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The expression of the p85α subunit of phosphatidylinositol 3-Kinase is induced by activation of the peroxisome proliferator-activated receptor γ in human adipocytes

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Abstract

Aims/hypothesis. Thiazolidinediones are new oral antidiabetic drugs that activate the nuclear receptor PPARγ. Our aim was to identify potential target genes of PPARγ in the human adipocyte in order to clarify how thiazolidinediones improve insulin sensitivity.
Methods. The effect of BRL 49653 (Rosiglitazone) on the mRNA expression of insulin receptor, insulin receptor substrate-1, p85α, p110α and p110β subunits of phosphatidylinositol 3-kinase, Glut 4 and hormone sensitive lipase was examined in isolated adipocytes. Target mRNA levels were determined by RT-competitive PCR.
Results. The BRL 49653 (1 μmol/l) increased the mRNA concentrations of p85αPI-3 K (264 ± 46 vs 161 ± 31 amol/μg total RNA, $p = 0.003$) whithout affecting the expression of the other mRNAs of interest. This effect was dose-dependent ($K_{0.5}$ = 5 nmol/l) and was reproduced by a specific activator of RXR, indicating that it was probably mediated by the PPARγ/RXR heterodimer. The BRL 49653 also increased the amount of p85αPI-3K protein in adipose tissue explants (71 ± 19%). In addition, BRL 49653 produced a more than twofold increase in insulin stimulation of phosphatidylinositol 3-kinase activity and significantly enhanced the antilipolytic action of insulin.
Conclusion/interpretation. This work demonstrates that the gene of p85αPI-3K is probably a target of PPARγ and that thiazolidinediones can improve insulin action in normal human adipocytes. Although the precise mechanism of action of BRL 49653 on PI3-Kinase activity is not completely clear, these findings improve our understanding of the insulin-sensitizing effects of the thiazolidinediones, possible drugs for the treatment of Type II (non-insulin-dependent) diabetes mellitus. [Diabetologia (2001) 44: 544–554]

Keywords Nuclear receptor, insulin resistance, gene regulation, RT-cPCR.

The thiazolidinediones are a new family of oral hypoglycaemic agents that have been shown to reduce blood glucose, insulin and triglyceride concentrations in insulin resistant Type II (non-insulin-dependent) diabetic patients [1, 2]. These agents appear to act mostly by increasing the sensitivity of peripheral tissues to insulin as has been demonstrated in animal models and in Type II diabetic patients treated with troglitazone [2]. Although the mechanism of this effect is not clear, it has been shown that thiazolidinediones are high affinity ligands of the peroxysome proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor family [3, 4]. Their capacity to bind and to activate PPARγ in vitro correlates with their antidiabetic action in vivo, suggesting that

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Abbreviations: PPAR, Peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator responsive element; IR, insulin receptor; IRS-1, insulin receptor substrate-1; p85αPI-3, p85α regulatory subunit of phosphatidylinositol 3-kinase; PI3-kinase, phosphatidylinositol 3-kinase; RT-cPCR, RT-competitive PCR.

PPARγ mediates the insulin sensitizing effect of these molecules [5, 6]. The PPARγ is predominantly expressed in differenciated adipocytes [7–9] suggesting that adipose tissue might be an important target for the effect of thiazolidinediones on insulin sensitivity. The PPARγ exists as two isoforms (γ1 and γ2) generated from the same gene by alternative promoter usage and mRNA splicing [10, 11], and differing in their 28 amino terminus amino acids in humans [11]. Therefore, both isoforms could bind the thiazolidinediones in the ligand binding site located in the E domain [12]. The PPARγ1 mRNA has been found in several tissues [7–9], including liver and skeletal muscle where the expression levels are very low [9]; PPARγ2 is expressed almost exclusively in adipose tissue [8, 9]. However, in human subcutaneous adipose tissue and isolated adipocytes, PPARγ1 mRNA is the major isoform, representing more than 80% of the PPARγ transcripts [9].

The PPARγ are considered key actors in adipocyte differentiation and lipid metabolism [12]. Forced expression of PPARγ into fibroblasts triggers their transformation into adipocytes [13]. However, the high levels of PPARγ expression in mature adipocytes [7–9] also indicate that they have a major role in maintaining the metabolic functions of the fully differentiated adipocytes. In agreement with this, most of the known PPARγ target genes encode adipose tissue proteins involved in lipid storage and metabolism, such as adipose fatty acid-binding protein [14], lipoprotein lipase [15], acyl-CoA-synthetase [16] or fatty acid transport protein [16]. To control the transcription of their target genes, the PPARγ heterodimerize with the 9-*cis*-retinoic acid receptor (RXR) [17] and then bind specific *cis*-acting elements called peroxisome proliferator responsive elements (PPREs) that generally consist of a hexameric nucleotide direct repeat spaced by one nucleotide (DR-1) [17–19].

Several possibilities have been proposed to explain the insulin sensitizing action of the thiazolidinediones [20]. Activation of PPARγ in adipose tissue could firstly increase the production of small adipocytes that are more sensitive to insulin; secondly, decrease the production of "adipocytokines" (i.e. tumor necrosis factor-α, leptin) that are thought to induce insulin resistance; and thirdly, reduce the concentrations of plasma lipid and fatty acid by increasing their clearance by adipocytes. All these effects can contribute to the improvement of whole body insulin sensitivity. However, most of these data have been obtained using animal models of cell cultures and the human target genes of PPARγ are not yet known. In addition the target genes identified so far in rodents are not directly involved in the mechanism of action of insulin and thus it not clear how changes in their expression can improve insulin sensitivity.

Therefore, in an attempt to clarify the mechanisms by which thiazolidinediones improve insulin sensitivity, we examined whether key genes of the insulin signalling pathways are target genes of PPARγ in human adipocytes. Isolated mature adipocytes and short incubation periods were used to avoid effects linked to PPARγ-induced cell differentiation. We incubated the cells with Rosiglitazone (BRL 49653), a potent PPARγ-selective thiazolidinedione [3, 4] and measured the rapid changes in the expression levels of candidate genes, namely the insulin receptor (IR), the insulin receptor substrate-1 (IRS-1), the p85α regulatory subunit of the phosphatidylinositol-3 kinase (p85αPI-3K), the p110α and β catalytic subunits of PI-3 K, the insulin-sensitive glucose transporter Glut 4 and the hormone sensitive lipase (HSL).

