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The Endogenous Cannabinoid System Stimulates Glucose Uptake in Human Fat Cells via Phosphatidylinositol 3-Kinase and Calcium-Dependent Mechanisms

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Background: The endogenous cannabinoid system participates in the regulation of energy balance, and its dysregulation may be implicated in the pathogenesis of obesity. Adipose tissue endocannabinoids may produce metabolic and endocrine effects, but very few data are available in human adipose tissue and in primary human fat cells.

Experimental Design: We measured expression of type 1 and type 2 cannabinoid receptors (CNR), enzymes of cannabinoids synthesis and degradation in human omental, sc abdominal, and gluteal adipose tissue from lean and obese subjects. Furthermore, we assessed the effect of CNR1 stimulation on glucose uptake and intracellular transduction mechanisms in primary human adipocytes. Then we assessed the reciprocal regulation between CNR1 and peroxisome proliferator-activated receptor-γ (PPARγ). Finally, we tested whether leptin and adiponectin are regulated by CNR1 in human adipocytes.

Results: We found that most genes of the endocannabinoid system are down-regulated in gluteal fat and up-regulated in visceral and sc abdominal adipose tissue of obese patients. Treatment of adipocytes with rosiglitazone markedly down-regulated CNR1 expression, whereas Win 55,212 up-regulated PPARγ. Win 55,212 increased (+50%) glucose uptake, the translocation of glucose transporter 4, and intracellular calcium in fat cells. All these effects were inhibited by SR141716 and wortmannin and by removing extracellular calcium. Win 55,212 and SR141716 had no effect on expression of adiponectin and leptin.

Conclusions: These results indicate a role for the local endocannabinoids in the regulation of glucose metabolism in human adipocytes and suggest a role in channeling excess energy fuels to adipose tissue in obese humans. **(*J Clin Endocrinol Metab* 92: 4810–4819, 2007)**

OBESITY IS A MAJOR concern with epidemic proportions in Western countries. It is a complex metabolic disorder characterized by a positive disequilibrium between energy intake and energy expenditure. The consequent expansion of the adipose organ, and in particular of visceral fat depots, increases the risk of developing obesity complications (*i.e.* insulin resistance, type 2 diabetes, atherosclerosis, steatohepatitis, and cardio- and cerebrovascular diseases), but the precise mechanisms underlying this association are poorly understood.

The endogenous cannabinoid system has been demonstrated to be an important regulator of energy homeostasis acting through central pathways whose endogenous ligands [2-arachidonoylglycerol (2AG) and anandamide] activate the type 1 cannabinoid receptor (CNR1) (1–4). CNR1 knockout mice display decreased body fat and are resistant to diet-induced obesity, and treatment of obese humans with a specific CNR1 antagonist decreases body weight (5–7). Evidence is accumulating to indicate that the endogenous cannabinoid system is present also in peripheral tissues. Enzymatic pathways for the synthesis of endogenous cannabinoids and CNR1 are expressed in peripheral tissues involved in energy metabolism (liver, adipose tissue, and skeletal muscle) (4, 7). Activation of CNR1 in the liver was shown to increase *de novo* synthesis of fatty acids by activating the transcription factor sterol regulatory element-binding protein 1c (SREBP-1c) in mice (8). CNR1 is up-regulated in adipose tissue of genetically obese Zucker rats, and treatment with the CNR1 antagonist SR141716 increases the expression of adiponectin both *in vivo* in mice and *in vitro* in 3T3-F442A cells (9).

However, most of the peripheral effects of cannabinoids were reported in rodents and in cell lines, whereas the role of the endocannabinoid system in humans has been poorly investigated. Endogenous cannabinoids are increased both in sc and visceral adipose tissue (VAT) of obese patients and in diet-induced obese mice (10, 11). However no direct evidence has been reported so far on the direct effects of endocannabinoids on glucose and lipid metabolism and on adipokine release in human fat cells. Therefore, the main aim of the present work is to explore the role of the cannabinoid

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Abbreviations: ABD-SAT, Abdominal sc adipose tissue; 2AG, 2-arachidonoyl-glycerol; au, arbitrary units; BMI, body mass index; CNR1, type 1 cannabinoid receptor; DAGL, sn-1-selective diacylglycerol lipase; 2DG, 2-deoxyglucose; FAAH, fatty acid amide hydrolase; GLT-SAT, gluteal sc adipose tissue; GLUT4, glucose transporter 4; MAGL, monoacylglycerol lipase; NAPE, *N*-acylphosphatidylethanolamine; NAPE-PLD, NAPE-selective phospholipase D; PI3-kinase, phosphatidylinositol 3-kinase; PPARγ, peroxisome proliferator-activated receptor-γ; VAT, visceral adipose tissue.

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system in human adipose tissue both *in vivo* and *in vitro* and, in particular, to assess the effect of the activation of CNR1 on glucose uptake and gene expression of adipokines and enzymes of lipid metabolism in human primary fat cells. Finally, the aim was to evaluate the regulation of genes involved in synthesis and degradation of endogenous cannabinoid and CNR1 in adipose tissue from different depots of lean and obese subjects. Our results show that CNR1 and enzymes involved in cannabinoid synthesis have a depot-specific dysregulation in obesity. The activation of CNR1 stimulates glucose uptake in primary human adipocytes, and this effect is mediated by increased influx of extracellular calcium and by phosphatidylinositol 3-kinase (PI3-kinase) activity.

Subjects and Methods

Subjects and adipose tissue biopsies

Gluteal sc adipose tissue (GLT-SAT) was obtained from nine lean [four males and five females, age 32 ± 3 yr, body mass index (BMI) 22.2 ± 0.9 kg/m^2, fat mass 21.8 ± 5.4%, waist circumference 73 ± 10 cm] and 12 sex- and age-matched obese subjects (five males and seven females, age 34 ± 3 yr, BMI 42.2 ± 4.0 kg/m^2, fat mass 40.9 ± 11.7%, waist circumference 110 ± 31 cm). Both groups had normal lipid profile and normal glucose tolerance, whereas obese patients were insulin resistant as measured by homeostasis model assessment index (3.6 ± 1.8 *vs.* 1.1 ± 0.5, $P < 0.001$). All patients were recruited in the outpatient clinic and among the hospital staff and were on an unrestricted dietary regimen. Body weight was stable during the 3 months preceding the study. In each subject, a venous blood sample was collected after overnight fasting between 0800 and 0900 h for biochemical and hormonal determinations. Plasma was immediately separated and frozen and stored at −80 C until subsequent analysis. Homeostasis model assessment index of insulin resistance was calculated as [insulin (mIU/liter) × glucose (mmol/liter)]/22.5. In all subjects, height and body weight were measured. Waist was measured in standing position halfway between costal edge and iliac crest, whereas hip was measured as the greatest circumference around the buttocks. Body composition was assessed by bioelectric impedance and data analyzed with the software provided by the producer (BIA-STA equipment and Bodygram software; Akern, Florence, Italy).

