a significant increase in HDL-cholesterol was found in the 20-mg treatment group compared with the group of patients undergoing placebo treatment. Chronic blockade of CB1 receptors in obese animals and humans also reduces, when present, the signs of the 'metabolic syndrome' as first defined by the National Cholesterol Education Program Adult Treatment Panel III (National Institutes of Health, 2001), i.e. high waist circumference (abdominal obesity), high triglyceridemia, low HDL cholesterol, high mean arterial pressure and high fasting glycaemia (and subsequently hyperinsulinemia) (Ravinet et al. 2003; Poirier et al. 2005; Van Gaal et al. 2005). The study of Van Gaal et al. (Van Gaal et al. 2005) demonstrated that rimonabant adds a further important and significant weight independent effect on lipid parameters to the positive effects derived from weight loss and waist reduction. In fact, as determined by statistical analysis, the effect of 20 mg rimonabant on both HDL-cholesterol and tryglicerides at 12 months has been shown to be partly independent of weight loss, being 60% of the increase in HDL-cholesterol and 45% of the reduction in trygliceride accounted for by weight loss, and the

remainder due to reasons not related to body weight changes (Van Gaal et al. 2005). Although Van Gaal et al. (Van Gaal et al. 2005) proposed that a rise in adiponectin might be responsible for these relevant positive changes in lipid profile, other mechanisms might enter into play.

One of the main objectives of this thesis is to understand if and how rimonabant may directly act on peripheral tissues, such as skeletal muscle.

AIM OF THE STUDY

The endocannabinoid system plays an important role in the regulation of energy balance (Pagotto et al. 2006). Recent studies, both in animal models and in humans, have shown that endocannabinoids and exogenous cannabinoids increase food intake. Pharmacological CB1 blockade with SR141716 reduces this hyperphagic effect, suggesting that CB1 is involved in the regulation of food intake (Williams and Kirkham 2002). Engeli and co-workers (Engeli et al. 2005) have shown that CB1 receptor is expressed in some peripheral human tissue relevant to the pathogenesis of obesity-associated metabolic disorders and also has observed an increased circulating AEA and 2-AG levels in obese women. Several lines of evidence suggest a peripheral role for endocannabinoids in the regulation of lipogenesis and body weight in adult animals. SR141716 consistently alters hormone and serum lipid levels associated with animal models of obesity (Bensaid et al. 2003; Liu et al. 2005).

However, it is not known whether these additional beneficial effects are merely the consequence of weight loss or also the result of peripheral action of CB1 blockers exerted directly on cells involved in lipogenesis and insulin production, i.e. white adipose tissue and pancreatic islet β-cells, respectively. White adipocytes express functional CB1 receptors, whose levels are higher in obese rats whose blockade leads to increased levels of adiponectin (Cota et al. 2003; Bensaid et al. 2003), an adipokine that enhance insulin sensitivity and glucose and lipid metabolism (Chandran et al 2003; Gil-Campos et al. 2004). The presence of CB1 mRNA in fat tissue from wild-type mice is consistent with a direct role of endocannabinoids in the regulation of lipogenesis. It has been also observed that 2-AG through CB1 and CB2 receptors regulates $[Ca^{2+}]_i$ signals in β-cells and, as a consequence, it decreases insulin secretion (Nakata and Yada 2007; Juan-Pico et al 2006).

Finally, little is known about CB1 receptor expression in the muscle. It is expressed in the murine soleus muscle as shown by Pagotto and co-workers (Pagotto et al 2006). Recently, Liu et al. has shown that SR141716 may directly affect glucose uptake in the isolated soleus muscle of genetically obese mice (Liu et al. 2005). The molecular mechanism of SR141716 induced glucose uptake remains unclear. Because of muscle is involved in insulin sensitivity and in glucose metabolism, the aim of my study has been to investigate the molecular mechanism through which SR141716 is able to stimulate glucose uptake in a skeletal muscle cell model, the L6 cells.

MATERIALS AND METHODS

Materials

Media, sera and antibiotics for cell cultures were from Invitrogen Ltd. (Paisley, United Kingdom). Phospho-PDK1, phospho-Ser473 PKB and phospho-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-PKCζ/PKCζ, p110, PTEN, SHIP2, PKB and phospho-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.). [γ-^{32}P] ATP (3,000 Ci/mmol), D-[U-^{14}C] Glucose (3mCi/mmol) and ECL reagents were from Amersham Biosciences (Arlington Heights, Ill.). Met-F-AEA and SR141716 were the generous gifts of Prof. Bifulco (Università degli Studi di Salerno, SA, Italy). Other reagents were from Sigma.

