Regulation of p85α phosphatidylinositol-3-kinase expression by peroxisome proliferator-activated receptors (PPARs) in human muscle cells

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Abstract Regulation of p85α phosphatidylinositol-3-kinase (p85αPI-3K) expression by peroxisome proliferator-activated receptor (PPAR) activators was studied in human skeletal muscle cells. Activation of PPARγ or PPARβ did not modify the expression of p85αPI-3K. In contrast, activation of PPARα increased p85αPI-3K mRNA. This effect was potentiated by 9-*cis*-retinoic acid, an activator of RXR. Up-regulation of p85αPI-3K gene expression resulted in a rise in p85αPI-3K protein level and in an increase in insulin-induced PI3-kinase activity. According to the role of p85αPI-3K in insulin action, these results suggest that drugs with dual action on both PPARγ and PPARα can be of interest for the treatment of insulin resistance. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Nuclear receptor; Thiazolidinedione; Fibrate; Insulin resistance

1. Introduction

The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily. Three different PPAR genes have been characterized that give rise to four distinct proteins (α, β, γl and γ2) [1,2]. PPARγl and γ2 are generated from the same gene by alternative promoter usage and mRNA splicing [3]. All PPARs heterodimerize with the 9-*cis*-retinoic acid (9-*cis*-RA) receptor (RXR) [4] and change the transcription rate of their target genes after binding to specific peroxisome proliferator-responsive elements (PPREs) [1,2]. PPARα is preferentially expressed in the liver [1,2,5,6], but significant levels of the protein have been also found in skeletal muscle, kidney and endothelial cells [7]. PPARα is mainly involved in the regulation of fatty acid oxidation, lipoprotein metabolism and cellular fatty acid uptake [1,2]. PPARβ (also designated PPARδ or NUC1) seems to be ubiquitously expressed [5,6] and its functions are still largely unknown. In contrast, PPARγ is highly expressed in adipose tissue where it is known to play a critical role in adipocyte differentiation and in fat deposition [8,9]. PPARγl, however, is also expressed at a significant level in other cells and tissues, like in colon [6], in

epithelial cells of the intestinal tract [10] or in macrophages [11]. In addition, PPARγl mRNA [6,12] and protein [12] can be detected in skeletal muscle, although the level of expression was several fold lower than in the adipose tissue. In contrast, PPARγ2 is expressed almost exclusively in adipocytes [6].

All PPARs are activated by naturally occurring fatty acids or their derivatives [1,2,13–15] and by several synthetic compounds [14–16]. Among them, the thiazolidinediones are a new class of oral antidiabetic agents that reduce blood glucose, insulin and triglyceride concentrations and increase insulin sensitivity in animal models and in type-2 (non-insulin-dependent) diabetic patients [17,18]. Thiazolidinediones are high affinity ligands of PPARγ, but the mechanism by which activation of PPARγ results in an improvement of insulin action is still largely unknown [8,9]. Proposed mechanisms involve PPARγ-induced differentiation of small insulin sensitive fat cells, higher rate of fatty acids trapping by adipocytes, or the reduction of adipocytokine expression [9]. In addition, we have recently found that activation of PPARγ by Rosiglitazone can increase insulin signaling in human adipocytes by inducing the expression of the p85α-regulatory subunit of phosphatidylinositol-3-kinase (p85αPI-3K) [19,20] and by increasing insulin-induced PI-3K and protein kinase B (PKB) activities [20]. Because the PI-3K/PKB signaling pathway is involved is almost all the metabolic effects of insulin, notably the translocation of glucose transporters [21], it was tempting to speculate that the observed up-regulation of p85αPI-3K expression by Rosiglitazone [19,20] could participate in an improvement of glucose uptake. However, the contribution of adipose tissue to whole-body glucose uptake is low and it is well recognized that skeletal muscles mainly contribute to insulin resistance in vivo [22]. Because a significant expression of PPARγ protein has been recently reported in human muscle [12], we aimed to verify whether the effect of Rosiglitazone on p85αPI-3K expression found in adipose tissue also takes place in skeletal muscle. For this purpose, we have investigated the regulation of p85αPI-3K gene expression by PPAR activators in human skeletal muscle cells in primary culture.