Subjects and methods

Products. Both BRL 49563 (5-(4-[2-Methyl-N-(2-pyridyl)amino) ethoxy] benzyl)-thiazolidine-2,4-dione) and LG 1069, a selective RXR agonist [21], was kindly provided by Dr Heyman and Dr M. Leibowitz (Ligand Pharmaceuticals, San Diego, Calif., USA).

Preparation of human isolated adipocytes. Abdominal subcutaneous adipose tissue (6 ± 1.5 g wet weight) was obtained during surgery with the informed consent of the patients, after approval of the protocol by the ethic committee of the Hospices Civils of Lyon (France). Adipose tissue samples were obtained either from healthy lean subjects or from non-diabetic obese subjects (see Results for details). The tissue was immediately immersed in Hanks medium, kept at 4 °C and cell preparation was started within 15 min after tissue sampling. Adipocytes were isolated by digestion with collagenase (1 mg/ml; type II, Sigma, La Verpillère, France) according to the method previously described [22] with the modifications [23]. Isolated adipocytes were incubated at 37 °C in DMEM (Life Technologies, Cergy Pontoise, France) containing 4% fatty acid free bovine serum albumin in a final volume of 1 ml. The average cell number in the incubation medium varied from 50,000 to 150,000 cells per ml. Cells were incubated 30 min at 37 °C before the various agents or vehicle were added. At the end of the incubation periods, infranatant was removed by aspiration and cells were immediately frozen in liquid nitrogen. We verified the viability and the metabolic integrity of the cells prepared and incubated under these conditions [23]. The effectiveness of BRL 49653 in human adipocytes was assessed by measuring the effects of the thiazolidinedione on the mRNAs of leptin and uncoupling protein 2, two genes that have been previously identified as targets of PPARγ in adipose cells [24, 25]. Leptin mRNA was significantly decreased by 30 ± 3% ($p = 0.027$) and uncoupling protein 2 mRNA increased by 44 ± 10% ($p = 0.008$) after 4 h of incubation with 10^{-6} mol/l BRL 49653 [26].

Adipose tissue explants. Abdominal subcutaneous adipose tissue samples were cut into small pieces under sterile conditions, rinsed once in DMEM, weighed and incubated (about 300 mg tissue/2 ml media) in DMEM supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (without bovine serum albumin and serum) at 37 °C under 5% CO_2, 95% O_2 atmosphere.

Total RNA preparation and quantification of the target mRNAs. Total RNA from the isolated adipocytes was prepared using the RNeasy total RNA kit from Qiagen (Courtaboeuf, France) as previously indicated [23]. Total RNA concentration was determined by absorbance measurement at 260 nm. The 260/280 nm absorption ratio of all preparation was between 1.8 and 2.0. The average yield of total RNA was about 1 μg/100,000 isolated cells.

The mRNA levels of the target genes were determined by reverse transcription reaction followed by competitive polymerase chain reaction (RT-cPCR). The RT-cPCR method consists, after specific reverse transcription reactions, in the co-amplification of the target cDNAs with known amounts of a specific competitor DNA in the same tube [27]. For the assays of IR, IRS-1, p85αPI-3K, Glut 4 and HSL mRNAs, the construction of the competitor DNA molecules, the sequence of the primers and the validation of the RT-cPCR assays have been reported elsewhere [28, 29]. In the case of p85αPI-3K, the assay was specific for the p85αPI-3K mRNA and did not determine the amount of mRNAs of the shorter spliced variants of p85α.

New competitor DNA molecules were constructed for the quantitation by RT-cPCR of the mRNAs encoding the p110α PI-3K and p110βPI-3K, the main isoforms of catalytic subunits of PI-3Kinase in insulin sensitive tissues [30]. For p110αPI-3K, a 517 nucleotide-long cDNA fragment was obtained by RT-PCR on human skeletal muscle total RNA using 5'-$_{79}$CCAAATGGAATGATAGTGAC$_{98}$-3' as sense primer and 5'- $_{593}$ACTATTACCCAAATCACCAC$_{574}$-3' as antisense primer. The competitor was obtained by deleting 61 bp using a two step PCR overlap extension method. For p110βPI-3 K, a 457 nucleotide-long, cDNA fragment was obtained by RT-PCR using 5'-$_{2712}$CTGTGCTGGCTACTGTGTA$_{2730}$-3' as sense primer and 5'-$_{3166}$TAGTAGTCCAG CTTTCCCTG$_{3147}$-3' as antisense primer. The competitor was obtained by deleting 49 bp. The checked sequences of the p110 αPI-3K and the p110βPI-3K cDNA fragments and the corresponding competitors matched the published sequences perfectly. The p110α and p110βPI-3K mRNA assays were validated using a large range of in vitro synthetised RNA (0.25 to 50 amol in the reaction) as recommended [27].

For the assays of the target mRNAs, the RT-cPCR reactions were carried out in the conditions previously indicated [27–29]. CY-5 5'-end labelled sense primers were used in the PCR and the products were analysed using a ALFExpress DNA sequencer (Pharmacia, Upsala, Sweden) as previously reported [29]. The coefficient of variation of the method was demonstrated to be between 5 and 10 % [27].

Quantification of p85αPI-3K and protein kinase B (PKB) protein concentrations by western-blotting. Adipose tissue explants were homogenized in a phosphate-buffered saline (PBS) lysis buffer containing 1 % Nodinet P-40 (Sigma), 0.5 % sodium desoxycholate (Sigma), 0.1 % SDS (Sigma) and supplemented with a freshly prepared cocktail of protease inhibitors (400 μg/ml 4-(2-aminoethyl)-benzenesulphonyl fluoride, 5 mg/ml EDTA, 1 μg/ml leupeptin and 1 μg/ml pepstatin) (ICN Pharmaceuticals, Orsay, France). After centrifugation of the lysate at 4 °C, protein concentration was measured with the Bio-Rad protein assay system. Proteins (5 μg for p85αPI-3 K and 200 μg for PKB) were separated by SDS-PAGE, transferred to polyvinylidine difluoride (PVDF) membrane (Millipore, France) and blocked over-night at 4 °C in blocking buffer (PBS, 0.1 % Tween-20, 1 % skim milk and 1 % bovine serum albumin). The p85αPI-3 K was identified using a rabbit polyclonal antibody that recognizes human, mouse and rat p85α subunit of the phosphatydilinositol-3 kinase (Upstate Biotechnology, N.Y., USA). Membranes were incubated for 1 h at room temperature with the antibody (1:1000), and next developed with an anti-rabbit IgG peroxydase conjugate (Sigma) diluted at 1:10,000. The complex was visualized using a chimioluminescent kit (Specichrom, Lyon, France). Films (Biomax ML, Kodak, Creteil, France) were analysed with a Vernon photometer-integrator. Protein kinase B α (Akt1) was identified using a goat polyclonal antibody from Santa Cruz Biotechnology (Sant Cruz, Calif., USA) and an anti-goat/sheep IgG alkaline phosphatase conjugate (Sigma) as secondary antibody. Signals were visualized using the Enzyme Catalyzed Fluorescence substrate for western blotting from Amersham Pharmacia Biotech (Buckinghamshire, UK) and quantified using a FluorImager SI and ImageQuant software (Molecular Dynamics, Sunnyvale, Calif., USA).