Methods

Cell Culture

L6 rat skeletal muscle cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 2 mM glutamine, 10,000 U/ml penicillin and 10 mg/ml streptomycin in a humidified CO_2 incubator. Cultures were maintained at 37 °C, in a humidified atmosphere containing 5% (v/v) CO_2. Cells were treated with 100 nM insulin, with different concentrations of Met-F-anandamide (0.1 μM, 1 μM, 10 μM) and of SR141716 (0.1 μM, 1 μM, 10 μM) for different time. For PI3K inhibition studies, cells were pretreated with 10 μM LY294002 for 30 minutes followed by combined treatment with LY294002 and 0.1 μM SR141716 for 16 hrs. For PKA inhibition studies, cells were pretreated with 15 μM H-89 for 30 minutes followed by combined treatment with H-89 and 0.1 μM SR141716 for 24 hrs. To study SR141716 effect on protein synthesis, the cells were incubated with 40 μg/ml cicloeximide in presence of 0.1 μM SR141716 for 24 hrs.

Western blot analysis and Immunoblotting

For Western blotting, the L6 cells, in presence or not of serum (16-24 hrs of starvation), after treatment with anandamide and SR141716, were solubilized in lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 50 mM NaF, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 100 μg of aprotinin/ml, 1 mM leupeptin) for 60 min at 4 °C. The lysates were clarified by centrifugation at 5,000 × *g* for 20 min at 4 °C. Solubilized proteins were then separated by SDS-PAGE and transferred onto 0.22 μm pore-size Immobilon-P membranes (Millipore Corp., Bedford, Mass). 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. Upon incubation with the primary and secondary antibodies,

immunoreactive bands were detected by ECL according to the manufacturer's instructions.

2-deoxy-D-glucose uptake

Cells were incubated in serum free Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.25% (w/v) bovine serum albumin for 18 hrs in the presence or absence of anandamide and SR141716 at different concentrations. The medium was removed and cells were further incubated for 30 min in glucose-free HEPES buffer (20 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $MgSO_4$, 5 mM KCl, 1 mM $CaCl_2$) and exposed or not to 100 nM insulin. Glucose uptake was measured by incubating cells with 20μM 2-deoxy-D-[^{3}H]glucose (1 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.

Real-time PCR

To analyse CB1 receptor expression, total RNA was isolated from L6 cells by using the Rneasy Kit (Qiagen Sciences) according to the manifacturer's instruction. For real.time RT-PCR analysis, 1 μg cell RNA was reverse transcrived 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'

Densitometry and statistical analysis

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.

Results and Discussion

Effect of CB1 modulation on glucose uptake

The Endocannabinoid system (ECS) is a crucial regulator of energy balance (Pagotto et al. 2006; Osei-Hyiaman et al. 2006). 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). However, whether the regulation of glucose metabolism represents a direct action of ECS targeting in peripheral tissues or a mere consequence of weight loss remains to be defined. We have addressed this issue by studying the effect of CB1 modulation on glucose uptake in the L6 cells, a well characterized model of differentiating skeletal muscle cells (Yaffe 1968; Klip et al. 1982).

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 cells. Detectable levels of the CB1 mRNA (Fig. 7) and protein (Fig. 8A, 8B) were observed. However, in the differentiated myotubes, CB1 mRNA and protein abundance was reduced by 35% ($P < 0.01$) and 45% ($P < 0.001$), respectively, compared to the undifferentiated myoblasts.

A

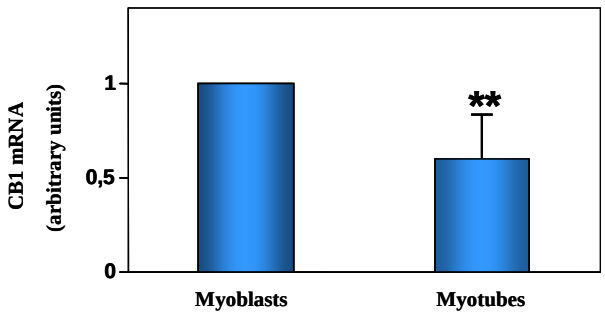


Figure 7: CB1 mRNA expression in myoblasts and myotubes. A. The abudance of mRNA for CB1 was determined by real-time PCR analysis of total RNA isolated from L6 myoblasts and L6 myotubes. Bars represent the mRNA levels in myotubes and are relative to those in myoblasts. Data are expressed as means ± SD of triplicate reactions for total RNAs from each cell type in five independent experiments. Asterisks indicate statistically significant differences (**, $p<0.01$).

