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The Cannabinoid CB1 Receptor Antagonist Rimonabant (SR141716) Inhibits Cell Proliferation and Increases Markers of Adipocyte Maturation in Cultured Mouse 3T3 F442A Preadipocytes

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ABSTRACT

Adipocyte cell proliferation is an important process in body fat mass development in obesity. Adiponectin or Acrp30 is an adipocytokine exclusively expressed and secreted by adipose tissue that regulates lipid and glucose metabolism and plays a key role in body weight regulation and homeostasis. Adiponectin mRNA expression in adipose tissue and plasma level of adiponectin are decreased in obesity and type 2 diabetes. In obese rodents, the selective CB_1 receptor antagonist rimonabant reduces food intake and body weight and improves lipid and glucose parameters. We have reported previously that rimonabant stimulated adiponectin mRNA expression in adipose tissue of obese fa/fa rats, by a direct effect on adipocytes. We report here that rimonabant (10–400 nM) inhibits cell proliferation of cultured mouse 3T3 F442A preadipocytes in a concentration-dependent manner. In parallel to this inhibitory effect on preadipocyte cell proliferation, rimonabant (25–100 nM) stimulates mRNA expression and protein levels of two late markers of adipocyte differentiation (adiponectin and glyceraldehyde-3-phosphate dehydrogenase) with a maximal effect at 100 nM, without inducing the accumulation of lipid droplets. Furthermore, treatment of mouse 3T3 F442A preadipocytes with rimonabant (100 nM) inhibits basal and serum-induced p42/44 mitogen-activated protein (MAP) kinase activity. These results suggest that inhibition of MAP kinase activity by rimonabant may be one of mechanisms involved in the inhibition of 3T3 F442A preadipocyte cell proliferation and stimulation of adiponectin and GAPDH expression. The inhibition of preadipocyte cell proliferation and the induction of adipocyte late "maturation" may participate in rimonabant-induced antiobesity effects, particularly the reduction of body fat mass.

Obesity is a complex metabolic disorder resulting from an imbalance between energy intake and expenditure. This dysregulation may have a genetic and/or behavioral origin, involving the quality and quantity of food intake as well as lifestyle (Carpino, 2000). Obesity is characterized by an increase in body weight and adipose tissue hyperplasia and hypertrophy with excessive fat storage (Hausman et al., 2001; Spiegelman and Flier, 2001). Adipose tissue is now considered an active endocrine organ that produces and secretes various hormones and biologically active proteins called adipocytokines, including leptin, adipsin, resistin, tumor necrosis factor α, interleukin 6, transforming growth factor β, angiotensinogen, plasminogen activator inhibitor-1, and adiponectin (Trayhurn and Beattie, 2001; Fain et al., 2004; Trayhurn and Wood, 2004). Through autocrine, paracrine, and endocrine mechanisms, these adipocytokines play multiple and crucial roles in several pathophysiological processe, such as obesity, and associated chronic diseases, including diabetes, dyslipidemia, cardiovascular disorders (atherosclerosis, hypertension, myocardial infarction, coronary diseases), and inflammation (Trayhurn and Beattie, 2001; Fortuno et al., 2003; Lyon et al., 2003; Rajala and Scherer, 2003; Reilly and Rader, 2003; Ruan and Lodish, 2003; Borst, 2004; Fain et al., 2004; Matsuzawa et al., 2004; Pischon et al., 2004; Trayhurn and Wood, 2004; Wisse, 2004).

In obesity, excessive visceral and abdominal body fat mass development is generally accompanied by changes in the structural and cellular composition of adipose tissue associated with dramatic dysregulation of synthesis and release of adipocytokines and of enzyme activities involved in lipid and

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ABBREVIATIONS: Acrp30, adipocyte complement-related protein of 30 kDa (adiponectin); SR141716, rimonabant; DMEM, Dulbecco's modified Eagle's medium; PTX, *Bordetella pertussis* toxin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBST, Tris-buffered saline/Tween 20; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase.

glucose metabolism (Dugail et al., 1988, 1992; Rolland et al., 1995; Fried and Russell, 1998; Guerre-Millo, 2002). This dysregulation of adipocytokines and adipoenzymes with resulting metabolic and behavioral dysfunctions may be the principal cause of obesity and associated diseases (Lyon et al., 2003; Rajala and Scherer, 2003; Reilly and Rader, 2003; Borst, 2004; Grundy, 2004; Matsuzawa et al., 2004; Pischon et al., 2004; Wisse, 2004). Structural and functional integrity of adipose tissue plays a pivotal role in maintaining metabolic regulation, body weight, and physiological homeostasis. In fact, biological abnormality of adipose tissue may be a key step contributing to the emergence of pathologic features that characterize the metabolic syndrome and particularly obesity associated diseases. Adipocyte cell proliferation (hyperplasia) is an important process in body fat mass development in obesity. Studies in humans and in animal models indicate that increase in adipocyte cell size (adipocyte hypertrophy) often precedes increase in adipocyte cell number (Hausman et al., 2001), and the development of hyperplastic adipose tissue is currently associated with the most severe form of obesity and associated diseases (Hirsch et al., 1989). Based on these observations, drugs capable of restoring structure and functionality of adipose tissue may represent a novel approach to the therapeutic treatment of obesity and associated diseases. Adipocyte complement-related protein of 30 kDa (Acrp30), or adiponectin, is an adipocytokine exclusively expressed and secreted by adipose tissue that has been shown to regulate lipid and glucose metabolism and to play a key role in body weight regulation and homeostasis. Adiponectin has also been reported to be involved in obesity and with associated metabolic diseases. In fact, adiponectin mRNA expression in adipose tissue and its plasma level are decreased in obesity and associated pathologies (Hu et al., 1996; Arita et al., 1999; Fruebis et al., 2001; Weyer et al., 2001).