Measurement of PI3-Kinase activity. Adipose tissue explants were pre-incubated for 12 h at 37 °C in presence of BRL 49 653 or vehicule, rinsed in DMEM, and further incubated for 2 and 15 min with insulin (0.1 μmol/l). At the end of the incubation, explants were homogenized by brief sonication in 2 ml of lysis buffer (140 mmol/l NaCl, 20 mmol/l TRIS-HCL pH 7.4, 10 mmol/l EDTA, 100 mmol/l NaF, 2 mmol/l Vanadate, 10 mmol/l Pyrophosphate, 1 % Nodidet P-40) supplemented with a freshly prepared cocktail of protease inhibitors, and solubilization was achieved by continuous stirring for 1 h at 4 °C. After a centrifugation at 12,000 g for 10 min, the infranatant was used for protein determination and for immunoprecipitation either with a specific antibody of the p85αPI-3 K (kindly provided by Dr. J-F Tanti) or with a specific antibody of IRS-1 (Santa Cruz Biotechnology, Calif., USA). Immunoprecipitations were done as described [31]. The PI3-Kinase activity was measured on the immunoprecipitate directly, in a reaction mixture (30 μl final volume) consisting of 40 mmol/l HEPES pH 7.5, 20 mmol/l $MgCl_2$, 50 μmol/l [γ-^{32}P]ATP (8 μCi) and phosphatidylinositols (10 μg/sample) as described [31]. The reaction was stopped after 20 min at ambiant temperature by addition of 40 μl of 4 N HCl. The phospholipids were extracted in 160 μl of chloroform:methanol (1:1, v:v). The lipid extracts were spotted on a thin layer chromatography plate and the chromatogram was developped with CHCl3, MeOH, 1N NH4OH (60:47:13.2) as described [31]. Following autoradiography, films were analysed and bands were quantified using a desktop image scanner and the NIH-Image software.

Determination of PKB phosphorylation. For the determination of the amount of PKBα phosphorylated on Ser 473, adipose tissue explants were treated as described above. After centrifugation of the homogenate, 200 μg of proteins were separated by SDS-PAGE, transferred to PVDF membrane and blocked over-night at 4 °C in blocking buffer (PBS, 0.1 % Tween-20, 4 % skim milk). Phosphorylated PKBα was identified and quantified using an anti-phospho Akt1/PKBα (Ser 473) from Upsate Biotechnology and an anti-goat/sheep IgG alkaline phosphatase conjugate (Sigma) as secondary antibody. Signals were visualized using the Enzyme Catalyzed Fluorescence substrate for western blotting from Amersham Pharmacia Biotech and quantified using a FluorImager SI and ImageQuant software (Molecular Dynamics, Sunnyvale, Calif., USA).

Measurement of lipolysis in adipose tissue explants. Adipose tissue explants were incubated for 12 h at 37 °C and rinced in DMEM without sodium pyruvate and with 4.5 g/l of glucose (Life Technologies, Cergy Pontoise, France) and then further incubated in 1 ml of this buffer. After pre-incubation for 30 min at 37 °C, isoproterenol (1 μmol/l) alone or in combina-

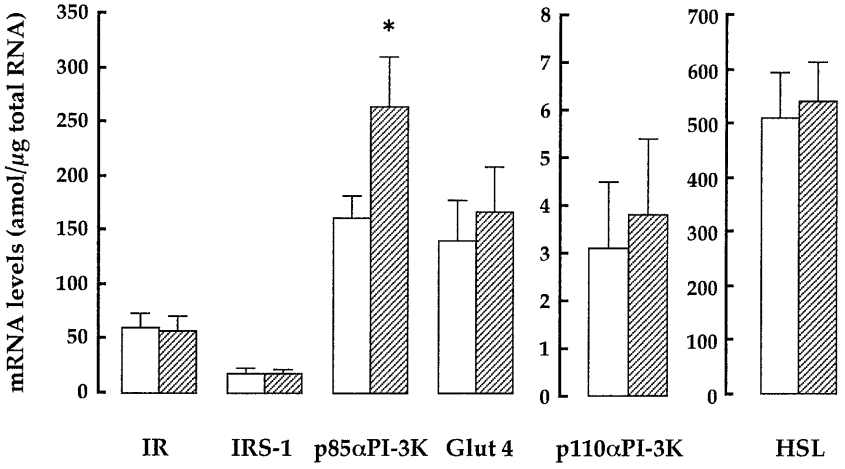

Fig. 1. Effect of BRL 49653 on the mRNA concentrations of insulin receptor (IR), IRS-1, p85αPI-3 K, p110αPI-3 K, Glut 4 and HSL in human isolated adipocytes. The mRNA levels of the target genes were determined by RT-cPCR in human adipocytes after 4 h of incubation in the absence (□) and in the presence of 1 μmol/l BRL 49653 (▨). In the absence of BRL 49653, cells were incubated with 0.1% dimethylsulfoxide (vehicle). The figure shows the mean ± SEM for 4 (IR, IRS-1, Glut 4 and HSL), 5 (p110αPI-3 K) or 12 (p85αPI-3 K) independent preparations of adipocytes (see details regarding the subjects in the text). *$p < 0.05$ with the *t* test for paired values

tion with various concentrations of insulin were added. Each condition was performed in triplicate. After 2 h of incubation, aliquots of the medium were removed to measure glycerol [32]. Explants of adipose tissue were heated overnight at 120 °C to measure the dry weight of the incubated sample.

Statistical Analysis. Data are presented as means ± SEM. Statistical analyses were done using the *t* test for paired values. A *p* value of less than 0.05 was considered statistically significant with the *t* test for paired values.