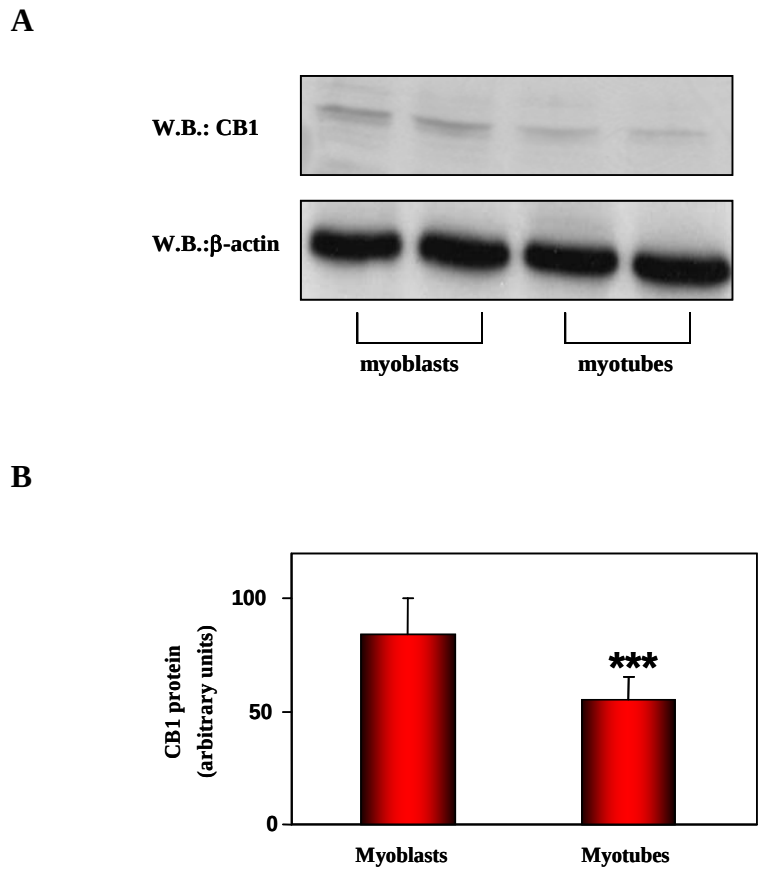


Figure 7: CB1 protein abundance in myoblasts and myotubes. A. Myoblasts and myotubes were solubilized and cell lysates were separated on SDS-PAGE and immuno-blotted with CB1 Ab. **B.** Filters obtained in B have been analyzed by laser densitometry. Asterisks indicate statistically significant differences (***, p<0.001).

Next, 2-deoxy-glucose (2-DG) uptake was measured in the myotubes in the absence or in the presence of increasing concentrations (0.1, 1.0 and 10 μM) of the metabolically stable anandamide analogue, 2-methyl-2'-F-anandamide (Met-F-AEA), and of the CB1 receptor inverse agonist SR141716 (Fig. 9A). Pre-incubation of L6 myotubes with Met-F-AEA for 16 hrs led to a dose-dependent inhibition of 2-DG uptake up to 50% decrease (at 1 μM Met-F-AEA, $P < 0.05$; at 10 μM Met-F-AEA, $P < 0.001$) compared to the basal levels. At variance, treatment of the cells with 0.1 μM SR141716 led to a significant 50% increase of 2-DG uptake ($p<0.001$), only slightly lower than that observed upon acute insulin stimulation (100 nM for 30 min). Raising SR141716 concentrations led to a

progressive reduction of 2-DG uptake, however, consistent with a partial agonist effect.

Time-course analysis revealed that the effect of 0.1 μM SR141716 was rapidly induced upon 30 min treatment and persisted up to 16 hrs (Fig. 9B) and beyond (up to 72 hrs, data not shown). At variance, earlier times of Met-F-AEA incubation (up to 5hrs) did not significantly change glucose uptake in L6 cells (Fig. 9B).