Rimonabant (SR141716), a selective CB_1 receptor antagonist (Rinaldi-Carmona et al., 1994), has been shown to possess potent antiobesity effects: it reduced food intake, body weight, and fat mass and improved lipid parameters and insulin sensitivity in obese rodents (Arnone et al., 1997; Chaperon et al., 1998; Colombo et al., 1998; Di Marzo et al., 2001; Ravinet-Trillou et al., 2002; Bensaid et al., 2003; Poirier et al., 2005). We have reported recently that rimonabant stimulates adiponectin mRNA expression in adipose tissue of obese fa/fa rats by a direct effect on adipocytes and reduces hyperinsulinemia associated with this animal model (Bensaid et al., 2003). These results demonstrated for the first time that rimonabant regulates hormones implicated in the control of lipid and glucose metabolism and that it could exert a metabolic "peripheral" action, which might account for its antiobesity effect (Bensaid et al., 2003).

The aim of the present study was to evaluate the effect of rimonabant on the cell proliferation, endocrine profile, and enzyme expression in cultured mouse 3T3 F442A preadipocytes and to identify possible molecular mechanisms involved in these effects.

Materials and Methods

Cell Culture. Mouse 3T3 F442A preadipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum and cultured as described previously (Green and Kehinde, 1973; Kuri-Harcuch and Green, 1977).

Cell Proliferation Assays. Mouse 3T3 F442A preadipocytes were seeded at 5×10^4 cells per 35-mm plastic dish containing DMEM supplemented with 10% calf serum and exposed or not to increasing concentrations of rimonabant (10–400 nM) added every day. Five days after seeding, cell cultures were harvested by trypsin incubation, and cell densities were determined with a Coulter counter (Beckman Coulter, Fullerton, CA). Triplicate dishes were used for each experimental point, and data are the mean ± S.E.M. of three different experiments.

RNA Preparation and Northern Blot Analysis. β-Actin and GAPDH cDNA probes were purchased from Clontech (Mountain View, CA). Adiponectin cDNA was produced as described previously (Bensaid et al., 2003). Total RNA was prepared from 3T3 F442A preadipocyte cultured in DMEM containing 10% calf serum and treated or not with rimonabant or *Bordetella pertussis* toxin (PTX) or with the combination of rimonabant and PTX, at indicated concentrations, using TRIzol reagent (Invitrogen, Carlsbad, CA). For Northern blot analysis, 20 μg of total RNA was electrophoresed, and transferred to a nylon membrane (Hybond N^+; GE Healthcare, Little Chalfont, Buckinghamshire, UK). The membranes were hybridized successively with Adiponectin, GAPDH, and β-actin probes labeled with [α-^{32}P]dCTP using a random priming kit (GE Healthcare). Membranes were scanned on a Storm PhosphorImager (GE Healthcare). Relative quantification of RNA expression levels was performed with the Image-Quant program (GE Healthcare). Results were normalized against the β-actin mRNA expression and were presented as a percentage of control values.

Western Blot Analysis of Adiponectin and GAPDH Expression. 3T3 F442A preadipocyte cellular proteins were dissolved in the lysis buffer (50 mM Tris-HCl, pH 7.5, 1% SDS, 10 mM EDTA, 100 mM NaCl, and 1% β-mercaptoethanol), containing protease inhibitors (Roche Diagnostics), and centrifuged at 12,000*g* for 15 min at 4°C. The supernatants were collected and protein concentrations were determined by BCA protein assay kit (Pierce). 3T3 F442A adipocyte cellular protein extracts (200 μg) and conditioned medium (lyophilized 100 μl) were analyzed on the Novex precast 4 to 20% Tris-glycine–SDS-polyacrylamide gels and transferred on polyvinylidene difluoride membranes as indicated by the manufacturer. The following steps were performed at room temperature. The membranes were blocked with TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk for 1 h and were then incubated for 3 h with specific antibodies rabbit anti-mouse Acrp30 or rabbit anti-mouse GAPDH (Affinity Bioreagents, Golden, CO) in TBST containing 5% nonfat dry milk. After three 10-min washes in TBST, membranes were then incubated for 30 min with horseradish peroxidase-conjugated anti-rabbit antiserum (Sigma, St. Louis, MO). Membranes were then washed three times for 10 min in TBST. The immunoreactivity was revealed with the ECL-plus chemiluminescent substrate (GE Healthcare). Membranes were scanned on a Kodak Image Station 440 CF (Eastman Kodak, Rochester, NY), and relative quantification of adiponectin and GAPDH protein levels were performed with 1D Image Analysis Software (Kodak).

Western Blot Analysis of the Regulation of p42/44 MAP Kinase Activity. Mouse 3T3 F442 preadipocyte cells were cultured in DMEM supplemented with 10% calf serum. At subconfluence, culture medium was changed and cells were exposed to serum-free DMEM. After 24 h of incubation, cells were treated for 10 min with serum at 0, 1, and 2% in the presence or absence of rimonabant (100 nM), PTX (100 ng/ml), or the combination of rimonabant/PTX. p42/44 MAP kinase activity (phospho-p42/44 MAPK) was determined according to the manufacturer's protocol (Cell Signaling Technologies, Beverly, MA). In brief, treated cell cultures were washed twice in phosphate-buffered saline buffer, lysed in 200 μl of SDS-polyacrylamide gel electrophoresis buffer, and immediately scarped and har-

vested. Cell extracts were sonicated and boiled at 95°C for 5 min, and 30 μl was submitted to Western blot analysis by using specific antibodies, rabbit anti phospho-p42/44 MAPK, or rabbit anti p42/44 MAPK. Membranes were then incubated for 60 min, at room temperature, with horseradish peroxidase-conjugated, anti-rabbit antiserum (1:2000). The immuno-reactivity was revealed with the Lumi-GLO chemiluminescent reagent (Cell Signaling Technologies). Membranes were scanned on a Kodak Image Station 440 CF, and relative quantification of phospho-p42/44 MAPK normalized against p42/44 MAPK protein levels was performed with 1D Image Analysis Software (Kodak). Values represent the mean ± S.E.M. from three independent experiments.