Results

Expression of p85αPI-3 K is increased by BRL 49653 in human isolated adipocytes. The mRNA expressions of IR, IRS-1, p85αPI-3 K, p110αPI-3 K, Glut4 and HSL in human isolated adipocytes after 4 h of incubation in the absence (vehicle) or in the presence of 1 μmol/l of BRL 49653 are shown in Figure 1. The mRNA expression of the target genes was not modified during incubation with vehicle alone (data not shown). The IR, IRS-1, Glut 4 and HSL mRNA concentrations were determined in 4 adipocyte preparations obtained from lean subjects (3 women and 1 men, age = 69 ± 2 years and BMI = 25 ± 1 kg/m2) and the expression of these 4 genes was not modified by the treatment with BRL 49653. Regarding the p110α and the p110β catalytic subunits of PI3-Kinase, we found that the mRNA abundance of the p110α form was about 50 times more important than the expression of the p110β form, in crude biopsies of subcutaneous adipose tissue of lean ($n = 3$) or obese ($n = 2$) subjects (4.9 ± 0.4 and 0.13 ± 0.03 amol/μg of total RNA for p110αPI-3 K and p110βPI-3 K mRNA, respectively, $n = 5$). A similar ratio was observed in isolated adipocytes, indicating that in fat cells, the p110αPI-3K is probably the main catalytic form involved in the signalling pathways. Therefore, we preferentially verified whether the expression of the p110αPI-3K was affected by treatment of human isolated adipocytes with BRL 49653. A 4-h incubation with BRL 49653 did not modify the mRNA concentrations of the p110αPI-3K ($n = 5$ preparations from lean subjects) (Fig. 1). Similarly, BRL 49653 did not seem to modify the mRNA expression of the p110βPI-3 K ($n = 2$ preparations, data not shown).

In contrast to these transcripts, BRL 49653 produced a significant 73 ± 14% increase in p85αPI-3 K mRNA abundance (220 ± 28 vs 128 ± 15 amol/μg total RNA with vs without BRL 49653, $p = 0.003$, $n = 12$). This effect was similarly found in adipocytes isolated from lean (75 ± 19%, $p = 0.004$, $n = 6$, 3 women and 3 men, age = 60 ± 7, BMI = 25 ± 1 kg/m^2) or from obese subjects (85 ± 22%, $p = 0.002$, $n = 6$, 4 women and 2 men, age = 54 ± 3, BMI = 33 ± 1 kg/m2) (Fig. 2). In cells from obese subjects, BRL 49653 did not modify the mRNA levels of the other target genes ($n = 2$–4, data not shown).

The BRL 49653-induced increase in p85αPI-3 K mRNA can be readily observed after 2 h, with a maximal effect obtained after 4 h and maintained until 6 h of incubation (data not shown). No effect of BRL 49653 on the other target mRNAs was observed after 6 h of incubation (data not shown). The effect on p85αPI-3 K mRNA was dependent upon BRL 49653 concentrations with a maximal response at 0.1 μmol/l and a half-maximal effect with about 5–10 nmol/l of the drug (data not shown).

The effect of BRL 49653 is probably mediated by the heterodimer PPARγ/RXR. To verify whether the in-

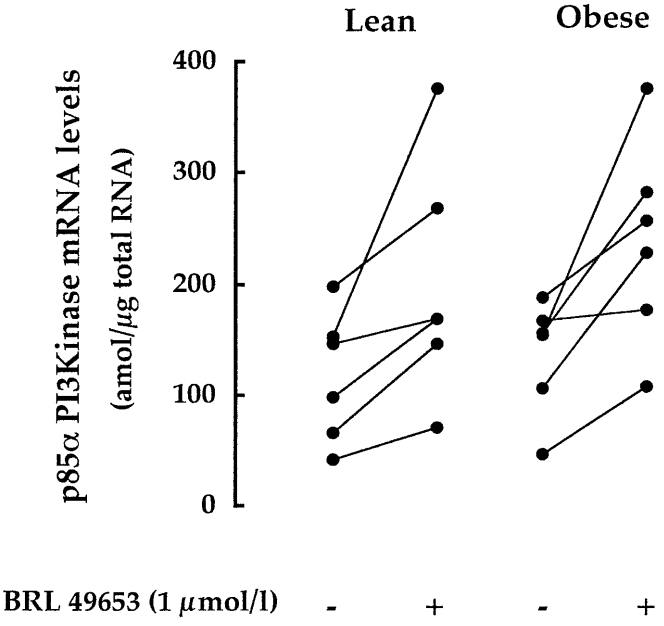

Fig. 2. Effect of BRL 49653 on the mRNA concentrations of p85αPI-3 K in adipocytes isolated from 6 lean and 6 obese subjects. The mRNA levels of p85αPI-3 K was determined by RT-cPCR in human adipocytes incubated for 4 h in the absence or in the presence of 1 μmol/l of BRL 49653

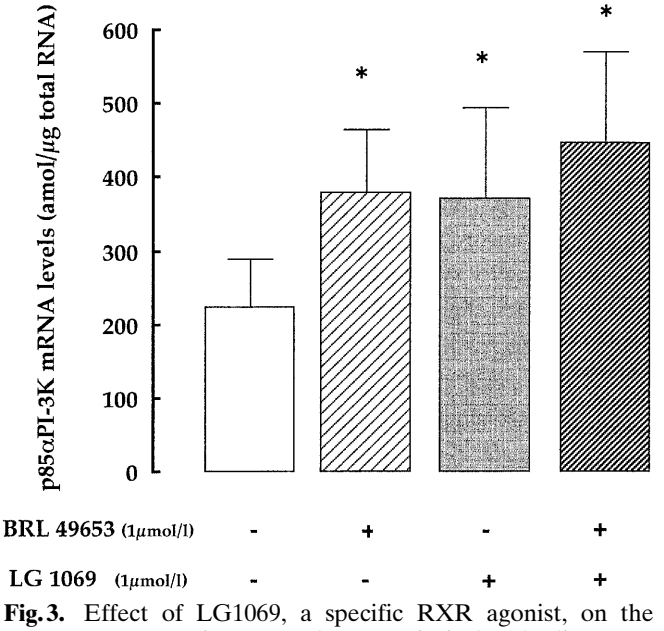

Fig. 3. Effect of LG1069, a specific RXR agonist, on the mRNA concentrations of p85αPI-3 K in isolated adipocytes. Isolated adipocytes were incubated for 4 h in the presence of the indicated agents (1 μmol/l). The mRNA concentrations of p85αPI-3 K were determined by RT-cPCR (means ± SEM, $n = 5$ independent preparations). * $p < 0.05$ compared with vehicle only with the *t* test for paired values