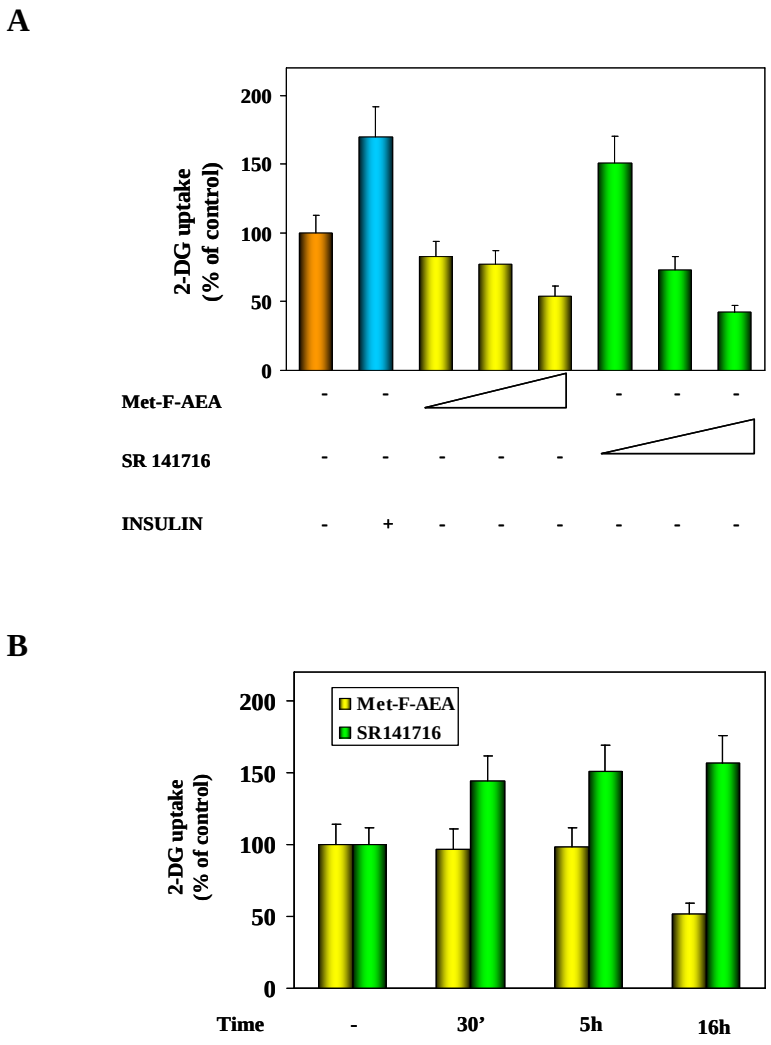


Figure 9: Glucose uptake upon Met-F-AEA and SR141716 incubation. A. Myotubes were exposed to 100 nM insulin and to raising concentrations of Met-F-AEA or of SR141716 for 16 hrs. Then the cells were assayed for 2-DG uptake. Bars represent mean ± SD of three different experiments in triplicate. **B.** .The cells were treated with 10 μM Met-F-AEA or with 0.1 μM SR141716 for different times. 2-DG uptake assay was performed as described in Materials and Methods. Bars represent mean ± SD of three different experiments in triplicate.

Thus, exposure of the myotubes to the anandamide analogue reduced glucose uptake in a dose-dependent manner. Conversely, the treatment with SR141716 significantly increased glucose uptake. This represents the first evidence, at the best of our knowledge, of a direct effect of CB1 modulation on glucose metabolism in skeletal muscle cells. 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 (Di et al. 2003; Wilson and Nicoll 2002; Williams and Kirkham 1999), and similar as in liver cells and adipocytes (Flier 2004; Wynne et al. 2005; Di Marzo et al. 2001), ECS may directly modulate nutrient metabolism in the skeletal muscle.

In particular, we show that CB1 exerts an inhibitory function on glucose uptake. Elevated levels of endocannabinoids, which have been found in obese animal models (Matias et al. 2006) and humans (Engeli et al. 2005; Bluher et al. 2006) may enhance CB1 activity and interfere with glucose metabolism in muscle cells. At the opposite, the pharmacologic block of CB1 by SR141716 enhances glucose uptake. 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. Intriguingly, however, 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 et al. 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 (data not shown). Consistent with its negative role in glucose uptake, CB1 expression decreases upon differentiation of L6 cells. In myotubes, which display a larger glucose uptake capacity, CB1 mRNA and protein levels are lower than those detected in undifferentiated myoblasts.

Protein expression profile upon CB1 modulation

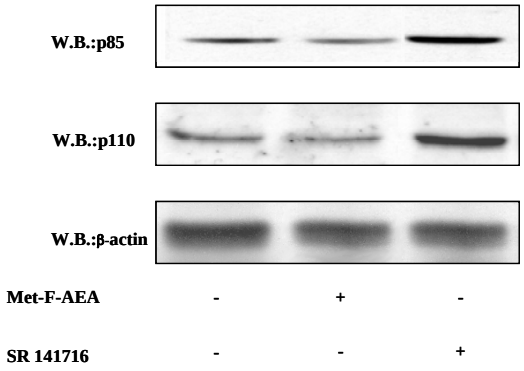
In order to address the mechanism by which CB1 may regulate glucose uptake in the L6 cells, protein lysates were obtained following treatment either with 10 μM Met-F-AEA or 0.1 μM SR141716 for 16hrs. Protein expression profiling was achieved by immunoblot with specific antibodies (Table I).