Analysis of Lipid Droplet Accumulation. Subconfluent mouse 3T3 F442A preadipocytes cultured in DMEM supplemented with 10% calf serum were exposed or not (control) for 10 days, to rimonabant (100 nM) added every day, or to differentiating medium containing insulin (5 μg/ml) used as a positive control for the ability of cultured 3T3 F442A preadipocytes to accumulate lipids. Cell accumulation of lipid droplets was monitored by microscopic analysis and confirmed by Oil Red O staining as described previously (Green and Kehinde, 1973).

Results

Inhibitory Effect of Rimonabant on Mouse 3T3 F442A Preadipocyte Cell Proliferation. To evaluate the effect of rimonabant on preadipocyte cell proliferation, mouse 3T3 F442A preadipocytes were seeded at a low initial density (5×10^4 cells per 35-mm dish) in 10% calf serum-supplemented DMEM and exposed or not to increasing concentrations of rimonabant (10–400 nM) added every day. Five days after cell seeding, final densities were determined by counting cells under a Coulter counter. As shown in Fig. 1, in the absence of rimonabant, mouse 3T3 F442A preadipocytes proliferated actively and reached a final density of $3.20 \pm 0.20 \times 10^5$ cells per 35-mm dish. The addition of rimonabant inhibited 3T3 F442A preadipocyte cell proliferation in a concentration-dependent manner with a half-maximal effect (IC_{50}) at 60 ± 5 nM. No cytotoxicity was observed in the culture treated with rimonabant, even at the highest concentration tested. At this rimonabant concentration (400 nM), final density of 3T3 F442A preadipocyte cell culture was $1.7 \pm 0.10 \times 10^5$ cells per dish, which represented 53% of the final density of the control (rimonabant-untreated) culture, but remained 3.5-fold higher than the initial seeding density: 5×10^4 cells per dish (Fig. 1).

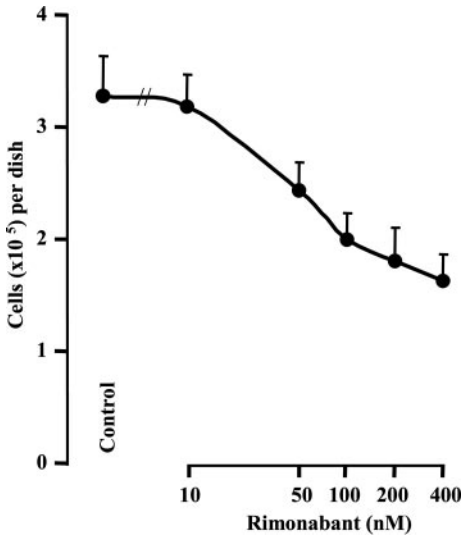

Fig. 1. Effect of rimonabant on cell proliferation of cultured mouse 3T3 F442A preadipocytes. Mouse 3T3 F442A preadipocytes seeded at a density of 5×10^4 cells per dish in DMEM supplemented with 10% calf serum were exposed or not (control) to increasing concentrations of rimonabant (10–400 nM) added every day. Five days after seeding, cells were harvested and counted with a Coulter counter. Values represent the mean ± S.E.M. of final cell densities from three independent experiments.