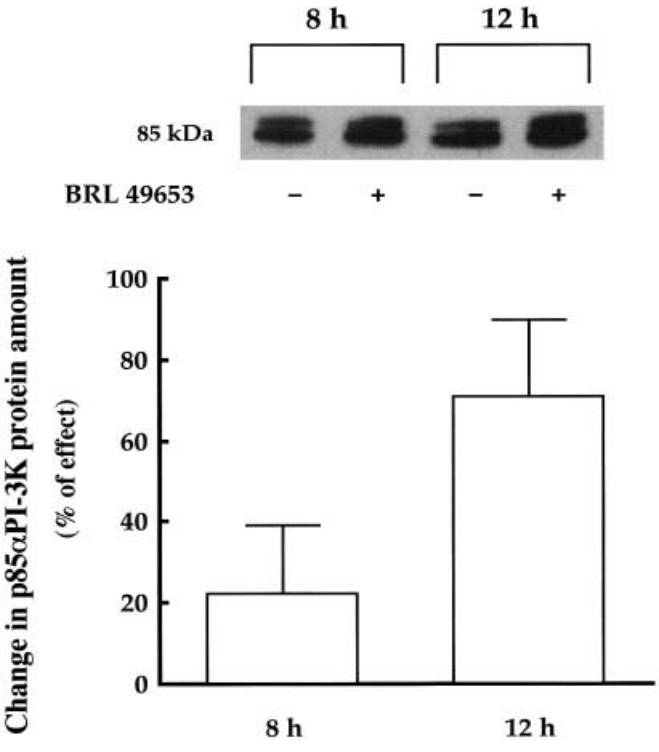

Fig. 4. Effect of BRL 49653 on p85αPI-3 K protein amounts in human subcutaneous adipose tissue explants. Western blots of p85αPI-3 K were performed in adipose tissue explants (300 mg in 2 ml of culture medium), incubated for 8 and 12 h with or without 1 μmol/l of BRL 49653. The blot shows a representative experiment and the bars represent the effect of BRL 49653 (mean ± SEM) in 3 separate experiments

duction p85αPI-3 K mRNA concentrations was mediated by the heterodimer PPARγ/RXR, we investigated the effect LG 1069, a specific activator of RXR, on p85αPI-3 K mRNA expression in isolated adipocytes. The LG 1069 (1 μmol/l, 4 h of incubation) increased of p85αPI-3 K mRNA to a similar extent as BRL 49653 did (62 ± 20% with LG 1069 and 82 ± 18% with BRL 49653, $n = 5$, no significant difference) (Fig. 3). The combination of BRL 49653 and LG 1069 tended to increase further p85αPI-3 K mRNA concentrations (116 ± 24%, $n = 5$) but the difference between the change induced by the two agonists together and the effect of BRL 49653 alone did not reach significance ($p = 0.225$). LG 1069 (1 μmol/l, 4 h of incubation) alone, or in combination with BRL 49653, did not modifiy the mRNA expressions of IR, IRS-1 or p110αPI-3K (data not shown).

BRL 49653 increased P85αPI3 K protein levels and enhanced PI3-Kinase activity. We then investigated the effect of BRL49653 on the p85αPI-3 K protein amount. Because of the limited viability of the isolated human adipocytes, the experiments were conducted in explants of adipose tissue. The BRL 49653 (1μmol/l) treatment increased p85αPI-3 K protein

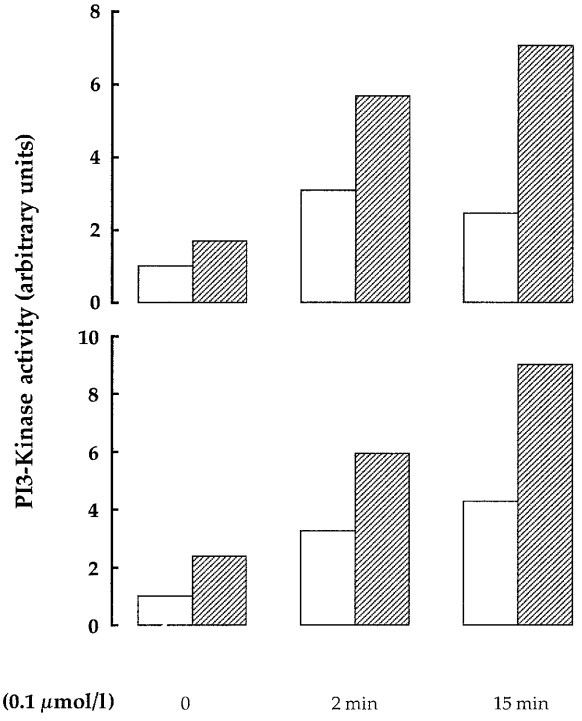

Fig. 5. Effect of BRL 49653 on PI3-Kinase activity. Human adipose tissue explants were treated (▨) or not (□) with BRL 49653 (1 μmol/l) for 12 h, and then stimulated by insulin (0.1 μmol/l) for 2 and 15 min. PI3-Kinase activity was determined on cellular proteins that were immunoprecipitated with a specific anti-p85αPI-3 K antibody. The figure shows two independent experiments and the results are presented as arbitrary units taking the basal PI3-Kinase activity of the untreated tissue as 1 unit

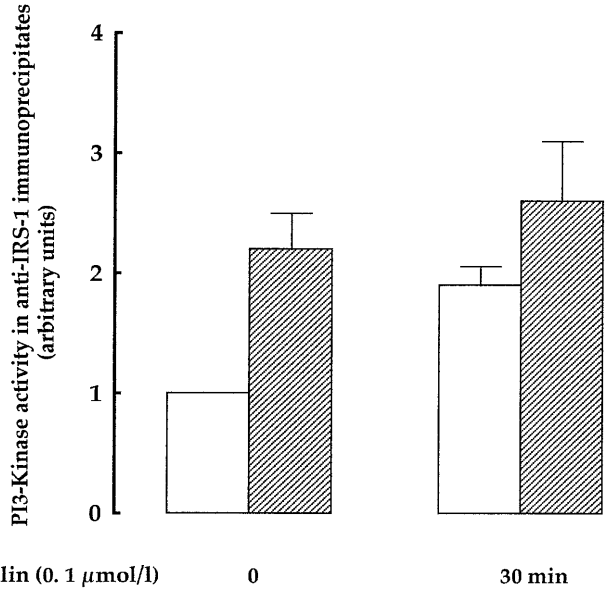

Fig. 6. Effect of BRL 49653 on IRS-1-associated PI3-Kinase activity. Adipose tissue explants were treated (▨) or not (□) with BRL 49653 (1 μmol/l) for 12 h, and then stimulated by insulin (0.1 μmol/l) for 15 min. The PI3-Kinase activity was determined on immunoprecipitates with a specific anti-IRS-1 antibody. The figure shows the means ± SEM of 3 independent experiments and the results are presented as arbitrary units taking the basal PI3-Kinase activity of the untreated tissue as 1 unit

concentration after 12 h of incubation (71 ± 19%, $n = 3$). This effect was less pronounced (22 ± 17%, $n = 3$) when the explants were incubated for 8 h (Fig. 4).