Protein	Met-F-AEA (% of control)	SR141716 (% of control)
IR	100	108
IRS-1	105	107
IRS-2	103	100
p85	37***	196***
p110	63*	168*
PDK1	100	99
PKCζ	109	112
AKT/PKB	77	123
PTEN	105	107
GLUT1	108	100
GLUT4	110	108

Table 1: The cells were incubated with 10 μM Met-F-AEA or with 0.1 μM SR141716 for 16 hrs. Filters were immuno-blotted with specific antibodies. Then they were analyzed by laser densitometry. The values in the table represent mean of three independent experiments in duplicate. Asterisks denote statistically significant difference of the samples obtained from treated vs. untreated cells (* $P < 0.05$; *** $P < 0.001$).

The intracellular content of the regulatory (p85α) and the catalytic (p110α) subunits of class I PI3K was decreased by 63% and 37%, respectively upon Met-F-AEA exposure (Table I and Fig. 10A). SR141716 increased by 2.0 and 1.7-fold, respectively, the expression levels both of the p85 and of p110. No significant changes, instead, were detected for the insulin receptor, IRS-1 and -2, the phosphoinositides-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 reciprocally changed by Met-F-AEA and SR141716, although differences did not reach statistically significant values (Table I). We then evaluated the timing of PI3K regulation by CB1 modulators. The treatment of L6 cells with SR141716 for 5 hrs led to a slight increase of p85 expression, which raised up to 24h and remained stable up to 72 hrs (Fig. 10B). At the opposite, Met-F-AEA treatment led to a progressive decrease of p85 expression (up to 48 hrs). No detectable change was observed upon 30 min exposure to SR141716 or to Met-F-AEA (Fig. 10B). Very similar results were obtained with p110 (data not shown).

A



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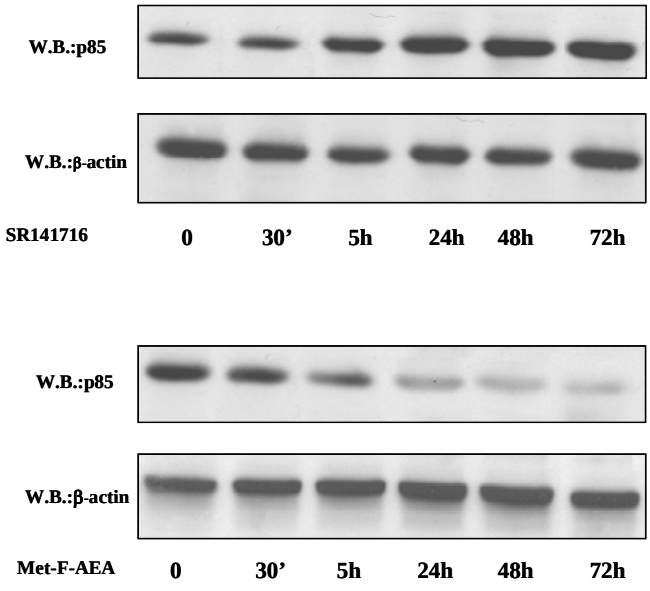


Figure 10: p85 and p110 regulation by Met-F-AEA and by SR141716. A. L6 myotubes were serum starved and incubated with 10 μM Met-F-AEA or with 0.1 μM SR141716 for 16 hrs. Cell lysates were then analyzed by p85, p110 and β-actin immunoblot. **B.** Cells were treated with 0.1 μM SR141716 (upper panel) or with 10 μM Met-F-AEA (lower panel) for different times. Cell lysates were then analyzed by p85 and β-actin immunoblot as indicated.

Regulation of p85 expression by CB1 activity

In order to assess whether CB1-mediated regulation of p85 expression occurred at the level of protein synthesis, L6 cells were treated with cycloheximide (CHX). At the baseline, CHX treatment reduced p85 cellular abundance (Fig. 11A). The treatment with SR141716 was still able to increase p85 immuno-detection, indicating that regulation occurred, at least in part, at post-translational level.

It has been previously shown that CB1 receptor may regulate cAMP intracellular levels and protein kinase A (PKA) activity (Howlett et al. 1986; Bidaut-Russell et al. 1990). Indeed, in parallel with increased p85 expression, SR141716 induced the phosphorylation of CREB on ser 133 in the L6 cells (Fig. 11B). We have therefore tested whether PKA inhibition reverted SR141716 effect on p85. To this end, L6 cells were treated with H-89 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. 11B).