After induction of local anesthesia, a G16 needle was inserted in the sc adipose tissue of the gluteal region and a 30- to 50-mg biopsy was obtained by gentle aspiration with a syringe. Tissue was immediately washed in cold saline, rapidly frozen in liquid nitrogen and stored at −80 C for subsequent RNA extraction. The procedure was well tolerated by all subjects. In a separate set of patients, biopsies of sc abdominal and omental adipose tissue (approximately 300 mg) were collected from patients undergoing elective abdominal surgery for gastric banding (eight obese patients, three males and five females, age 41 ± 4 yr, BMI 45.0 ± 2.1 kg/m^2) or nonmalignant abdominal disease (seven lean patients, three males and four females, age 46 ± 5 yr, BMI 23.9 ± 1.1 kg/m^2) that included pancreatic pseudocysts and abdominal aortic aneurysm. All patients were free from malignant disease, major renal and hepatic dysfunction, and diabetes or endocrine-metabolic disorders other than obesity. The purpose of the study was explained to the patients, and written informed consent was obtained. The protocol was approved by the institutional ethics committee. After collection, adipose tissue samples were washed in cold saline, frozen in liquid nitrogen, and stored at −80 C for subsequent analysis.

Isolation of human preadipocytes and adipose differentiation in vitro

Abdominal visceral fat was obtained from five subjects undergoing elective surgery for minor abdominal diseases. One set of cell culture experiments was performed from each subject. The stromal vascular fraction was isolated from adipose tissue by collagenase type II digestion (1 mg/ml; Sigma-Aldrich, St. Louis, MO) in DMEM/F12 at 37 C for 1 h. Cell suspension was centrifuged (350 × *g* for 8 min), and pellet containing stromal cells was resuspended in erythrocyte-lysing buffer, washed, and seeded in DMEM/F12 supplemented with 10% fetal bovine serum (0.7 × 10^6 cells per well in 24-well plates). After 16–20 h for cell attachment, cultures were re-fed with a serum-free adipogenic medium containing DMEM/F12 supplemented with 33 μmol/liter biotin, 17 μmol/liter pantothenate, 10 μg/ml human transferrin (Sigma-Aldrich), 66 nmol/liter insulin (Lilly Research, Indianapolis, IN), 100 nmol/liter dexamethasone, 1 nmol/liter T_3, and 0.25 mmol/liter 3-isobutyl-1-methylxanthine (Sigma-Aldrich) with or without 10 μmol/liter rosiglitazone. 3-isobutyl-1-metylxanthine and rosiglitazone were removed after 3 d, and the medium was changed three times per week. Preadipocytes maintained in DMEM/F12 alone were used as controls. The cells were harvested after 3, 6, 9, 12, and 15 d of differentiation. Acute experiments for gene expression experiments were performed in fully differentiated adipocytes (*i.e.* after 15 d of differentiation). Cells were stimulated for 3 h with the CNR agonist Win 55,212 (1 μM), the CNR1-specific antagonist SR141716 (rimonabant) (1 μM), or both in combination. Win 55,212 and SR141716 were dissolved in dimethylsulfoxide and added to cell culture as 1-μl aliquots.

Glucose uptake in primary human fat cells

After incubation with serum-free DMEM/F12 for 3 d, human adipocytes were incubated with or without insulin (2 μM), Win 55,212 (CNR agonist, 1 μM), SR141716 (CNR1 antagonist, 1 μM), wortmannin (100 nmol/liter), or EGTA (1 mmol/liter) alone or in combination for 1 h. Adipocytes were then washed with Krebs-Ringer buffer (130 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, 25 mM HEPES, pH 7.4). Glucose uptake was initiated by the addition of 2-deoxy-[^{3}H]*d*-glucose at the final assay concentration of 1.5 μCi/ml for 15 min at 37 C. Glucose uptake was terminated by two washes with ice-cold PBS. Cells were solubilized with 1% Triton X-100, and radioactivity was determined by scintillation counting (Wallac, PerkinElmer, Boston, MA). Fifty microliters of each sample were used for total protein quantification with Coomassie protein assay kit (Pierce, Rockford, IL). Insulin-induced glucose uptake was normalized to total protein content and expressed as percentage of mean basal value.

$[Ca^{2+}]_i$ measurement in human adipocytes

Primary cultures of adipocytes were grown on glass coverslips and incubated with 2 μM fura-2/AM for 30 min at 37 C in the dark. After incubation, cells were washed twice in the standard medium and suspended in 1 ml of a solution containing (mM) 140 NaCl, 5.0 KCl, 1.0 $MgCl_2$, 25 HEPES, and 5 glucose (pH 7.4) at 37 C, with or without the addition of 1.0 mM $CaCl_2$ (for standard or Ca^{2+}-free medium, respectively). Coverslips were transferred to the recording chamber, maintained at 37 C, and placed on the stage of an inverted epifluorescence microscope as previously described (12). Cells were stimulated with Win 55,212 (1, 10, and 100 μM) and SR141716 (1 μM) alone or in combination. Images of fura-2-loaded adipocytes with the excitation wavelength alternating between 340 and 380 nm were captured with a cooled CCD camera. After subtraction of background fluorescence, the ratio of fluorescence intensity at the two wavelengths was calculated. Ratio levels in groups of five to 10 individual cells per coverslip were analyzed using MetaFluor software package (Universal Imaging Corp., West Chester, PA). All graphs are averaged responses from groups of individual cells from representative single experiments. All experiments have been repeated on three separate occasions.

RNA isolation and quantitative RT-PCR

Total RNA was extracted using RNeasy Minikit; (QIAGEN, Hilden, Germany) following the supplier's instructions. One microgram of RNA was treated with DNase and removal reagents (Ambion, Austin, TX) and reverse transcribed for 1 h at 37 C in a 50-μl reaction volume containing 1× RT buffer, 150 ng random hexamers, 0.5 mmol/liter deoxynucleotide triphosphates, 20 U RNAsin ribonuclease inhibitor, and 200 U Moloney murine leukemia virus RT (Promega Corp., Madison, WI). Primer sequences and reaction conditions are illustrated in supplemental data (published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). PCR was carried out

using a DNA Engine (Opticon 2 continuous fluorescence detection system; MJ Research, Waltham, MA). Reactions were performed two times with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 5 ng cDNA as previously described (13). Standard curves for rRNA 18S, sn-1-selective diacylglycerol lipase (DAGL), monoacylglycerol lipase (MAGL), *N*-acylphosphatidylethanolamine (NAPE), fatty acid amide hydrolase (FAAH), and CNR1 were obtained using cDNAs from adipose tissue, whereas cDNAs from peripheral blood mononuclear cells were used for CNR2.