2. Materials and methods

2.1. Products

Rosiglitazone was kindly provided by SmithKline Beecham Pharmaceuticals (Harlow, UK). The PPARβ agonist L-165041 was a gift from Drs. J. Berger and D. Moller from Merck and Co., Inc. (Rahway, NJ, USA). Wy-14643 were obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA). All fatty acids and other drugs were obtained from either Sigma (L'isle d'Abeau, France) or

Biomol Research Laboratories. Culture media were from Life Technologies (Cergy Pontoise, France) or from Biomedia (Boussens, France).

2.2. Primary culture of human skeletal muscle cells

Biopsies of the lumbar mass (erector spinae) muscle (about 1 g) were taken, with the consent of the patient, during surgical procedure. The experimental protocol was approved by the Ethics Committee of Lyon Hospitals. In the present study, biopsies were taken from healthy subjects (three men, three women, age $= 49 \pm 5$ years, body mass index ranging from 24 to 31 kg/m^2) with no familial or personal history of diabetes or dyslipidemia. The satellite cells were isolated from the muscle biopsy by trypsin digestion and were grown as previously described in detail [23]. Confluent myoblasts were allowed to differentiate into myotubes and were always used 12 to 16 days after induction of the differentiation process [23].

2.3. Quantification of p85αPI-3K mRNA

Total RNA was prepared using the RNeasy kit (Qiagen, Courtaboeuf, France), following the instructions of the manufacturer. The yield of total RNA averaged 1.3 μg/well from a 6-well culture plate, corresponding to approximately 250 000 myoblasts per well at confluence, before induction of the differentiation process.

The absolute mRNA concentration of p85αPI-3K was determined using the reverse transcription-competitive polymerase chain reaction (RT-cPCR) assay as previously described [23]. The RT reaction was performed with 0.1 μg of total RNA in the presence of the specific antisense primer and a thermostable reverse transcriptase enzyme to warrant optimal synthesis of first strand cDNA [24]. Cy-5 5′-end labeled sense primer was used during the cPCR to generate fluorescent PCR products that were analyzed using an automated laser fluorescence DNA sequencer (ALFexpress, Pharmacia, Uppsala, Sweden) in 4% denaturing polyacrylamide gels.

2.4. Quantification of p85αPI-3K protein by Western blotting

Myotubes were homogenized in a phosphate-buffered saline (PBS) lysis buffer containing 1% Nonidet P-40 (Sigma), 0.5% sodium desoxycholate (Sigma), 0.1% SDS (Sigma) and supplemented with a freshly prepared cocktail of protease inhibitors (ICN Pharmaceuticals, Orsay, France). Proteins (5 μg) were separated by SDS–PAGE and transferred to polyvinylidine difluoride (PVDF) membrane (Millipore, France). Quantification of p85αPI-3K protein amount was performed as previously described [23], using a rabbit polyclonal antibody (Upstate Biotechnology, New York, USA).

2.5. Determination of PI3-kinase activity

Myotubes were homogenized in a lysis buffer (140 mM NaCl, 20 mM Tris–HCl pH 7.4, 10 mM EDTA, 100 mM NaF, 2 mM vanadate, 10 mM pyrophosphate, 1% Nonidet P-40) supplemented with a
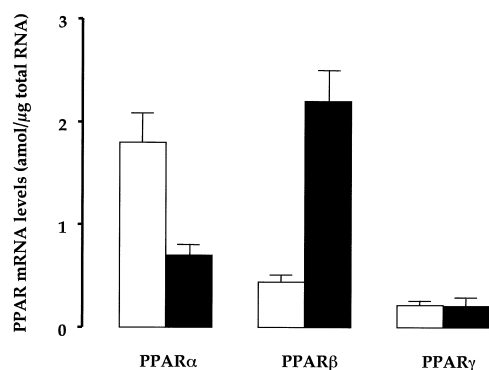
Fig. 1. Comparison of PPARα, β and γ mRNA expression between human skeletal muscle biopsies and primary cultures of muscle cells. The concentrations of the different PPAR mRNAs were determined in muscle (vastus lateralis) biopsies (open boxes, $n = 6$) and in differentiated myotubes (black boxes, $n = 5$) obtained from healthy subjects using the RT-cPCR assay previously described and validated [6]. The vastus lateralis muscle biopsies were obtained from subjects participating in a separated study [29]. Data are presented as the mean ± S.E.M.
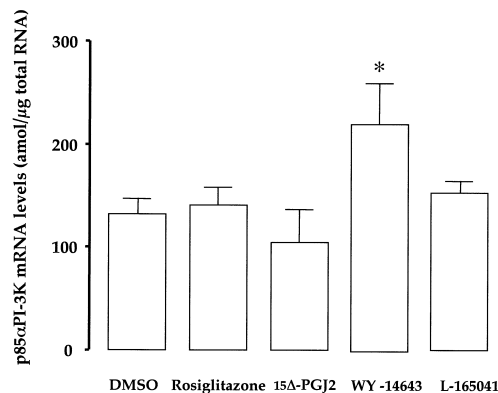
Fig. 2. Effect of different PPAR activators on p85αPI-3K mRNA levels in human myotubes. The mRNA level of p85αPI-3K was determined after 6 h of incubation with 10^{-6} M of the indicated activators. The final concentration of DMSO (vehicle) was kept at 0.1% in all conditions. The figure shows the mean ± S.E.M. for five to eight different experiments involving five independent preparations of muscle cells. *$P < 0.05$ vs. vehicle.