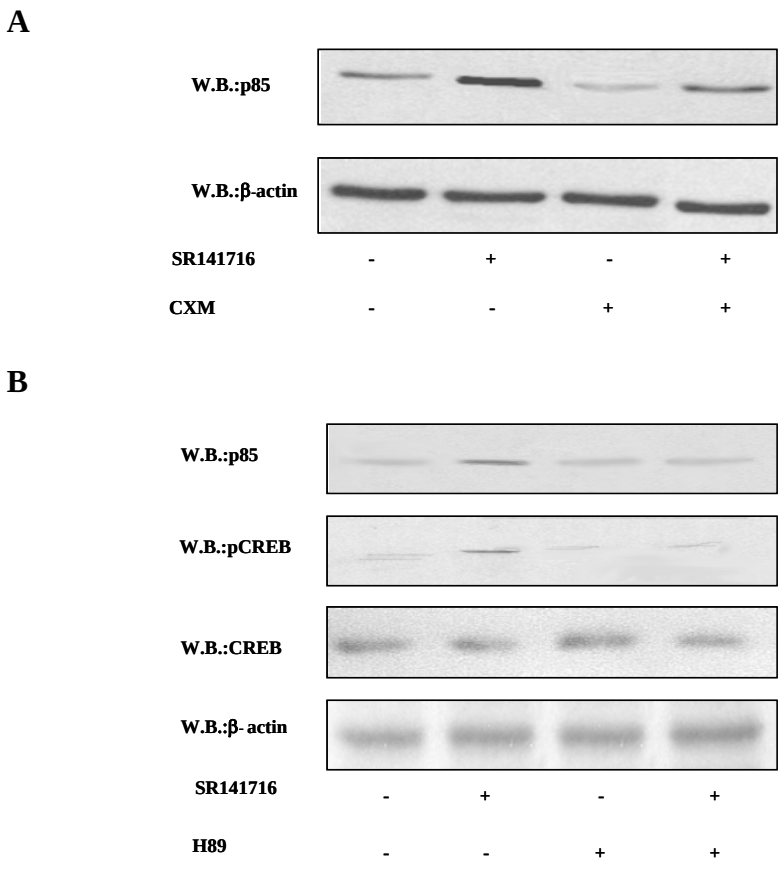
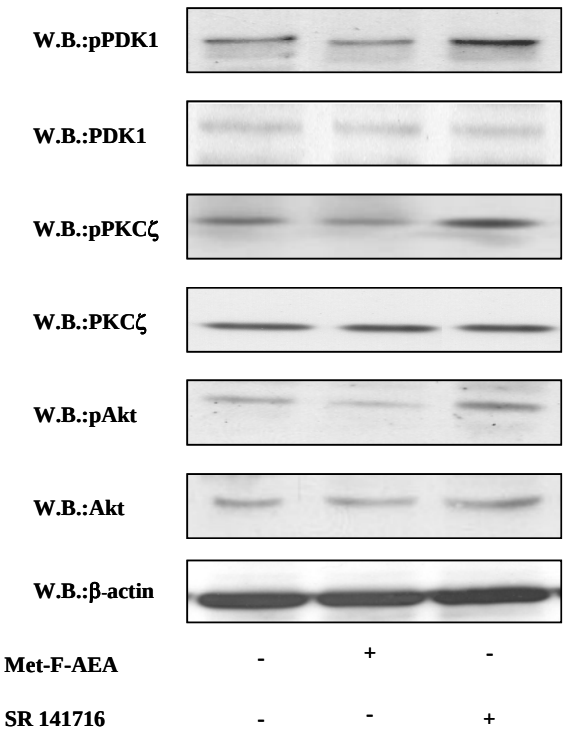


Figure 11: SR141716 regulates p85 levels via PKA. A. Cells were serum starved and incubated with 40 μg/ml cycloheximide (a protein synthesis inhibitor) in absence or in presence of SR141716 for 24 hrs. Cell lysates were then analyzed by immunoblot with p85 and β-actin antibodies. **B.** Cells were pretreated with 15 μM H-89, a PKA inhibitor, and incubated with 0.1 μM SR141716 for 24 hrs. Cell lysates were then analyzed by immunoblot with p85, CREB, pCREB and β-actin antibodies.

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 Met-F-AEA (10 μM) or with SR141716 (0.1 μM). Immuno-detection of the phosphorylated forms of PDK1, Akt/PKB and PKCζ was taken as readout for PI3K activity (Fig. 12A). Decreased phosphorylation of PDK1, Akt/PKB and PKCζ was detected when the cells were incubated with Met-F-AEA for 16 hrs. 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 hrs (Fig. 12B).