Immunodetection of CNR1 and glucose transporter 4 (GLUT4) translocation by confocal microscopy

Human adipocytes were cultured as described above on glass coverslips and were incubated for 30 min in the absence (control) or presence of 2 μM insulin, 1 μM Win 55,212, or 1 μM SR141716 alone or in combination as indicated. After incubation, cells were fixed with 4% paraformaldehyde in PBS for 20 min, washed with PBS, and treated with 0.25% Triton X-100 in PBS for 5 min. GLUT4 was detected by incubation (4 μg/ml, 1 h) with a polyclonal primary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) using standard procedures. After washing with PBS, binding of primary antibodies was detected with Alexa-488-conjugated secondary antibodies (Molecular Probes, Invitrogen, Milan, Italy). The cells were then examined for localization of fluorescence with a Leica confocal laser scanning microscope. Images were generated by the maximal projection of a stack of sections from the middle of each cell. Cells that are positive for GLUT4 translocation show an increase in plasma membrane-associated fluorescein staining that is visualized as a ring around the cell. We measured the mean value of fluorescence in the following areas: two diametrically opposed cell membrane areas representing GLUT4 translocated onto the cell surface and one in the intracellular space between these two points, representing nontranslocated GLUT4. The ratio between the mean of these two compartments was calculated. Measures were performed in at least 50 cells per coverslip.

The presence of CNR1 immunoreactivity on human preadipocytes and differentiated adipocytes was studied by immunocytochemistry. Cells were cultured as described above on glass coverslips and fixed in acetone-chloroform solution. Cells were preincubated for 10 min in nonimmune rabbit serum and then incubated for 30 min with 2 μg/ml CNR1 antiserum (Santa Cruz Biotechnology). Cells were washed three times in PBS for 5 min, incubated for 30 min with the secondary antibody, and washed in PBS. Immunocytochemical visualization was performed by peroxidase-antiperoxidase complex. The chromogen diaminobenzidine was added for 5 min. All passages were at room temperature. Negative control studies were performed using the same procedure except that the primary antibody was omitted. Finally, cells were stained with hematoxylin and eosin and mounted in synthetic resin. For immunohistochemical determination of CNR1, formalin-fixed sc adipose tissue was sectioned on a microtome into 7-μm slices and deparaffinized, and endogenous peroxidase was blocked by a 10-min incubation with 3% H_2O_2 in PBS. Sections were preincubated for 10 min with rabbit antihuman serum and exposed to a 1:50 dilution of polyclonal antihuman CNR1 antibody (101500 CNR1 polyclonal antibody; Cayman Chemical Co., Ann Arbor, MI). Sections were then exposed to a secondary biotinylated IgG (ScyTek Laboratories, Logan, UT) and visualized by incubation for 3 min with a peroxidase substrate solution containing the chromogen diaminobenzidine. Slides were then washed, counterstained with hematoxylin, shed in water and in alcohol, and mounted in synthetic resin.

Statistical analysis

Results are expressed as mean ± SE. Variables data were tested for normal distribution using the Kolmogorov-Smirnov test, and different groups were compared by ANOVA with Bonferroni correction. Statistical analysis was carried out using the MDAS 2.0 (Medical Data Analysis System) software package (EsKay Software, Pittsburgh, PA). P values < 0.05 were considered significant.

Results

Regulation of endocannabinoid system in adipose tissue of obese subject is depot specific

To assess the regulation of the endogenous cannabinoid system in human obesity, we measured mRNA expression of CNR1 and enzymes of local synthesis and degradation of endogenous cannabinoids in adipose tissue of lean and obese subjects obtained from three different depots. CNR1 mRNA was markedly down-regulated in GLT-SAT of obese subjects compared with controls. Expression of enzymes of biosynthesis and degradation of 2AG, DAGLα, and MAGL, respectively, was similarly reduced, whereas a nonsignificant trend to reduction was found for NAPE-selective phospholipase D (NAPE-PLD), the enzyme for biosynthesis of anandamide. Also the tissue anandamide-degrading enzyme FAAH was markedly down-regulated in GLT-SAT of obese subjects (Fig. 1A). Conversely NAPE-PLD, CNR1, and MAGL were found to be expressed at higher levels in obese subjects both in abdominal sc adipose tissue (ABD-SAT) and in VAT, whereas DAGL and FAAH mRNA were higher in obese subjects in ABD-SAT only (Fig. 1, B and C).

When expression levels in ABD-SAT and VAT were compared, NAPE-PLD was found to be expressed at higher levels in VAT compared with ABD-SAT both in lean [108 ± 25 *vs.* 48 ± 10 arbitrary units (au), $P < 0.01$] and obese subjects (270 ± 45 *vs.* 151 ± 18 au, $P < 0.01$), whereas no difference was found for FAAH. Also, DAGLα mRNA was similarly expressed in ABD-SAT and VAT, whereas MAGL was higher in ABD-SAT than in VAT in lean (38 ± 5 *vs.* 73 ± 15 au, $P < 0.01$) and obese subjects (53 ± 7 *vs.* 110 ± 13 au, $P < 0.01$). CNR1 expression was higher in VAT in obese subjects only (170 ± 30 *vs.* 80 ± 18 au, $P < 0.05$). CNR2 was expressed at low level in adipose tissue of both obese and lean subjects with no significant differences between lean and obese subjects and between different adipose depots.

Taken together, these data indicate that enzymes of both synthesis and degradation of endocannabinoids and CNR1 are down-regulated in GLT-SAT of obese subjects and up-regulated in ABD-SAT and VAT of obese subjects.

CNR1 is up-regulated during adipocytes differentiation and is down-regulated by rosiglitazone

CNR1 was not expressed in primary preadipocytes before differentiation and was rapidly expressed after the addition of the adipogenic medium. This was revealed by quantitative PCR analysis (Fig. 2A) and confirmed by immunocytochemistry (supplemental figure). Differentiation of cells in the presence of the peroxisome proliferator-activated receptor-γ (PPARγ) agonist rosiglitazone clearly down-regulated CNR1 expression and up-regulated FAAH expression in differentiated adipocytes (Fig. 2C). CNR2 was expressed at low levels in preadipocytes and became negligible after adipose differentiation (Fig. 2B).