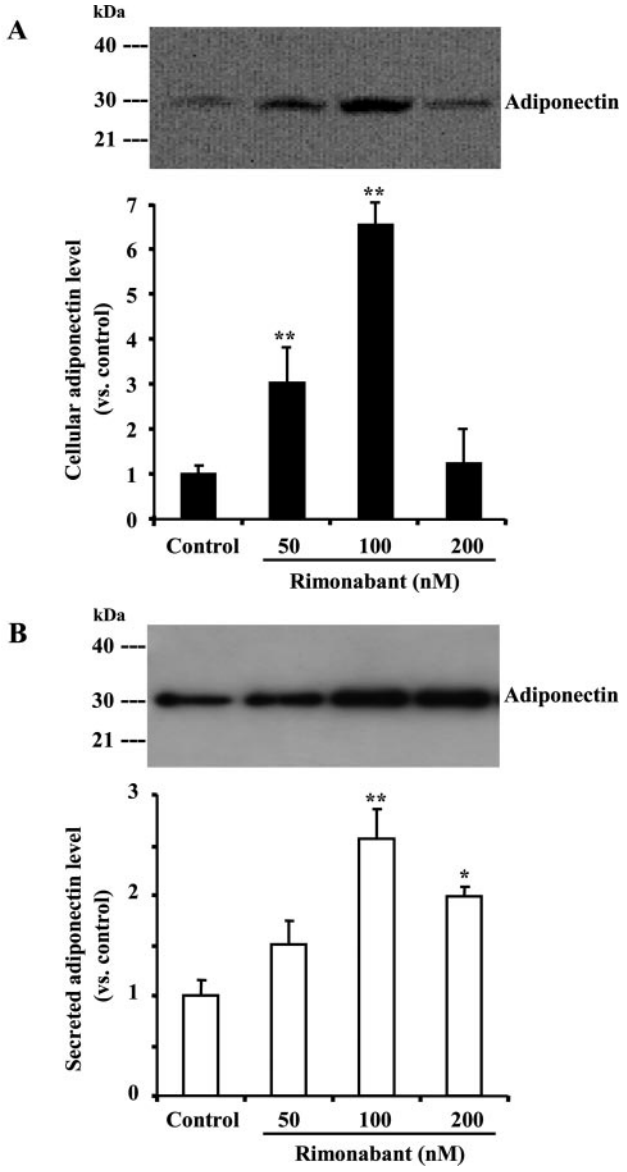

Fig. 2. Effect of rimonabant on adiponectin protein levels in cellular extract and in conditioned medium of cultured mouse 3T3 F442A preadipocytes. Subconfluent mouse 3T3 F442A preadipocytes cultured in DMEM supplemented with 10% calf serum were exposed or not (Control) to various concentrations of rimonabant (50–200 nM) added every day. After 4 days of treatment, the medium was changed and cell cultures were exposed for an additional 24 h to serum-free medium. Cellular protein extracts (200 μg) (A) and secreted proteins from conditioned medium (24 h) (B) were analyzed by Western blot using rabbit anti-mouse Acrp30 antibody at 1:1000 (Affinity Bioreagents), as described under *Materials and Methods*. Top, representative Western blot analysis. Bottom, bar graphs showing results of quantification analysis of adiponectin protein level in cellular extract and conditioned medium using image analysis Software (Kodak). Values represent the mean ± S.E.M. from three independent experiments. *, $p < 0.05$; **, $p < 0.01$, compared with control.

Rimonabant Increases Adiponectin and GAPDH mRNA and Protein Expression in Cultured Mouse 3T3 F442A Preadipocytes. To investigate the action of rimonabant on endocrine function and on enzyme content of mouse 3T3 F442A preadipocytes, we studied its effect on adiponectin (hormone) and GAPDH (enzyme) mRNA and protein expression in these cells. Four days of treatment of subconfluent cultures of 3T3 F442A preadipocytes with rimonabant (50 or 100 nM) increased, in a concentration-dependent manner, cellular and conditioned medium (secreted) levels of adiponectin protein with a maximal effect at 100 nM (Fig. 2). The cellular and conditioned medium levels of adiponectin protein in cultures treated with rimonabant (100 nM) were 6.5- and 2.5-fold higher than those observed in control cultures, respectively. These results agree with those reported previously showing that rimonabant stimulated adiponectin mRNA expression and increased cellular adiponectin level in these cells (Bensaid et al., 2003). At a higher concentration (200 nM) of rimonabant, these rimonabant-effects were not as pronounced as those observed using lower concentration of rimonabant (Fig. 2). Furthermore, the treatment of subconfluent cultures of 3T3 F442A preadipocytes with rimonabant (100 nM) induced a rapid increase in GAPDH mRNA expression. As shown in Fig. 3, GAPDH mRNA expression, compared with that of control cultures, increased by 2.1- and 3.5-fold after 30 and 60 min of rimonabant (100 nM) incubation, respectively. After 60 min of treatment, rimonabant (from 25 to 100 nM) increased GAPDH mRNA expression, in a concentration-dependent manner, with a maximal effect at 100 nM (Fig. 3). In parallel, after 4 days of treatment, and like adiponectin, rimonabant (from 50 to 200 nM) increased, in a concentration-dependent manner, the cellular content of GAPDH protein in cultured 3T3 F442A preadipocytes, with a maximal effect at 100 nM and a more modest effect at 200 nM (Fig. 4).

Rimonabant Does Not Induce Lipid Droplet Accumulation in Cultured Mouse 3T3 F442A Preadipocytes. The treatment for 10 days of subconfluent mouse 3T3 F442A preadipocytes with rimonabant (100 nM) added every day did not induce lipid droplet accumulation as monitored by Oil Red O staining. In contrast, subconfluent mouse 3T3 F442A preadipocytes exposed to differentiating medium, in the presence of insulin (5 μg/ml) added every 2 days, used as an internal positive control for the maintain of cell culture integrity, showed lipid droplet accumulation. This lipid accumulation was visible at day 4 of differentiating medium incubation and increased rapidly thereafter to reach a maximal level on day 10, as monitored by microscopic analysis and confirmed by Oil Red O staining (Fig. 5).

Rimonabant Inhibits Basal and Serum-Induced MAP Kinase Activities in Cultured Mouse 3T3 F442A Preadipocytes. To elucidate potential molecular mechanisms involved in the rimonabant-induced inhibition of mouse 3T3 F442A preadipocyte cell proliferation and increase of adiponectin and GAPDH expression, the effect of rimonabant on

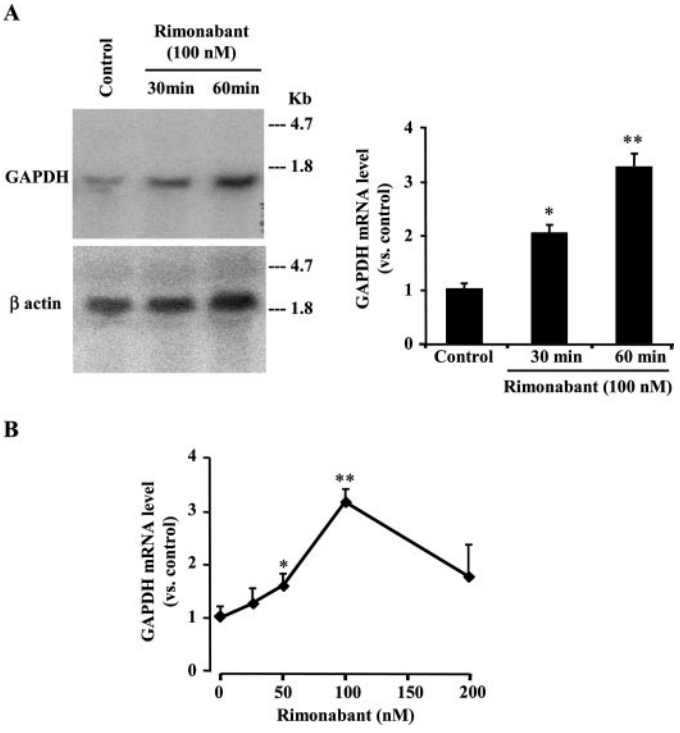

Fig. 3. Effect of rimonabant on GAPDH mRNA expression in mouse 3T3 F442A preadipocytes. Mouse 3T3 F442A preadipocytes were cultured in DMEM supplemented with 10% calf serum. At subconfluence, cells were exposed or not (control) to rimonabant (100 nM). At indicated times, total cellular mRNA was prepared and analyzed by Northern blot as described under *Materials and Methods*. A, representative Northern blot analysis. Bar graphs show results of quantification analysis of GAPDH expression normalized against the expression levels of β-actin by using the ImageQuant software (GE Healthcare). Values represent the mean ± S.E.M. from three independent experiments. *, $p < 0.05$; **, $p < 0.01$, compared with control. B, concentration-dependent effect of rimonabant (at 60 min of the treatment) on GAPDH mRNA expression in cultured mouse 3T3 F442A preadipocytes. Values represent the mean ± S.E.M. from three independent experiments. *, $p < 0.05$; **, $p < 0.01$, compared with control (in the absence of rimonabant).