To determine whether this positive effect of BRL 49653 on p85αPI-3 K mRNA and protein concentrations could change PI3-Kinase activity, human adipose tissue explants were pre-incubated alone or in presence of BRL 49653 (1 μmol/l) for 12 h. They were then stimulated with insulin for 2 and 15 min, respectively. The PI3-Kinase activity was measured in cellular proteins that were immunoprecipitated with an anti-p85αPI-3 K antibody. Figure 5 represents insulin stimulation of PI3-Kinase activity in two independent adipose tissue preparations. In each experiment, basal PI3-kinase activity was increased (about twofold) by the treatment of the adipose tissue explants with BRL 49653. Addition of insulin to control explants resulted in about a threefold stimulation of PI3-Kinase activity. Pre-incubation of the tissue with BRL 49653 markedly potentiated the stimulation of PI3-Kinase activity by insulin both at 2 min (fivefold over basal) and at 15 min (eightfold over basal) (Fig. 5). We also measured PI3-Kinase activity in immunoprecipitates obtained with an anti-IRS-1 antibody. The PI3-Kinase activity associated with IRS-1 in the basal state was about twofold greater in adipose tissue explants treated with BRL 49653 (1 μmol/l, 12 h) than in untreated tissue (Fig. 6). Under these conditions, stimulation with insulin (0.1 μmol/l for 15 min) further increased the activity of PI-3 kinase (2.6 ± 0.5 vs 1.9 ± 0.1 arbitrary units, $n = 3$) in explants that had been treated with BRL 49653 but the magnitude of the effect was less important than in untreated tissues (2.2 ± 0.3-fold increase in response to insulin, $n = 3$) (Fig. 6).

BRL 49653 increased PKB phosphorylation. The PKB is thought to play a role in various insulin effects as a downstream target of PI3-Kinase. The basal and insulin-stimulated phosphorylation of serine 473 of PKBα was investigated in human adipose tissue explants both treated or not treated with BRL 49653 for 12 h. BRL 49653 treatment did not modify the total amount of PKBα determined by western blotting ($n = 5$ independant experiments, data not shown). In the basal state (i.e. without insulin), the amount of phosphorylated PKBα was similar in adipose tissue explants both treated or not treated with BRL 49653 (Fig. 7). Stimulation by insulin resulted in a time dependent increase in the phosphorylation of PKBα. After 30 min of incubation, the phosphorylation of PKBα increased 3 times (3 ± 0.1, $n = 3$) in untreated

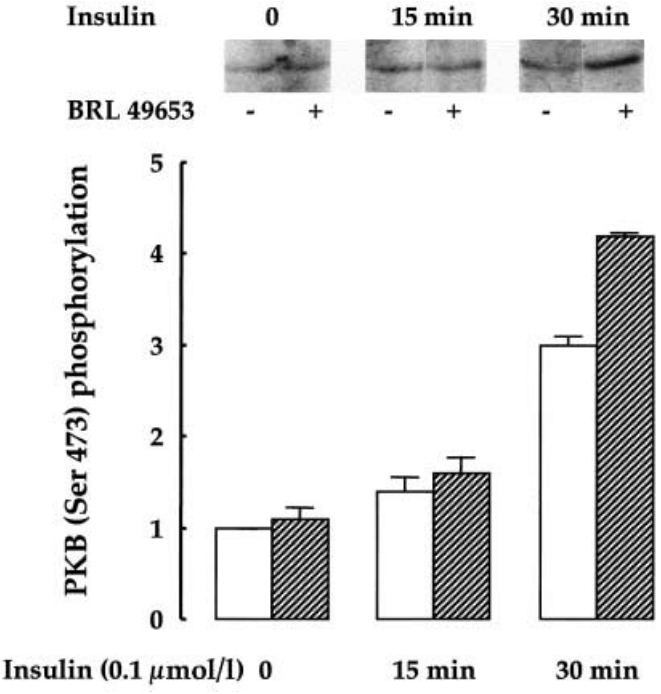

Fig. 7. Effect of BRL 49653 on PKB phosphorylation. The amount of phosphorylated PKBα was determined by western blotting using a specific antibody for the Ser 473 phosphorylated form. Adipose tissue explants were treated (▨) or not (□) with BRL 49653 (1 μmol/l) for 12 h, and then stimulated by insulin (0.1 μmol/l) for 15 or 30 min. The blot shows a representative experiment and the bars represent the results (mean ± SEM) of 3 separate experiments. Data are presented as arbitrary units taking the basal amount of phosphorylated PKB in untreated tissue as 1 unit

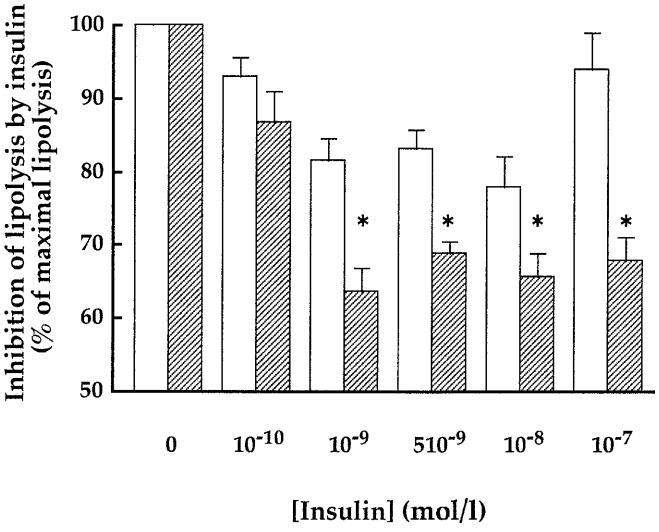

Fig. 8. Effect of BRL 49653 on the antilipolytic action of insulin. Lipolysis was measured in adipose tissue explants pre-incubated (▨) or not (□) for 12 h with BRL 49653 (1 μmol/l). Explants were incubated with 1 μmol/l of isoproterenol (maximal lipolysis) and various concentrations of insulin for 2 h. The amount of glycerol released in the medium was quantified spectophotometrically. The results are presented as percentage of the maximal lipolysis. The absolute lipolytic rates are indicated in the text. Data are means ± SEM for 5 independent tissue preparations. * $p < 0.05$ with the t test for paired values for the difference between treated and untreated tissue

tissues and more than 4 times (4.2 ± 0.02, $n = 3$) in explants treated with BRL 49653 (Fig. 7).