CNR1 activation induces glucose uptake and GLUT4 translocation in human primary adipocytes

Glucose uptake was assessed with the 2-deoxyglucose (2DG) technique in fully differentiated human fat cells. In-

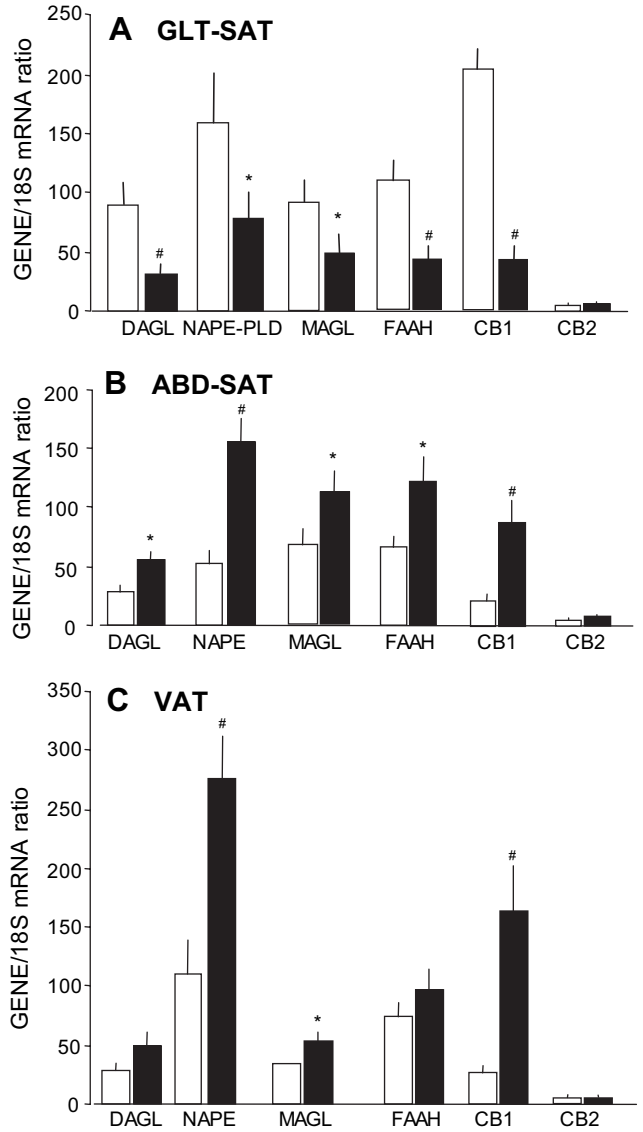

FIG. 1. Quantification of mRNA expression of CNR1 and CNR2 and enzymes of anandamide and 2AG synthesis (NAPE-PLD and DAGLα) and degradation (FAAH and MAGL) in tissue extracts from GLT-SAT (A), ABD-SAT (B), and VAT (C) of lean (*white bars*) and obese (*black bars*) subjects. Each *bar* represents mean ± SEM of seven to nine samples for the control group and eight to 12 samples for the obese group (see *Subjects and Methods* for details). *, $P < 0.05$ *vs.* lean subjects; #, $P \leq 0.01$ *vs.* lean subjects.

sulin induced a 100% increase of 2DG uptake by adipocytes, whereas a 50% increase was observed when cells were stimulated with the CNR agonist WIN 55,212. The effect was blocked by coincubation of cells with the CNR1 antagonist SR141716. No additive or synergistic effect was observed when cells were incubated with both insulin and WIN 55,212 (Fig. 3).

Moreover, when GLUT4 translocation to plasma membrane was quantified by quantitative analysis, a significant translocation of GLUT4 from the intracellular compartment to plasma membrane was found both in adipocytes incubated with insulin and in those treated with the CNR agonist WIN 55,212. Again, the effect of WIN 55, 212 was blocked by coincubation with the CNR1 antagonist SR141716 (Fig. 3).

CNR1 activation increases $[Ca^{2+}]_i$ in human adipocytes

Treatment of fat cells with WIN 55,212 induced a rapid increase of $[Ca^{2+}]_i$ (Fig. 4) that was terminated by the addition of the Ca^{2+}-chelating agent EGTA and was completely absent in Ca^{2+}-free medium (Fig. 4). These data demonstrate that $[Ca^{2+}]_i$ activated by WIN 55,212 is due to an influx of Ca^{2+} from the extracellular medium. Furthermore, preincubation of adipocytes with the specific CNR1 antagonist SR141716 inhibited the rise of $[Ca^{2+}]_i$ induced by WIN 55,212 (Fig. 4). Interestingly, the increase of $[Ca^{2+}]_i$ stimulated by WIN 55,212 was blocked when adipocytes were preincubated with wortmannin, a specific inhibitor of PI3-kinase (Fig. 4).

CNR1-induced glucose uptake is mediated by PI3-kinase and intracellular calcium

To investigate further the intracellular mechanisms underlying the effect of CNR1 activation on glucose uptake, we used the specific inhibitor of PI3-kinase wortmannin and a calcium-free model of cell culture. Uptake of 2DG and GLUT4 translocation induced by WIN 55,212 was prevented by EGTA, whereas EGTA had no effect either on basal and insulin-stimulated glucose uptake or on GLUT4 translocation (Fig. 5). Finally, to assess whether PI3-kinase was involved in WIN 55,212-induced stimulation of glucose uptake, we preincubated adipocytes with wortmannin, an irreversible inhibitor of PI3-kinase activity. Pretreatment with wortmannin completely blocked the effect of both WIN 55,212 and insulin on 2DG uptake and GLUT4 translocation (Fig. 5). Collectively, these data indicate that the presence of extracellular calcium and PI3-kinase activity are necessary for CNR1-stimulated glucose uptake. They suggest that plasma membrane calcium channels are activated through PI3-kinase and that both PI3-kinase and the rise of $[Ca^{2+}]_i$ are necessary for CNR1-induced increase of glucose uptake.

Cannabinoids stimulate PPARγ expression in adipocytes but do not modify expression of adipokines

When primary human fat cells were stimulated with WIN 55,212 in an early phase of differentiation (third day), a significant increase of PPARγ expression was observed, whereas the effect was no longer evident when fully differentiated (10–12th day) cells were stimulated (Fig. 2D). Treatment of primary human adipocytes with WIN 55,212, SR141716, or both in combination had no effect on adiponectin and leptin expression (Fig. 2E).