A



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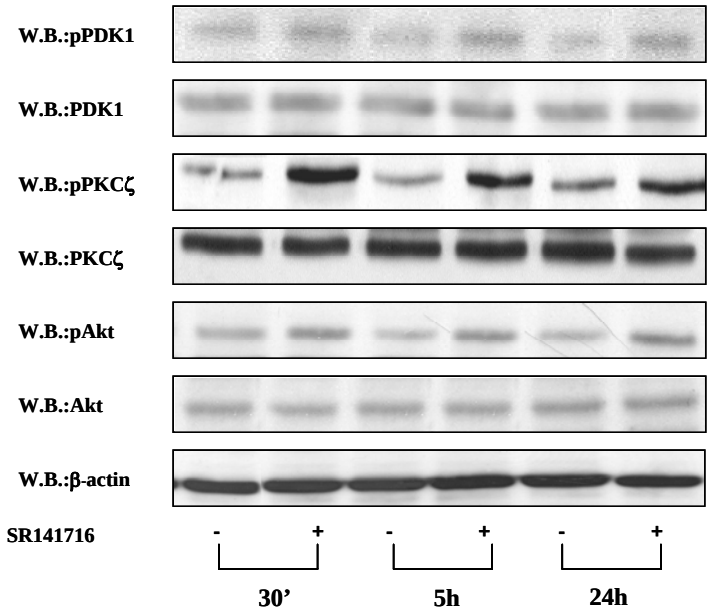
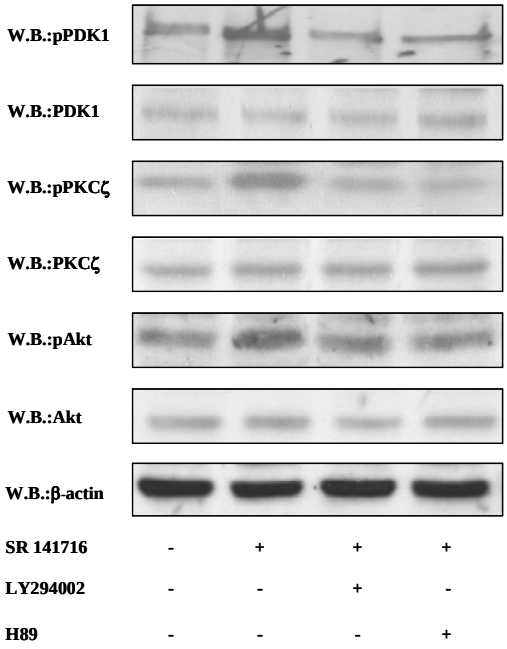


Figure 12: Effect of CB1 modulation on PI3K signalling. A. L6 myotubes were incubated with 10 μM Met-F-AEA or with 0.1 μM SR141716 for 16 hrs. Cells were then solubilized and phosphorylation and expression of PDK1, PKCζ and Akt were analyzed. The immunoblots shown are representative of three independent experiments. **B.** Cells were treated with 0.1 μM SR141716 for different times. The cell extracts were then solubilized and subjected to SDS-PAGE followed by immunoblotting with the indicated antibodies.

L6 myotubes were therefore pre-treated with 10 μM LY294002, in order to block PI3K activity, or with 15 μM H-89, in order to block PKA activity, and stimulated with 0.1 μM SR141716. In both conditions, SR141716 failed to induce phosphorylation of PDK1, Akt/PKB and PKCζ (Fig.13A). Also, the effect of SR141716 on 2-DG uptake was blunted following LY294002 and H89 pre-treatment (Fig. 13B).

A



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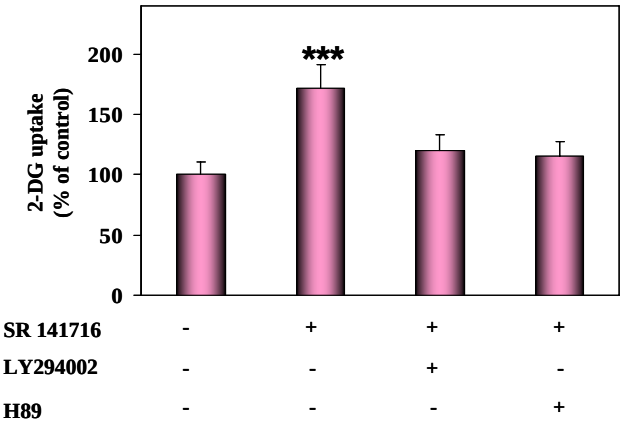

Figure 13: SR141716 increases glucose uptake, activating PKA and PI3K. A. Cells were serum starved and incubated with LY294002 (a PI3K inhibitor) or with H-89, in presence of 0.1 μM SR141716 for 16 hrs. Then cell extracts were subjected to SDS-PAGE followed by immunoblotting with specific anti phospho-antibodies. **B.** L6 myotubes were treated as described in A. Then the cells were assayed for 2-DG uptake. Bars represent mean ± SD of three different experiments in triplicate.