BRL 49653 enhanced the antilipolytic response to insulin. The antilipolytic action of insulin was then investigated in human adipose tissue explants both incubated or not incubated with 1 μmol/l of BRL 49653 for 12 h. Treatment of tissue with BRL 49653 induced a slight, but significant, decrease in the basal (non-stimulated) lipolytic rates (340 ± 67 vs 249 ± 38 nmol of glycerol released in 2 h/g tissue dry weight, without vs with BRL 49653, $p = 0.043$, $n = 5$). Isoproterenol (1 μmol/l) increased five- to sevenfold the basal lipolytic rates (1,592 ± 299 and 1,749 ± 353 nmol of glycerol released in 2 h/g tissue dry weight, without and with BRL 49653, respectively, $n = 5$). There was no significant difference in the maximal lipolytic rates in tissues treated or not treated with BRL 49653 ($p = 0.225$). Figure 8 shows that the antilipolytic action of insulin was significantly higher after treatment with BRL 49653 for all the concentrations of insulin 10^{-9} mol/l or more. The concentration of insulin that produced half-maximal inhibition of lipolysis did not seem to be affected ($\pm 10^{-10}$ mol/l), but was difficult to assess with precision because of the low effect of the hormone. The action of BRL 49653 was more pronounced in the presence of 0.1 μmol/l of insulin but this result was mainly due to the lack of effect of insulin, at this concentration, on the lipolysis rate in untreated tissue (Fig. 8).

Discussion

This work demonstrates that the activation of PPARγ by the thiazolidinedione BRL 49653 increases the expression of the p85α regulatory subunit of phosphatidylinositol 3-kinase (p85αPI-3 K) at the mRNA and the protein levels in human adipocytes. The effect on p85αPI-3 K mRNA concentration can be observed a few hours after BRL 49653 addition and can be reproduced by the activation of RXR, the partner of PPARγ. The increased expression of this adapter protein is associated with an increase in the stimulation by insulin of PI3-Kinase activity and PKBα phosphorylation. Furthermore, the antilipolytic action of insulin, which requires the activation of the PI3-Kinase pathway [33], is also significantly increased in human adipose tissue incubated with BRL 49653.

Thiazolidinediones improve insulin action in insulin resistant animals and in Type II diabetic patients [1, 2]. This effect is most probably mediated by PPARγ, a nuclear receptor preferentially expressed

in adipose tissue [9, 20]. However, the target genes of this nuclear receptor are still not well known in human tissues. We have hypothetized that PPARγ controls the expression of genes coding key enzymes of insulin action. This hypothesis was tested in human isolated adipocytes, a cell model that has been successfully used to study the short-term regulation of gene expression by insulin [23]. In addition, this model of mature cells allowed us to study the role of PPARγ without interfering with its action on adipocyte differentiation.

Insulin receptor, IRS-1, p110αPI-3 K, Glut 4 and HSL mRNA levels were not affected by the treatment of isolated adipocytes with BRL 49653, suggesting that these genes are not acutely regulated by PPARγ. It has been reported that insulin binding to adipocytes from obese mice treated for 14 days with BRL 49653 was increased twofold without change in receptor affinity [34]. However, it was not known whether this observed change in insulin receptor number was a specific effect of the drug or a secondary action arising from the reduction in hyperinsulinaemia. In cultured adipocytes, thiazolidinediones increased the expression of the glucose transporters Glut 1 and Glut 4 and enhanced insulin stimulated glucose uptake [35]. In vivo too, treatment of insulin resistant KKAy mice and Zucker *fa/fa* rats with pioglitazone [36, 37] or *ob/ob* mice with BRL 49653 [34] increase adipose tissue expression of Glut 4, suggesting that Glut 4 could be a target gene of PPARγ. In human adipocytes, our data suggest that Glut 4 is not a target gene of PPARγ. However, it is possible that longer incubation times are required to observe a change in Glut 4 expression. In addition, we have previously observed that activation of RXR, in combination with BRL 49653, could increase Glut 4 mRNA expression in isolated adipocytes [26], suggesting that the transcription of Glut 4 gene can be activated by the heterodimer RXR/PPARγ.

The main result of our study is that the expression of the p85α regulatory subunit of PI3-Kinase (p85αPI-3 K) was increased during the treatment of human adipose tissue with a thiazolidinedione. The p85αPI-3 K is an adapter protein that links the p110 catalytic subunits of PI3-Kinase to upstream signalling molecules (such as the IRS) when phosphorylated on specific tyrosine residues [38]. Therefore, the rapid induction of p85αPI-3 K expression observed in the present work could have consequences in the transmission of the insulin signal in the cells. In agreement with this, we found that insulin-stimulated PI3-Kinase activity and phosphorylation of PKBα increased in adipose tissue that had been treated with BRL 49653. Several studies already suggest that thiazolidinediones can affect the PI3-Kinase pathway of insulin action, both in vivo in rodent [39, 40] and in vitro in cell lines [41]. However, none of these studies have identified a target of the thiazolidinediones. Recently, a gene coding a new signalling molecule, the c-Cbl-associated protein (CAP), was identified as a possible target gene of PPARγ and the thiazolidinediones in 3T3-L1 adipocytes [42]. This adapter protein has also been involved in an alternate signalling pathway required for the stimulation of glucose transport by insulin [43]. The regulation of the expression of CAP by the thiazolidinediones could thus participate in the beneficial effects of these drugs in vivo. However, the expression and the regulation of CAP has not yet been reported in human tissues.