freshly prepared cocktail of protease inhibitors. Immunoprecipitation with the antibody of the p85αPI-3K (Upstate Biotechnology) and determination of PI3-kinase activity on the immunoprecipitate were performed as previously described [20].

3. Results

At the mRNA level, PPARγ was expressed at the lowest level among the members of the PPAR family, both in human skeletal muscle and in primary muscle cells in culture (Fig. 1). Only PPARγ1 mRNA was measurable in muscle biopsies or in differentiated myotubes. The mRNA of PPARγ2, the adipocyte-specific isoform, was not detectable (data not shown). There was no difference in PPARγ mRNA levels in differentiated myotubes and in muscle biopsies. The cultured cells were however characterized by modifications in the mRNA expression of the other PPARs (Fig. 1). PPARα mRNA levels were slightly lower in myotubes than in muscle biopsies, and PPARβ expression was about 4-fold higher in the cultured cells.

We have previously shown that treatment of human adipocytes with Rosiglitazone for 6 h resulted in a significant induction of p85αPI-3K mRNA expression. Fig. 2 shows that such effect did not take place in human myotubes. Addition of 15-deoxy-$\Delta^{12,14}$ prostaglandin J$_2$ (15ΔPGJ$_2$), a proposed natural ligand of PPARγ, also did not modify the mRNA expression of p85αPI-3K (Fig. 2). It should be added that the mRNA levels of p85αPI-3K were not affected after 24 h of incubation with either Rosiglitazone or 15ΔPGJ$_2$ (data not shown). Activation of PPARβ with L-165041, also did not modify significantly the mRNA levels of p85αPI-3K in human myotubes (Fig. 2). In contrast, activation of PPARα by Wy-14643 produced an about 2-fold increase in p85αPI-3K mRNA levels ($87 \pm 36\%$, $P = 0.03$) after 6 h of incubation (Fig. 2).

To verify whether the induction of p85αPI-3K mRNA levels was mediated by the heterodimer PPARα/RXR, we investigated the effect of 9-cis-RA alone and in combination with Wy-14643. As shown in Fig. 3, the treatment of myotubes for 6 h with 9-cis-RA alone significantly increased the concentration of p85αPI-3K mRNA ($121 \pm 24\%$, $P < 0.001$). The com-
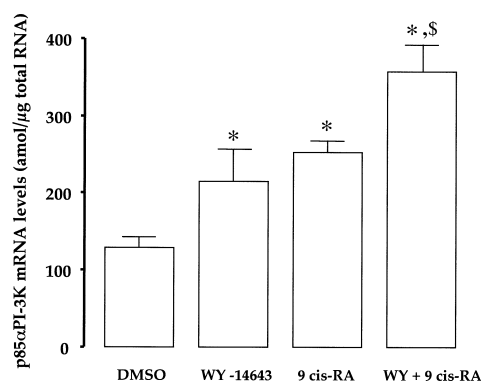
Fig. 3. Effect of the PPARα/RXR heterodimer activation on p85αPI-3K mRNA levels. The mRNA levels of p85αPI-3K were determined after 6 h of incubation with 10^{-6} M of the indicated activators. The final concentration of DMSO (vehicle) was kept at 0.1% in all conditions. The figure shows the mean ± S.E.M. for five independent preparations of muscle cells. $*P < 0.05$ vs. vehicle and $^\$P < 0.05$ vs. Wy-14643 or 9-*cis*-RA alone.