Several lines of evidence indicate that CB1 regulation of glucose uptake occurs through PI3K signalling. First, dose- and time-dependent decreases of both the regulatory (p85) and the catalytic (p110) subunits were observed following Met-F-AEA treatment. Second, these effects were paralleled by inhibition of the activity of several PI3K downstream molecules (PDK1, PKCζ and Akt/PKB). Third, low concentrations of SR141716 caused an increase of PI3K expression and activity. Fourth, the inhibition of PI3K activity counteracted the effect of SR141716 on glucose uptake.

Our findings show that the molecular events, involved in CB1 regulation of PI3K, involve modulation of PKA activity. Indeed, in parallel with p85 and p110 expression, SR141716 induces 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 has been reported that CB1 is coupled to $G_{i/0}$ proteins (Demuth and Molleman 2006). Then, engagement of CB1 by endogenous ligands causes inhibition of adenylate cyclase and reduction of cellular cAMP levels (Demuth and Molleman 2006). 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, since changes of PI3K cellular abundance still occurred in the presence of protein synthesis inhibitors. It has been recently described in several cell types that PKA directly phosphorylates p85 on Ser83 (Cosentino et al. 2007). This is crucial to transduce cAMP mediated effects (Cosentino et al. 2007; De Gregorio G. et al. 2007).

However, the acute stimulatory effect of SR141716 can not be accounted for by changes in PI3K subunits. CB1 modulation therefore may also affect PI3K activity, independent of its expression. It could be inferred that CB1 modulates PI3K by a dual mechanism: i) a short term mechanism, which directly stimulates PI3K activation, and ii) a long term mechanism, mediated by the enhanced PI3K expression. Both effects are largely mediated by PKA activation.

Thus, CB1 receptor exerts an inhibitory action on glucose uptake, which is 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 (Fig.14).

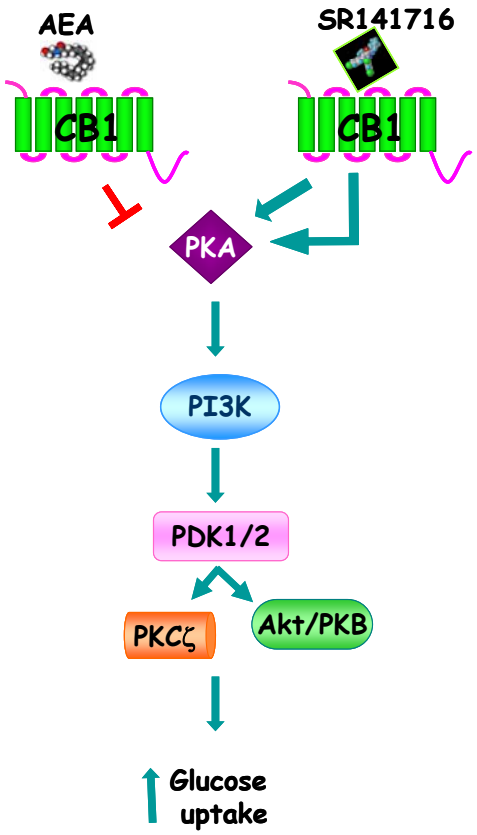

Figure 14: Molecular mechanism by which CB1 modulation may regulate glucose uptake. When anandamide (AEA) binds CB1, PKA activity is inhibited and thus glucose uptake is suppressed. SR141716 binding to CB1 activates PKA and PI3K, which, in turn, stimulate glucose uptake.

CONCLUSIONS

The increasing information on the physiological role played by the endogenous cannabinoids in energy balance and metabolism has led this signalling system to become an attractive model for novel therapeutic approaches. The intense pharmacological research based on this information has yielded potent selective drugs targeting the endogenous cannabinoid system, which include SR141716 (Rimonabant). In this thesis, I have provided evidence that endogenous cannabinoids (anandamide, AEA) impair glucose transport in skeletal muscle cells model. At variance, Rimonabant can stimulate glucose uptake. In the same way, AEA interferes with PI3K signalling system. In fact, anandamide decreases expression of both the regulatory (p85) and the catalytic (p110) subunits and inhibits activation of the main enzymes involved in glucose metabolism, such as PDK1, PKB and PKCζ. At the opposite, SR141716 increases p85 and p110 expression and stimulates phosphorylation of PDK1, PKB and PKCζ. Moreover, the molecular events, involved in CB1 regulation of PI3K, involve modulation of PKA activity. In fact, SR141716 effect on glucose transport is reverted by pharmacological inhibition of PI3K and PKA.

Then, CB1 receptor exerts an inhibitory action on glucose uptake, which is 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.

This represents a promising starting point to develop novel strategies to fight metabolic disorders lacking effective therapies, such as obesity and diabetes.

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