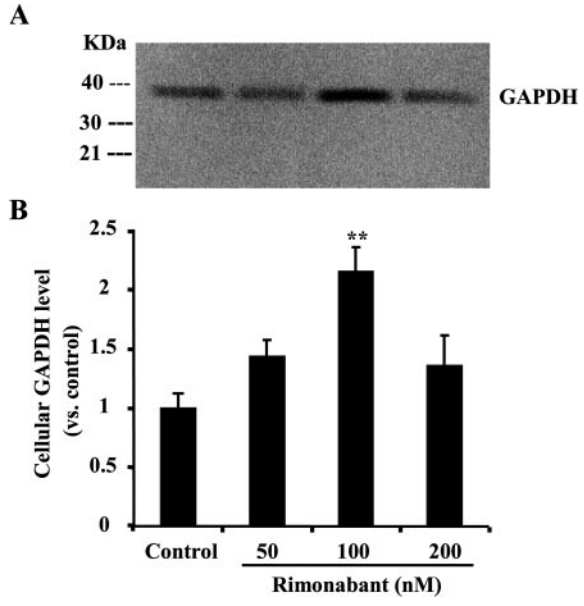

Fig. 4. Effect of rimonabant on GAPDH protein level in cultured mouse 3T3 F442A preadipocytes. Subconfluent mouse 3T3 F442A preadipocytes cultured in DMEM supplemented with 10% calf serum were exposed or not (control) to various concentrations of rimonabant (50–200 nM) added every day. After 4 days of treatment, cellular protein extracts (200 μg) were analyzed by Western blot using rabbit anti-GAPDH antibody at 1:2000 (Affinity Bioreagents). A, representative Western blot analysis. B, bar graph shows results of quantification analysis of cellular GAPDH level, using image analysis software (Kodak). Values represent the mean ± S.E.M. from three independent experiments. **, $p < 0.01$, compared with control.

MAP kinase activity was tested. For this purpose, subconfluent mouse 3T3 F442A preadipocytes exposed to serum-free DMEM for 24 h were treated for 10 min with calf serum at 0, 1, and 2% in the presence or absence of rimonabant (100 nM). Western blot analysis of MAP kinase activities, by using rabbit anti-phospho-p42/44 MAPK, showed that the addition of calf serum at 1 or 2% induced a strong stimulation of MAPK activity in mouse 3T3 F442A preadipocytes (Fig. 6). The stimulation rate of MAPK activity, compared with that of serum-free cultures, was 9.5- and 13.1-fold higher in the presence of 1 and 2% of calf serum, respectively. In contrast, rimonabant (100 nM) treatment strongly inhibited MAPK activity in mouse 3T3 F442A preadipocytes (Fig. 6). Rimonabant inhibited 90% of basal MAPK activity in mouse 3T3 F442A preadipocyte cell cultures exposed to serum-free medium. Furthermore, rimonabant inhibited 75 and 50% of MAPK activity induced by 1 and 2% of calf serum supplemented medium, respectively (Fig. 6). The effect of rimonabant-increasing concentrations on MAP kinase activity induced by 1% calf serum was studied and showed a biphasic profile depending on used rimonabant concentrations (Fig. 7). In fact, at low concentrations (10, 50, and 100 nM), rimonabant inhibited MAP kinase activity in a concentration-dependent manner with a maximal effect at 100 nM. The extent of MAP kinase activity-inhibition produced by rimonabant at 10, 50, and 100 nM was 15, 40, and 75%, respectively, compared with control cultures. It is surprising that at high concentrations (200 nM and 400 nM), rimonabant slightly

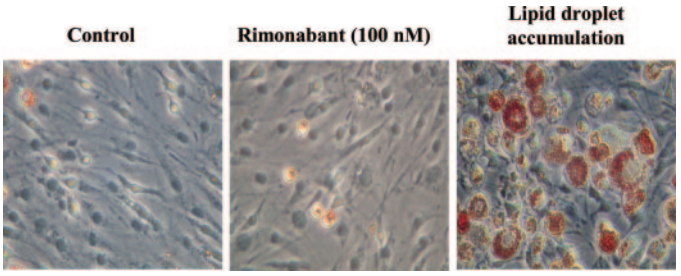

Fig. 5. Lack of lipid accumulation in cultured mouse 3T3 F442A preadipocytes treated with rimonabant. Subconfluent mouse 3T3 F442A preadipocytes cultured in DMEM supplemented with 10% calf serum were exposed or not (control) to rimonabant (100 nM) added every day. As a positive control of lipid droplet accumulation, other control 3T3 F442A preadipocyte cultures were exposed to differentiating condition in the presence of insulin (5 μg/ml) added every 2 days. After 10 days of treatment, cell accumulation of lipid droplets was monitored by microscopic analysis and confirmed by Oil Red O staining.