Discussion

After the discovery that the endogenous cannabinoids are involved in the hypothalamic regulation of food intake and in peripheral lipogenesis in rodents (2, 4, 8), the endogenous cannabinoid system has become a promising target for a pharmacological approach to obesity and diabetes (14). Four phase III clinical trials on the effects of rimonabant, a CNR1 antagonist, have been published so far, and all confirm that

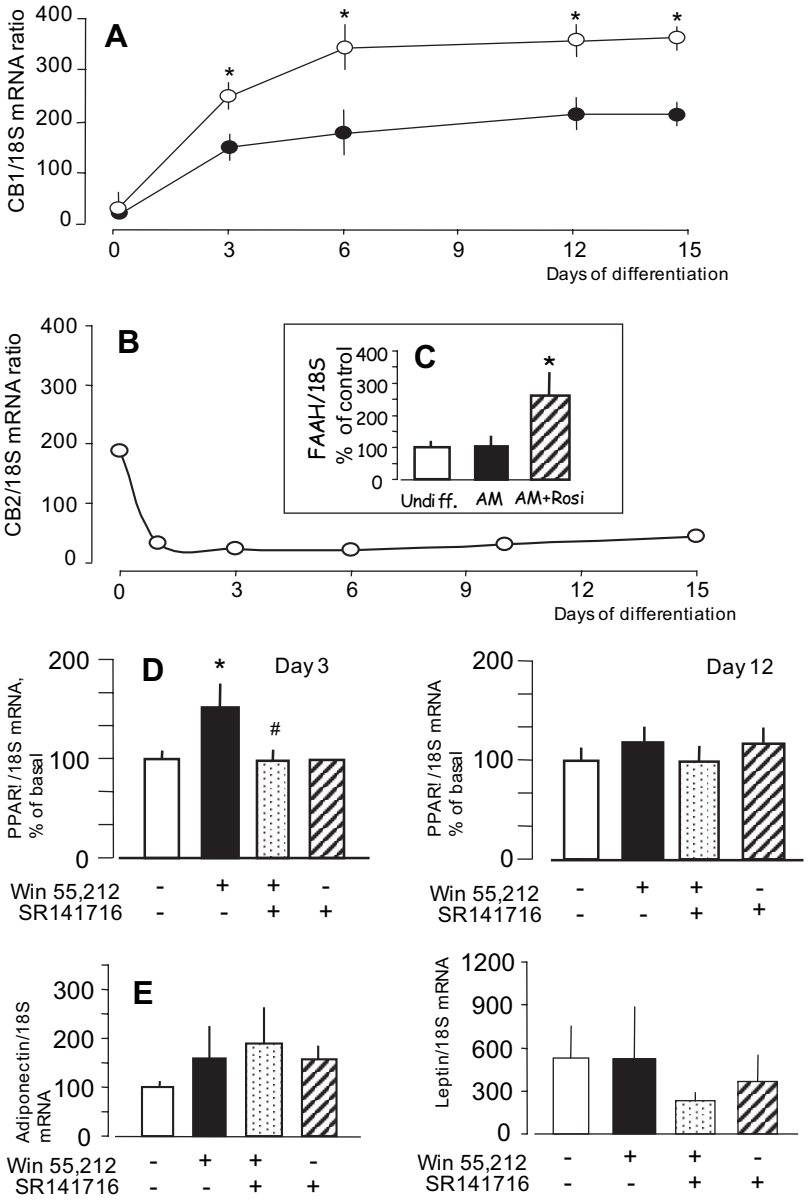

FIG. 2. A, Expression of CNR1 in human primary fat cells before differentiation (d 0) and during adipogenic differentiation. Preadipocytes were isolated from the stromal vascular fraction of adipose tissue and differentiated *in vitro* with an adipogenic medium in the presence (●) or absence (○) of rosiglitazone (10 μM) for the first 3 d of differentiation. Each data point represents mean ± SEM of six experiments for each condition. *, $P < 0.01$ *vs.* cells differentiated with rosiglitazone. B, Expression of CNR2 in human primary fat cells before differentiation (d 0) and during adipose differentiation. Each data point represents mean ± SEM of six experiments. C (*inset*), Expression of FAAH in human primary fat cells before differentiation (*white bar*) and during adipose differentiation in the absence (*black bars*) or presence (*dashed bars*) of rosiglitazone (10 μM). Each data point represents mean ± SEM of six experiments for each condition. *, $P < 0.01$ *vs.* cells differentiated without rosiglitazone. D, CNR1 stimulation by WIN 55,212 stimulates PPARγ expression in primary human adipocytes when stimulated in early differentiation, whereas no effect was observed after 12 d of differentiation. *, $P < 0.01$ *vs.* control; #, $P < 0.01$ *vs.* WIN 55,212. E, CNR1 treatment has no effect on adiponectin and leptin expression in human primary adipocytes.

blocking CNR1 reduces body weight in obese subjects and ameliorates cardiovascular risk factors in obese and diabetic patients (2, 6, 15, 16). However, although several studies in rodents and in 3T3-F442A adipocytes suggest a direct effect of endogenous cannabinoid on adipose tissue and adipocyte function (4, 9, 10, 17), no information is available, so far, in human fat cells. Triglyceride storage in fat cells and adipose tissue expansion needs an adequate supply of both fatty acids and glucose to fat cells. Lipoprotein lipase-mediated flux of fatty acids from very-low-density lipoprotein and chylomicrons to fat cells provides lipid substrates supply. Insulin-dependent GLUT4 translocation and glucose transport are key steps to produce the glycolytic intermediate α-glycerol-phosphate, which is essential for triglyceride assembly. Both these pathways are under the control of insulin. Thus, an adequate insulin sensitivity and the activation of its downstream machinery is a fundamental prerequisite to allow adequate fuel channeling to fat cells (18).

Our data show that CNR1 is not expressed in undifferentiated preadipocytes and that it is rapidly up-regulated in early stages of differentiation. Similar results were also reported by other authors in different experimental models (19, 20). Conversely, CNR2 is expressed in preadipocytes and is down-regulated to undetectable levels upon differentiation. Low levels of expression of CNR2 mRNA were found also in adipose tissue biopsies, suggesting that preadipose cells, infiltrating macrophages, or vascular cells could be the source of CNR2 mRNA in adipose tissue extracts. Our data are in agreement with those of Spoto *et al.* (20), who found CNR1, but not CNR2 mRNA, in human adipose tissue. They do not agree with those of Roche *et al.* (19), who reported CNR2 expression higher than CNR1 in human adipose tissue coming from liposuction interventions. Some methodological details may possibly explain the discrepancy, and higher CNR2 expression may be due to infiltration by immune cells or contamination of adipose samples with blood cells.