bination of Wy-14643 and 9-*cis*-RA further increased p85αPI-3K mRNA levels (285 ± 72%, $P < 0.001$). The change induced by the combination of the two activators was significantly higher ($P < 0.03$) than the change obtained with Wy-14643 or 9-*cis*-RA alone, indicating a potentiation of the effect when the two partners of the PPARα/RXR heterodimer were activated. Interestingly, a combination of Rosiglitazone and 9-*cis*-RA also induced an increase in p85αPI-3K mRNA levels (107 ± 23%, $n = 6$), but the magnitude of the effect was similar to what was obtained with 9-*cis*-RA alone (121 ± 24%). The lack of potentiation when Rosiglitazone and 9-*cis*-RA were used in combination further suggested that PPARγ was not involved in the regulation of p85αPI-3K expression in human muscle cells.

The observed up-regulation of p85αPI-3K mRNA by PPARα/RXR heterodimer was followed by a significant increase in p85αPI-3K protein levels after 12 h of incubation (Fig. 4). However, when the myotubes were incubated with Wy-14643 alone, the amount of p85αPI-3K protein was not significantly affected while treatment with 9-*cis*-RA promoted an about 2-fold increase (1.8 ± 0.1-fold, $P < 0.002$, $n = 4$). The combination of Wy-14643 and 9-*cis*-RA increased p85αPI-3K protein to a slightly, but significant, higher level (1.9 ± 0.1, $P < 0.002$ vs. vehicle or Wy-14643 and $P < 0.05$ vs. 9-*cis*-RA alone).

The effect of Wy-14643 and 9-*cis*-RA on total PI3-kinase activity was determined in anti-p85αPI-3K immunoprecipitates. Fig. 6 represents insulin stimulation (10 min) of PI3-kinase activity in three independent cell preparations after 12 h of incubation with Wy-14643 and 9-*cis*-RA. Treatment with the activators did not modify the basal PI3-kinase activity,
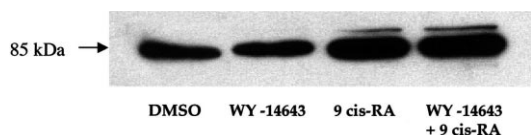

Fig. 4. Effect of PPARα/RXR heterodimer activation on p85αPI-3K protein levels in human myotubes. Western blots of p85αPI-3K were performed in differentiated myotubes incubated for 12 h with 10^{-6} M of the indicated activators. The figure shows a representative experiment that was reproduced in four preparations of human myotubes.
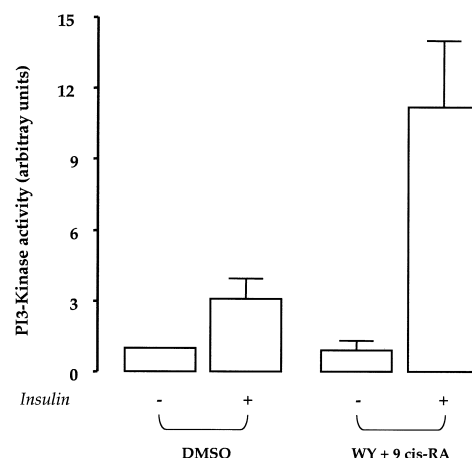

Fig. 5. Effect of PPARα/RXR heterodimer activation on insulin-induced PI3-kinase activity. Myotubes were incubated for 12 h with 10^{-6} M of Wy-14643 and 9-*cis*-RA, and PI3-kinase activity was measured in anti-p85αPI-3K immunoprecipitates before and after 10 min of stimulation with insulin (10^{-7} M). The figure shows the mean ± S.E.M. for three independent preparations of human myotubes and the results are presented as arbitrary units taking the basal PI3-kinase activity of the untreated cells as 1 unit.