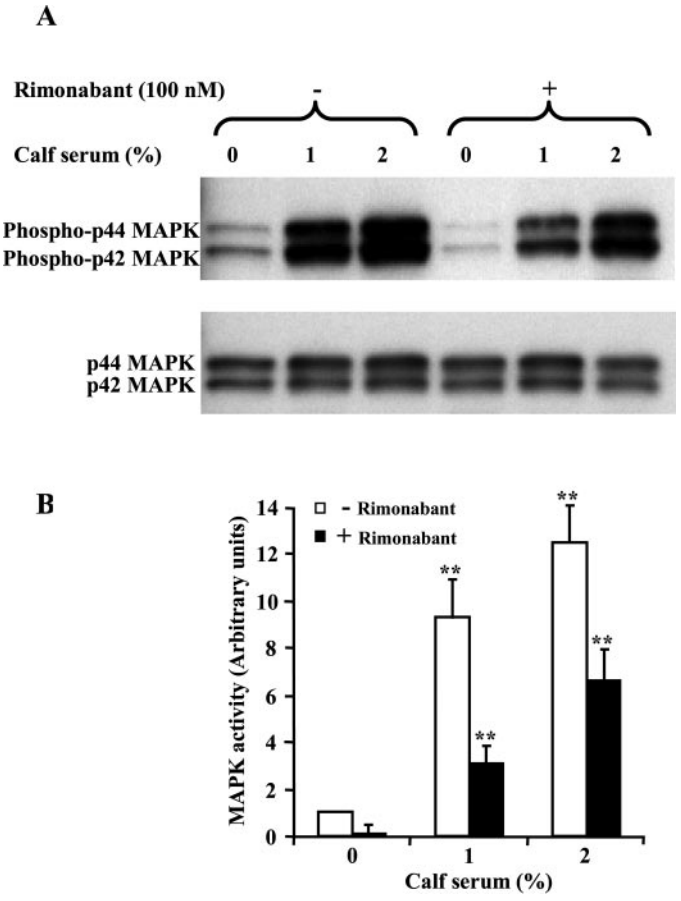

Fig. 6. Inhibition of MAP kinase activity in cultured mouse 3T3 F442A preadipocytes by rimonabant. Mouse 3T3 F442 preadipocytes were cultured in DMEM supplemented with 10% calf serum. At subconfluence, culture medium was changed and cells were exposed to serum-free DMEM for 24 h. Then, cells were treated for 10 min with calf serum at 0, 1, and 2% in the presence or absence of rimonabant (100 nM). MAP kinase activity was determined as described under *Materials and Methods*. A, representative MAP kinase activity (Phospho-p42/44 MAPK) analysis. B, bar graph shows results of quantification analysis of MAP kinase activity normalized against p42/44 MAPK cellular level, using image analysis software (Kodak). Values represent the mean ± S.E.M. from three independent experiments. **, $p <$ 0.01, compared with rimonabant-untreated cell cultures.

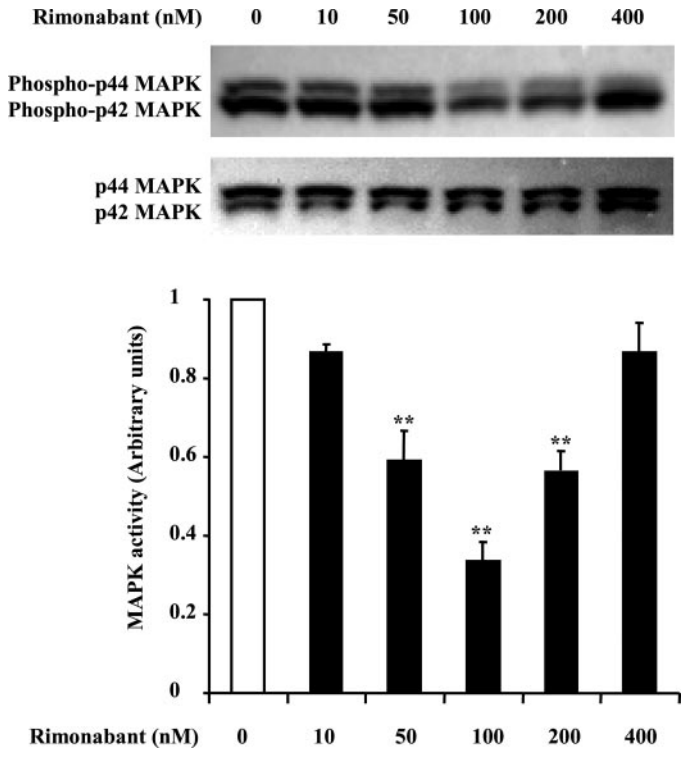

Fig. 7. Concentration-dependent effect of rimonabant on MAP kinase activity in cultured mouse 3T3 F442A preadipocytes. Mouse 3T3 F442 preadipocytes were cultured in DMEM supplemented with 10% calf serum. At subconfluence, culture medium was changed and cells were exposed to serum-free DMEM for 24 h. Then, cells were treated for 10 min with calf serum at 1% in the absence or in the presence of rimonabant (10–400 nM). MAP kinase activity was determined as described under *Materials and Methods*. A, representative MAP kinase activity (Phospho-p42/44 MAPK) analysis. B, bar graph shows results of quantification analysis of MAP kinase activity normalized against p42/44 MAP kinase cellular level, using image analysis software (Kodak). Values represent the mean ± S.E.M. from three independent experiments. **, $p <$ 0.01, compared with rimonabant-untreated cell cultures.

reduced MAP kinase activity. Indeed, the extent of rimonabant-inhibition of MAP kinase activity was 45 and 15% at concentrations 200 and 400 nM, respectively (Fig. 7). This loss in the potency of rimonabant at high concentrations, to inhibit MAP kinase activity, is probably due to a saturation of signaling pathway. This hypothesis needs complementary studies to be elucidated.