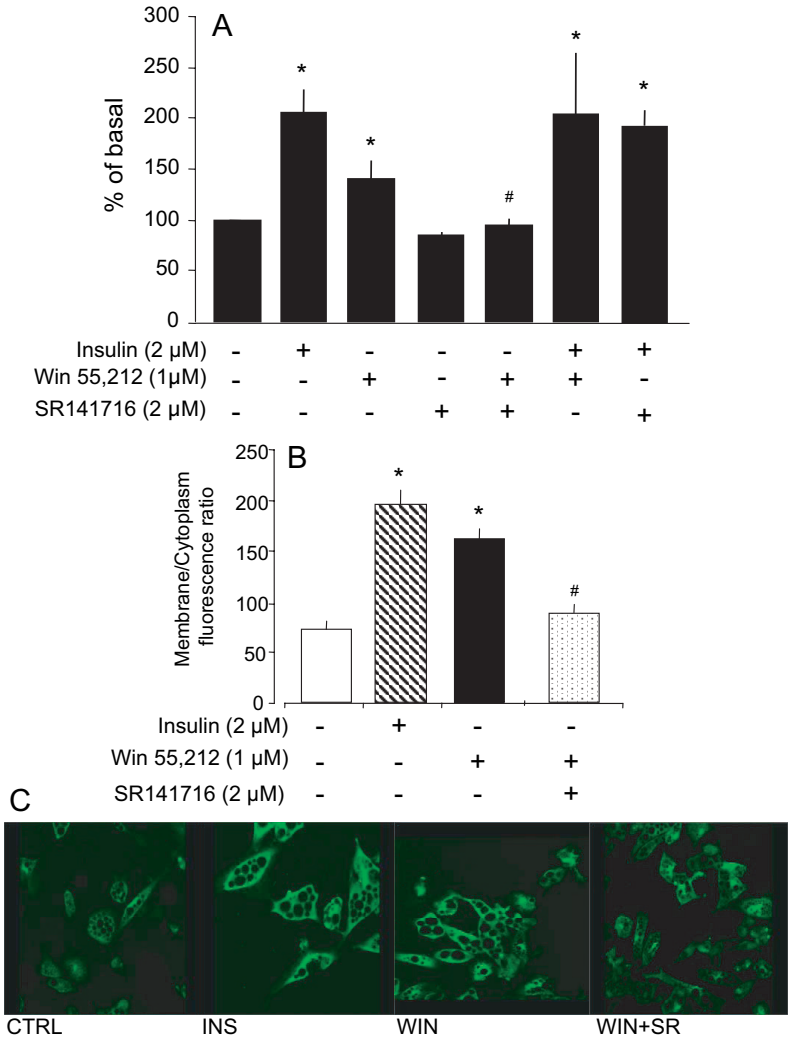

FIG. 3. A, Glucose uptake in human primary fat cells. Cells were treated for 30 min with insulin, Win 55,212, or SR141716 alone or in combination as indicated. Glucose uptake was evaluated as the uptake and accumulation of tritiated 2DG in fat cells. B and C, GLUT4 plasma membrane translocation is induced by insulin and Win 55,212. In basal conditions (control), GLUT4 immunoreactivity was localized in the intracellular compartment, whereas a shift of fluorescence to the plasma membrane compartment was observed after cells had been preincubated with insulin or Win 55,212. The effect of WIN 55,212 was abolished when cells were coincubated with the CNR1 antagonist SR141716, whereas no additive effect was observed when insulin and Win 55,212 were combined. Translocation from the intracellular compartment to plasma membrane was assessed as the ratio between fluorescence in the plasma membrane and the intracellular compartment (see *Subjects and Methods* for details). *, $P < 0.05$; #, $P < 0.01$ *vs.* control. Each *bar* represents the mean ± SEM of five to seven separate experiments.

Our study is the first to demonstrate the effect of endocannabinoids on glucose uptake in human adipocytes, which was about 50% of the effect produced by insulin. Because adipocytes express the enzymatic pathways to produce endogenous cannabinoids, it appears that glucose uptake may be locally modulated by endocannabinoids. Increased glucose uptake was mediated by increased translocation of GLUT4 on plasma membrane. It is noteworthy that the CNR1-mediated stimulation of glucose uptake and GLUT4 translocation was inhibited by wortmannin, an inhibitor of PI3-kinase, and by a rise of intracellular calcium. This suggests that insulin and endocannabinoids may share a common intracellular signaling pathway that include PI3-kinase activation and the rise of $[Ca^{2+}]_i$. In addition, it was recently reported, in 3T3-L1 cells, that anandamide enhances insulin-stimulated glucose uptake through a nitric oxide-dependent mechanism (21).

The role of cannabinoid receptors in the regulation of intracellular calcium fluxes has been extensively studied in neurons where CNR1 is coupled to a G_i protein and its activation reduces intracellular calcium (22). However, several studies reported that in several cell types, endocannabinoids may increase or potentiate intracellular calcium and that plasma membrane calcium flux is also under the control of PI3-kinase and inositol 3-phosphate (23–28). Membrane lipids and arachidonic acid may influence K-channel activity leading to membrane depolarization, calcium release from intracellular stores, and GLUT4 translocation in adipocytes (29, 30). Because human fat cells also have voltage-dependent K channels, it is also possible that the rise of $[Ca^{2+}]_i$ may be mediated in part by inhibition of K flux (31).

Although Win 55,212 is a ligand for both CNR1 and CNR2, we can conclude that the effects we found on fat cells are CNR1 mediated. As a matter of fact, we did not find any CNR2 expression on differentiated adipocytes, and the effects on glucose uptake, GLUT4 translocation, and the rise of $[Ca^{2+}]_i$ were fully reversed by the specific CNR1 antagonist SR141716.

Our results show for the first time an insulin-like effect of CNR1 activation on human adipocytes. Previous data in 3T3-L1 cells suggested that the activation of CNR1 could stimulate lipogenesis by directly activating lipoprotein lipase activity in mouse adipocytes (4) and by increasing PPARγ expression in 3T3-F442A adipocytes (10). Also, in our experimental model, treatment with WIN 55,212 up-regulated PPARγ expression, a central regulator of the adipogenic pro-