Our results suggest that the thiazolidinediones increase insulin action (PI3-Kinase activity and PKB phosphorylation) in human adipocytes and that among the different components of the insulin signalling pathway that have been tested, the gene encoding the p85α subunit of PI3-Kinase could be one of their molecular targets. However, several studies have previoulsy indicated that concommitant increases in the p85α regulatory subunit and in the p110 catalytic subunit of PI3-Kinase are required to obtain an increase in insulin action. For example, the overexpression of the inter-SH2 region of p85αPI-3 K in 3T3-L1 adipocytes led to increased glucose transport and glycogen synthase activity only when the p110α catalytic subunit was co-expressed [44]. In an other study, a fourfold induction of the p85αPI-3 K level, without a change in the p110PI-3 K, resulted in a decrease in PI3-kinase activity in dexamethasone-treated L6 cells [45], suggesting that the excess of p85αPI-3 K protein competed with the p85αPI-3 K.p110PI-3 K complexes for association with IRS-1 [45]. It can be concluded from these data that a large increase (overexpression or fourfold induction) in p85αPI-3 K produces a dominant negative effect on insulin action, at least in cultured cell lines. Here, in human isolated adipocytes, we found that BRL 49653 induced the expression of p85αPI-3 K but not the expression of the p110α or the p110β catalytic subunits of PI3-Kinase. Thus, a relatively small (less than twofold) increase in the p85αPI-3 K appeared to be associated with enhanced insulin action without a concommitant change in the expression of the catalytic subunits. If the change in p85αPI-3 K expression was directly responsible for the insulin sensitizing effect of BRL 49653, it is to be assumed that the initial amount of p85αPI-3 K was rate-limiting for the activation by insulin of the PI3-Kinase pathway in human adipocytes. However, the observed relative abundance of the mRNAs encoding the p85α and the p110α (about 20 times more p85αPI-3 K mRNA) did not support this hypothesis. Therefore, even though the relative protein abundance of the p85α and the p110α is not known, it is likely that other effects of BRL 49653 participate in the potentialization of insulin action in human adipocytes, in addition to the induction of p85αPI-3 K expression. These could include direct effects of the thiazolidinedione on oth-

er components of insulin action, such as on CAP expression [42], or indirect effects that could, for example, modify the phosphorylation state or the activity of other proteins involved in the insulin signalling pathways. However, whatever the exact mechanism of action of BRL 49653 was, our study demonstrates, for the first time in normal human cells, that thiazolidinediones can potentialize insulin action in adipocytes and that the gene encoding the p85α subunit of PI3-Kinase is one of their molecular targets, probably through the activation of PPARγ.

The effect of BRL 49653 on p85αPI-3 K expression did indeed appear to be mediated by PPARγ. Firstly, the concentration of BRL 49653 producing half-maximal induction of p85αPI-3 K mRNA was in agreement with its reported $K_{0.5}$ for binding to PPARγ [3] and for stimulation of gene expression in transactivation assay [46]. Secondly, the activation of RXR by a specific agonist [21] reproduced the effect of BRL 49653. These data strongly suggest that p85αPI-3 K is a target gene of the heterodimer PPARγ/RXR in human adipocytes. Several possible PPREs can be found in the promoter region of the human p85αPI-3 K gene (E. Lefai and H. Vidal, unpublished results). Studies are in progress to verify whether they are involved in the transcriptional regulation of the gene.

PPARγ is considered a key actor in adipocyte differentiation [14, 20]. Forced expression of PPARγ into fibroblasts triggers their transformation into adipocytes [13] and thiazolidinediones promote adipocyte differentiation in vivo and in vitro [20, 37]. Interestingly, it was recently demonstrated that the differentiation process of adipocytes required the activation of PI3-Kinase in 3T3 cells [47]. It is tempting to speculate that, in addition to a possible role in insulin action, the observed induction of p85αPI-3 K expression and increase in PI3-Kinase activity in response to BRL 49653 could participate in maintaining the differentiated state of the adipocytes.

One of the main effects of insulin in adipose tissue, particularly in humans, is the inhibition of lipolysis. The antilipolytic effect of insulin is mediated, at least in part, by activating the PI3-Kinase/PKB pathway [33, 38, 48]. Stimulation of this pathway by insulin leads to the phosphorylation and activation of the phosphodiesterase 3B, resulting in a decrease in intracellular cAMP, which in turn, leads to a reduction in PKA activity. This subsequently results in a decrease in HSL phosphorylation and thus to inactivation of the enzyme. In KKAy mice, treatment with pioglitazone for 4 days has been shown to increase phosphodiesterase 3B expression in adipose tissue [49], suggesting that reduction of the circulating concentrations of free fatty acid by thiazolidinedione could be a consequence of a decrease in adipose tissue lipolysis. However, this study does not clarify whether the change in phosphodiesterase expression is a direct effect of pioglitazone or a secondary event resulting from the improvement of insulin resistance during the treatment. We found that incubation of human adipose tissue explants with BRL 49653 enhanced the antilipolytic effect of insulin. In agreement with previous data in cultured cells [50], BRL 49653 did not affect the isoproterenol-induced maximal lipolytic rates. Inhibition of lipolysis by insulin was investigated after 12 h of treatment with BRL 49653, when the amount of the p85αPI-3 K protein was significantly increased and when the insulin-induced PI3-Kinase activity and PKB phosphorylation were enhanced in adipose tissue. Therefore, our results strongly suggested that the induction of p85αPI-3 K by BRL 49653 could participate in the insulin sensitizing effect of the thiazolidinedione in adipose tissue.

The thiazolidinediones are a new class of drugs for the treatment of Type II diabetes mellitus [1, 2]. The rate of lipolysis from adipose tissue is generally increased in Type II diabetic patients [51]. If the effect of BRL 49653 (Rosiglitazone) observed in vitro in adipocytes also occurs in vivo, this could contribute to an improvement in the lipid profile of the diabetic patients. Furthermore, an altered stimulation by insulin of PI3-Kinase activity has been clearly documented in subjects with Type II diabetes or obesity [52]. We have also recently shown that the regulation of p85αPI-3 K mRNA expression by insulin, and during fasting, is impaired in muscle and adipose tissue of Type II diabetic patients [29, 53]. Under such conditions, it may be assumed that the improvement of insulin sensitivity in diabetic patients treated with thiazolidinediones could result, at least in part, from the effect of these molecules on p85αPI-3 K gene expression and on PI3-Kinase activity, as shown in the present work.

In conclusion, we report in this study the identification of one of the target genes of the thiazolidinediones in human mature adipocytes. The mRNA and protein concentrations of the p85αPI-3 K are significantly increased by BRL 49653, probably through a mechanism involving an activation of the nuclear receptor PPARγ. BRL 49653 also enhances the insulin stimulation of PI3-Kinase activity and the antilipolytic response to insulin after a few hours of treatment. Although the precise mechanism of action of BRL 49653 on PI3-Kinase activity is not yet clear, these findings improve our understanding of the insulin-sensitizing effects of the thiazolidinediones, potential drugs for the treatment of Type II diabetes mellitus.

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