Effects of PTX on the Regulation of Adiponectin and GAPDH mRNA Expression and MAP Kinase Activity in Cultured Mouse 3T3 F442A Preadipocytes. Pretreatment of cultured 3T3 F442A preadipocytes by PTX (100 ng/ml) increased adiponectin and GAPDH mRNA expression. The potency of this effect was similar to that observed in cultures treaded by rimonabant (100 nM) alone. The addition of rimonabant (100 nM) to the culture of 3T3 F442A preadipocytes pretreated by PTX did not induce supplementary effect (Fig. 8A). Furthermore, and in the same way, pretreatment of cultured 3T3 F442A preadipocytes by PTX (100 ng/ml) inhibited MAP kinase activity. The extent of this MAP kinase inhibition induced by PTX was comparable with that induced by rimonabant (100 nM) treatment. The addition of rimonabant (100 nM) to the culture of 3T3 F442A preadipocytes pretreated by PTX did not induce supplementary effect (Fig. 8B). These results agree with those reported previously in Chinese hamster ovary stably transformed with CB1 receptor (Bouaboula et al., 1997).

Discussion

Adipose tissue is now recognized to be a dynamic tissue and an important endocrine organ, secreting biologically active molecules called adipocytokines that are involved in the regulation of energy metabolism and body weight homeostasis. It has been reported, in numerous studies, that these adipocytokines play a crucial role in several pathophysiological processes, including obesity and associated diseases (Trayhurn and Beattie, 2001; Fortuno et al., 2003; Lyon et al., 2003; Rajala and Scherer, 2003; Reilly and Rader, 2003; Ruan and Lodish, 2003; Borst, 2004; Fain et al., 2004; Grundy, 2004; Matsuzawa et al., 2004; Pischon et al., 2004; Trayhurn and Wood, 2004; Wisse, 2004). In obesity, adipose tissue development is due to adipocyte cell proliferation (hyperplasia) and adipocyte cell size increase (hypertrophy) with excessive fat storage inducing marked changes in cellular composition and distribution of adipose tissue with alteration of its endocrine function. This may be one of principal causes of obesity-associated diseases together with their progressive and chronic features. Adipocyte cell proliferation and differentiation represent two closely related cell processes that control adipose tissue homeostasis. One of the first events occurring during adipose tissue development in obesity is the re-entry of arrested preadipocytes into a proliferating state. After several rounds of clonal expansion, cells arrest proliferation again and undergo terminal adipocyte differentiation with energy storage as fat (triglycerides) and adipocyte size increase (Prins and O'Rahilly, 1997; Fajas et al., 2001; Hausman et al., 2001; Spiegelman and Flier, 2001; Trayhurn and Beattie, 2001). These events may be initiated by the availability of excessive energy, especially that resulting from an imbalance between energy intake and expenditure, which may be determined by the interaction between genetic, environmental and psychosocial factors and by changes in lifestyle (Carpino, 2000; Hausman et al., 2001; Spiegelman and Flier, 2001). The selective CB_1 receptor antagonist, SR141716 (rimonabant), has potent antiobesity effects in