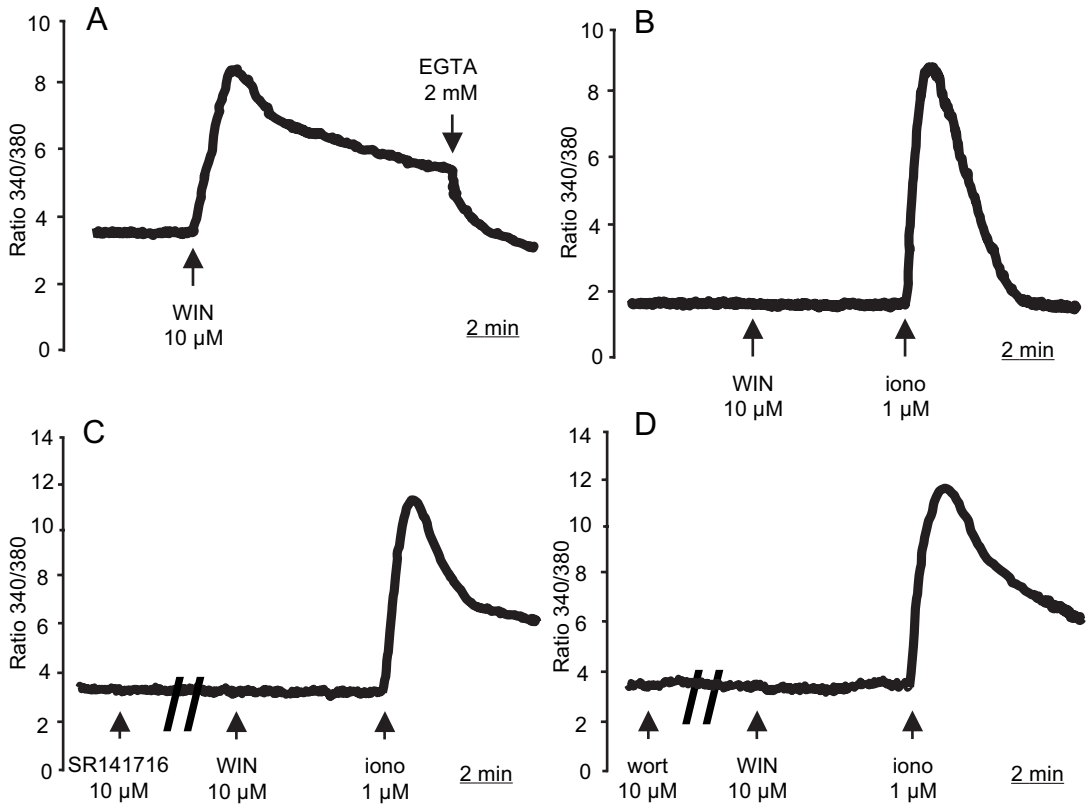

FIG. 4. WIN 55,212 increases $[Ca^{2+}]_i$ in human adipocytes, activating influx of Ca^{2+} from the extracellular medium. Human adipocytes, grown on glass coverslips, were loaded with fura-2/AM as described in *Subjects and Methods*. A, Adipocytes were suspended in Ca^{2+}-containing medium and then stimulated with WIN 55,212; B, adipocytes were suspended in Ca^{2+}-free medium (no Ca^{2+} added and EGTA 0.1 mM) and then stimulated with WIN 55,212; C, adipocytes were preincubated in the presence of SR141716 for 15 min before addition of WIN 55,212; D, adipocytes were suspended in Ca^{2+}-containing medium and preincubated in the presence of wortmannin for 15 min before addition of WIN 55,212. Where indicated, WIN 55,212 (10 μM), EGTA (2.0 mM), wortmannin (10 μM), and ionomycin (1 μM) were added. Traces represent the mean of all traces obtained from adipocytes during a typical experiment of three.

gram. It is noteworthy that the up-regulation of PPARγ by CNR1 was evident only in early differentiated adipocytes and was no longer present when CNR1 was stimulated in late differentiated adipocytes. This suggests a crucial role for endogenous cannabinoid in the adipogenic drive in early terminal adipogenesis. The relationship between endogenous cannabinoid and the regulation of the adipogenic program is further supported by the finding that CNR1 is down-regulated, and FAAH is up-regulated, when differentiation is stimulated by the PPARγ ligand rosiglitazone. The concomitant down-regulation of CNR1 and up-regulation of FAAH indicate a reduction of the endocannabinoid tone in the culture, and these data fit well with the reduction of 2AG induced by ciglitazone in partially differentiated adipocytes reported by Matias *et al.* (10). The relationship between endogenous cannabinoid, PPARs, and fat cell differentiation is not fully elucidated. Treatment of 3T3-F442A preadipocytes with rimonabant inhibits preadipocyte proliferation and increases the expression of molecular markers of late adipose differentiation without inducing triglyceride accumulation (32). Conversely, stimulation of differentiating adipocytes with the CNR agonist HU-210 stimulated PPARγ expression and increased lipid droplets accumulation in 3T3-F442A cells, and this effect was reversed by SR141716 (10). Thus, it is possible to speculate that endogenous cannabinoids cooperate with PPARγ to drive early stages of fat cell maturation. A negative feedback between PPARγ and endogenous cannabinoid would control lipid deposition, whereas leptin at late stages of differentiation would inhibit both endocannabinoids and PPARγ (10). In addition, the differentiation of smaller adipocytes under thiazolidinedione treatment could also inhibit the local endocannabinoid system as previously suggested (10). A possible interpretation of the reciprocal regulation between PPARγ and CNR1 is that endogenous cannabinoid activation precedes and stimulates PPARγ activation, and the feedback regulation of CNR1 is part of the homeostatic mechanism. However, it should be emphasized that anandamide binds and activates directly PPARγ and induces adipogenesis also by this mechanism (33) and that other lipid mediators not binding to CNR, *i.e.* oleoylethanolamide and palmitoylethanolamide, are putative ligands for PPARs (34, 35). Furthermore, it was recently reported that CNR1 is also regulated by PPARδ, thus making the picture even more complex (36). Hence, additional studies are needed to investigate the precise time course and the relationship between the local endogenous cannabinoid system and PPARγ activation.

It has been reported that CNR1 is overexpressed in muscle of insulin-resistant high-fat-fed animals (3) and that glucose uptake is increased in isolated soleus muscle of rimonabant-

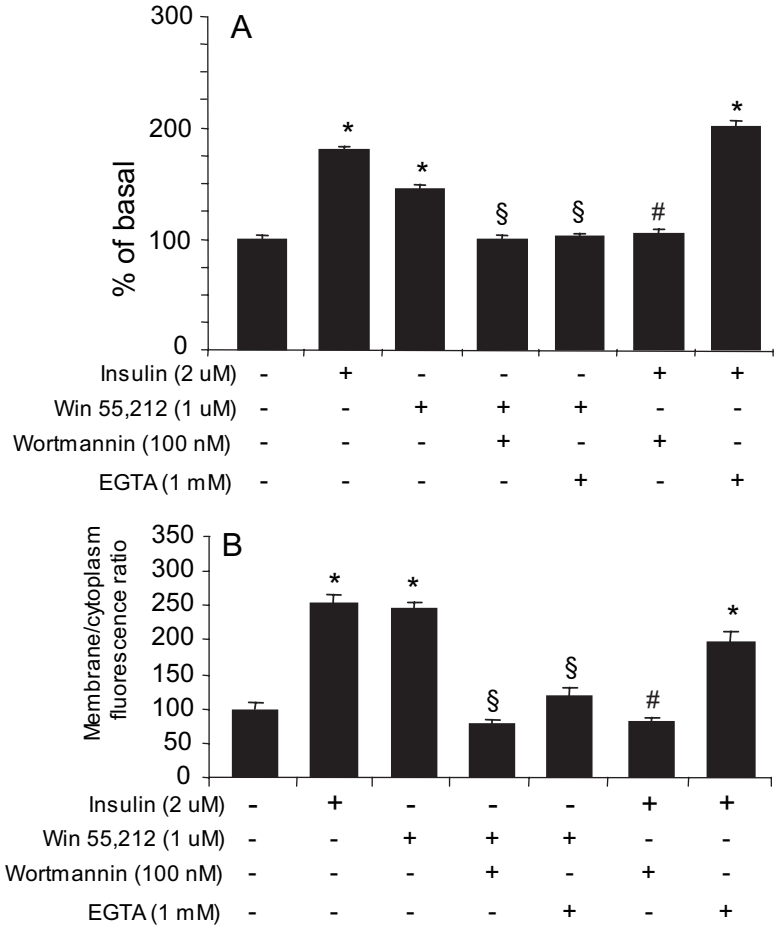

FIG. 5. Effect of wortmannin and EGTA on insulin- and CNR-stimulated glucose uptake (A) and GLUT4 translocation (B) in primary human adipocytes after 10 d of differentiation. Insulin, WIN 55,212, wortmannin, or EGTA was added as indicated. Treatment of cells with the specific PI3-kinase inhibitor wortmannin prevented both insulin-stimulated and CNR-stimulated glucose uptake and GLUT4 translocation. Culture of cells in a calcium-free medium inhibits CNR-induced but not insulin-stimulated glucose uptake and GLUT4 translocation. *, $P \leq 0.01$ compared with control; #, $P < 0.01$ compared with insulin; §, $P \leq 0.01$ compared with WIN 55,212.

treated ob/ob mice (37). It is possible to hypothesize that, in a condition of energy overload, the endocannabinoid system overactivation in skeletal muscle could induce local insulin resistance, whereas the increased CNR1-endocannabinoid activity and its insulin-mimetic properties in adipose tissue could favor preferential fuel channeling to adipose tissue rather than to muscle.

The lack of effect of endocannabinoids on adiponectin expression in human fat cells is in contrast with data reported *in vivo* in rodents and *in vitro* in 3T3-L1 cells (9, 10). In fact, it was shown that the CNR1 agonist reduced, and the CNR1 antagonist rimonabant increased, adiponectin expression *in vitro*. Also, *in vivo* treatment of rats with rimonabant for 4 d increased adiponectin expression in adipose tissue (9). A recent clinical trial in human obese patients reported that part of the adiponectin increase at the end of the 1-yr treatment with rimonabant was independent of weight loss (5). Therefore, it was postulated that rimonabant could have a direct effect on circulating adiponectin. However, a recent experiment to specifically address this issue *in vivo* in rats demonstrated that treatment for 14 d with rimonabant has no effect *per se* on adiponectin and that increase of adiponectin expression is solely mediated by the reduction of food intake and body weight (38). Moreover, Lofgren *et al.* (39) reported, in a cohort of 92 lean and obese subjects, that there is no association of adipose tissue mRNA expression of CNR1 and circulating and adipose tissue expression of adiponectin. Our results on human fat cells are in agreement with these data and do not support a direct role of endogenous cannabinoids on adiponectin in human adipose tissue. In addition, the lack of effect on leptin expression suggests that the effect on food intake of endocannabinoids and their antagonist is due essentially to a central hypothalamic action and is not mediated by changes of leptin secretion by adipose tissue. However, our experiments were carried out in adipocytes from normal subjects, and it is not possible to rule out that adiponectin and leptin are regulated by endocannabinoids in adipocytes of obese subjects.

Our data extend and clarify previous results on the regulation of endocannabinoid system in different adipose tissue depots. It was reported that CNR1 and FAAH expression is down-regulated in adipose tissue of obese subjects (11, 40). More recently, Matias *et al*. (10) reported that 2AG levels are increased in VAT from obese subjects compared with sc adipose tissue of both obese and lean subjects. Our experiments were designed to assess different elements of the adipose tissue endocannabinoid system (CNR1, CNR2, and enzymes of synthesis and degradation) from VAT, ABD-SAT, and GLT-SAT. Results clearly show that dysregulation of the endogenous cannabinoid system in adipose tissue is depot specific. In fact, we found a down-regulation of CNR1 and FAAH in GLT-SAT, in agreement with previous data, and extend those data to the down-regulation of MAGL and DAGL. However, an opposite, *i.e.* increased, change of these

genes was found in VAT and ABD-SAT of obese subjects, in line with the finding of increased 2AG levels previously reported (10) and with the positive correlation between circulating 2AG and visceral adiposity (41). However, CNR1 expression was also reported to be down-regulated or unchanged in sc adipose tissue of obese subjects (39, 40). This discrepancy may be due to the different patient selection of the two studies or to different sampling of sc adipose tissue in the truncal region. In fact, sc fat contains two layers of adipose tissue (superficial and deep) that have different biochemical and functional properties (42). Different sampling of these two regions may lead to different results on expression profiles. Taken together, our and previous data support the hypothesis of overactivation of the endocannabinoid system in central adipose tissue in human obesity and support a similarity of biological characteristic between intraabdominal adipose tissue and sc truncal adipose tissue compared with lower-body adipose tissue (43).

However, although our *in vitro* data clearly show that CNR1 activation increases PPARγ, the association between endogenous cannabinoid overactivation and adipose tissue expansion in obesity needs additional studies to address the specific point of whether the increased local cannabinoid tone precedes and is responsible for adipose tissue expansion or is a consequence of it.

In conclusion, these studies demonstrate an insulin-mimetic action of endocannabinoids on glucose uptake that is mediated by activation of CNR1, PI3-kinase, and the increase of intracellular calcium. The metabolic effect of CNR1-mediated endocannabinoids, the reciprocal interaction with PPARγ, and the up-regulation of the endocannabinoid system in abdominal adipose tissue of obese subjects strengthen the role of local endocannabinoids in the regulation of adipose tissue in physiology and pathology.

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