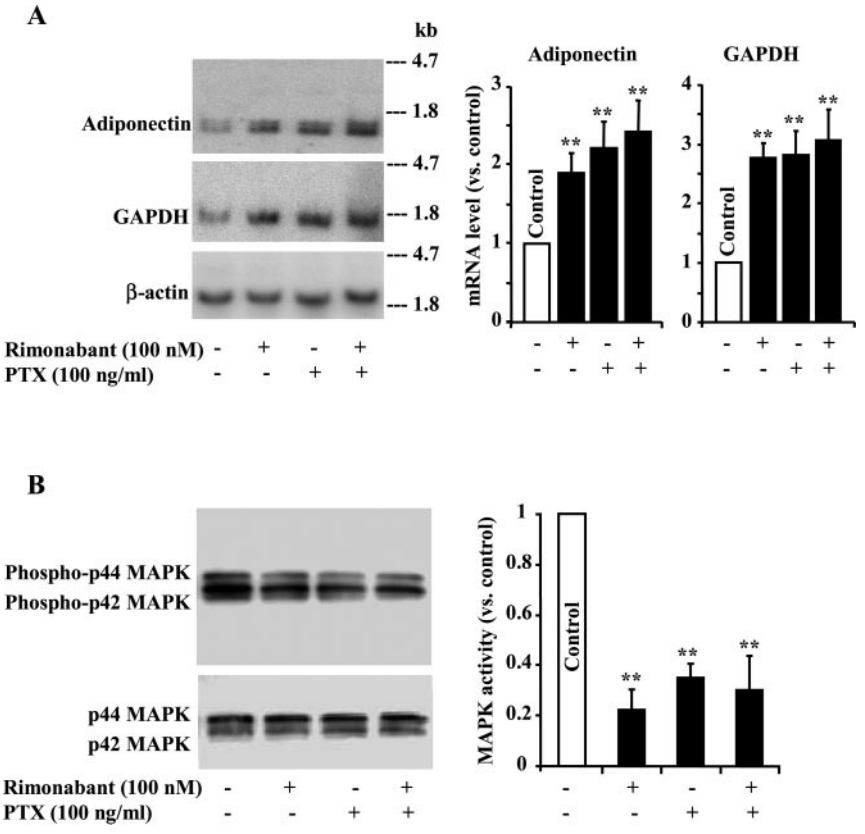

Fig. 8. Effects of PTX on the regulation of adiponectin and GAPDH mRNA expression and MAP kinase activity in cultured mouse 3T3 F442A preadipocytes. A, mouse 3T3 F442A preadipocytes were cultured in DMEM supplemented with 10% calf serum. At subconfluence, cells were exposed or not (control) to rimonabant (100 nM), PTX (100 ng/ml), or the combination of rimonabant and PTX. After 60 min of treatment, total cellular mRNA were prepared and analyzed by Northern blot as described under *Materials and Methods.* Representative Northern blot analysis is showed and bar graphs show results of quantification analysis of adiponectin and GAPDH expression normalized against the expression levels of β-actin by using ImageQuant program (GE Healthcare). Values represent the mean ± S.E.M. from three independent experiments. **, $p < 0.01$, compared with control cultures. B, mouse 3T3 F442 preadipocytes were cultured in DMEM supplemented with 10% calf serum. At subconfluence, culture medium was changed and cells were exposed to serum-free DMEM for 24 h. Then, cells were treated for 10 min with calf serum at 1%, in the absence (Control) or in the presence of, rimonabant (100 nM) or PTX (100 ng/ml) or the combination of rimonabant and PTX. MAP kinase activity was determined as described in Fig. 6. Representative MAP kinase activity (Phospho-p42/44 MAPK) analysis is shown, and bar graphs show results of quantification analysis of MAP kinase activity normalized against p42/44 MAPK cellular level, using image analysis software (Kodak). Values represent the mean ± S.E.M. from three independent experiments. **, $p < 0.01$, compared with control cultures.

rodents (Arnone et al., 1997; Chaperon et al., 1998; Colombo et al., 1998; Di Marzo et al., 2001; Ravinet-Trillou et al., 2002; Bensaid et al., 2003; Poirier et al., 2005), which may result from its metabolic "peripheral" action on adipose tissue. This action of rimonabant involved, at least in part, a CB1 receptor-mediated response producing a local stimulation of adiponectin expression (Bensaid et al., 2003).

In the present study, we investigated the effect of rimonabant on the proliferation of cultured mouse 3T3 F442A preadipocytes. Our results show that rimonabant treatment inhibits cell proliferation of cultured mouse 3T3 F442A preadipocytes, a process that has been shown to be the first and principal step of adipose tissue development (Hausman et al., 2001; Spiegelman and Flier, 2001). To assess the effect of rimonabant on endocrine function and enzyme content of cultured mouse 3T3 F442A preadipocytes, we also studied the effect of rimonabant on the expression of adiponectin (an adipo-hormone) and GAPDH (an enzyme) involved in lipid and glucose metabolism. We showed that rimonabant treatment of cultured mouse 3T3 F442A preadipocytes increased the expression of adiponectin and GAPDH, two late markers of adipocyte cell maturation. Lipid accumulation in adipocytes is also another marker of adipocyte maturation and final stage of differentiation (Ailhaud, 1997; Prins and O'Rahilly, 1997; Fajas et al., 2001; Hausman et al., 2001; Spiegelman and Flier, 2001). Excessive fat accumulation in adipocytes is the second step involved in adipose tissue development and may be associated with the endocrine and structural dysfunctions of adipose tissue in obesity and related diseases. For this reason, we studied the effect of rimonabant on lipid accumulation in cultured mouse 3T3 F442A preadipocytes. Results showed that rimonabant did not induce lipid accumulation in cultured mouse 3T3 F442A preadipocytes.

Together, our results show that rimonabant, although inhibiting cell proliferation, induces the expression of two late markers of adipocyte maturation (adiponectin and GAPDH) without inducing lipid accumulation in cultured mouse 3T3 F442A preadipocytes. However, arrested adipocyte cell proliferation is generally followed by maturation and differentiation of adipocytes with accumulation of lipids (Hausman et al., 2001; Trayhurn and Beattie, 2001). Our hypothesis is that, in this cellular animal model, rimonabant inhibits preadipocyte cell proliferation and induces an uncoupling of the association between the inhibition of adipocyte cell proliferation and lipid accumulation (Deslex et al., 1987; Ailhaud, 1997). This atypical property may account for the potent activity of rimonabant in obesity treatment. Complementary research is needed to clarify this hypothesis.

On the other hand, treatment of mouse 3T3 F442A preadipocytes with rimonabant (100 nM) inhibited basal and serum-induced p42/44 MAP kinase activities. These results agree with those reported previously, showing the involvement of MAP kinase activities in the CB_1 receptor signaling pathway (Bouaboula et al., 1997; Galve-Roperh et al., 2002) and suggest that the inhibition of MAP kinase activity induced by rimonabant may be one of the mechanisms involved in the inhibition of cultured 3T3 F442A preadipocyte cell proliferation and in the stimulation of adiponectin and GAPDH expression. The MAP kinase pathway involvement in the induction of adiponectin has been previously reported in mouse 3T3 L1 preadipocytes (Engelman et al., 1998). However, its involvement in the up-regulation of GAPDH remains to be elucidated. These effects of rimonabant may be mediated via antagonism of a local endocannabinoid tone or through the inverse agonist activity of this compound and involve functional CB1 receptors that have been reported previously to be expressed in rodent (Bensaid et al., 2003) and human (Guillot et al., 2003) adipocyte cells.

Finally, our results show clearly that rimonabant, at low concentrations (from 25 to 100 nM), increased adiponectin and GAPDH expression and reduced MAP kinase activity in cultured 3T3 F442A preadipocytes; however, at higher concentrations (200 or 400 nM), these rimonabant effects were observed only slightly or not at all in this cell type. This loss of rimonabant potency, at higher concentrations, may be due to a saturation of signaling pathway.

Indeed, PTX treatment increased adiponectin and GAPDH expression and inhibited MAP kinase activity in cultured 3T3 F442A preadipocytes. These PTX effects were comparable with those observed with rimonabant treatment. Furthermore, no additional effects were obtained when rimonabant was added to the culture of 3T3 F442A preadipocytes pretreated with PTX. This suggests that in cultured 3T3 F442A preadipocytes, the regulation by rimonabant of adiponectin and GAPDH expression and of MAP kinase activity is mediated by the CB1 receptor coupled to PTX-sensitive G_i protein.

Whatever the mechanism involved in the action of rimonabant, the inhibition of preadipocyte cell proliferation and the induction of adipocyte late "maturation" without fat accumulation may participate in the antiobesity effects of rimonabant, particularly in the reduction of body fat mass and in the restoration of adipose tissue homeostasis and its endocrine function.

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