but dramatically increased the effect of insulin (11 ± 2.8 vs. 3 ± 0.8-fold increase over basal with vs. without treatment for 12 h with Wy-14643 and 9-*cis*-RA) (Fig. 5). These data demonstrate thus that the increase in p85αPI-3K expression
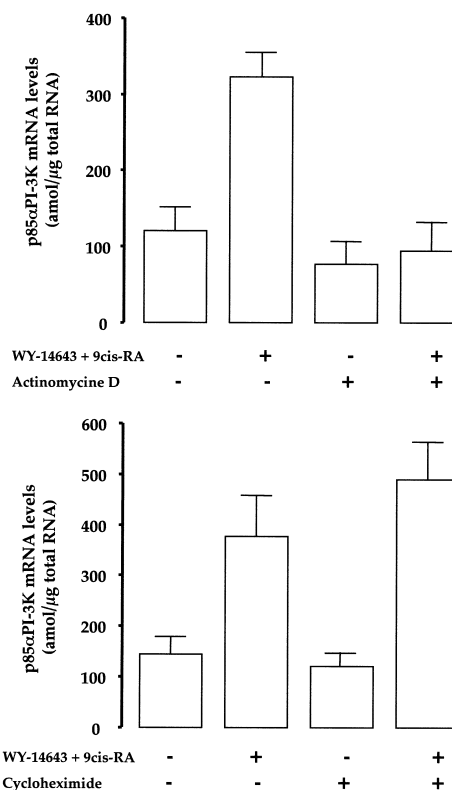

Fig. 6. Effect of actinomycin D and cycloheximide on the induction of p85αPI-3K mRNA. The upper panel shows that addition of actinomycin D (10^{-6} M) completely prevented the induction of p85αPI-3K by Wy-14643 and 9-*cis*-RA (incubation time = 6 h). In contrast, the lower panel shows that cycloheximide (10^{-6} M) did not alter the effect of Wy-14643 and 9-*cis*-RA. Data are presented as means ± S.E.M. for four different experiments.

(mRNA and protein levels) was associated with a marked potentiation of insulin signaling in human muscle cells.

Fig. 6 shows that the effect of Wy-14643 and 9-*cis*-RA on the mRNA expression of p85αPI-3K could be completely prevented by actinomycin D, a potent inhibitor of RNA polymerase II, suggesting that the PPARα/RXR heterodimer affects the transcriptional regulation of p85αPI-3K gene. Furthermore, the rise in p85αPI-3K mRNA in response to Wy-14643 and 9-*cis*-RA was not affected by cycloheximide (Fig. 6), indicating that protein synthesis is not required in the mechanism of action of these molecules on p85αPI-3K gene. These results suggested that the PPARα/RXR heterodimer directly controls the expression of the p85αPI-3K gene at the transcriptional level in human muscle cells in culture.

4. Discussion

We have recently reported that activation of PPARγ by Rosiglitazone increases the expression of p85αPI-3K in human isolated adipocytes without affecting insulin receptor, insulin receptor substrate (IRS)-1 or the p110α and β catalytic subunits of PI-3K [19,20]. This specific induction of p85αPI-3K by Rosiglitazone was amplified in the presence of a RXR activator, strongly suggesting that the gene encoding p85αPI-3K is a target of the heterodimer PPARγ/RXR in human adipocytes. We found, in the present work, that this regulation of p85αPI-3K expression by thiazolidinediones did not take place in human differentiated muscle cells. The activation of PPARγ (with Rosiglitazone or with 15ΔPGJ2) did not modify the mRNA expression of p85αPI-3K. This marked difference from what we have previously observed in human adipocytes could be a consequence of the very low expression level of PPARγ in muscle compared to the fat cells. Such difference in the response to thiazolidinediones between adipocytes and muscle cells has been already observed in the case of the uncoupling protein 2 (UCP-2) gene, the expression of which is up-regulated in human adipocytes [19] and unaffected in human myotubes [25]. Similar tissue-specific effects have been also reported for the genes encoding the fatty acid transporter protein-1 (FATP-1) and the lipoprotein lipase in rodents adipose and muscle cell lines [26,27]. It is likely that the very low level of PPARγ in muscle cells, and, in contrast, its very high level in adipocytes may explain these observations and the preferential effect of the thiazolidinediones in fat cells.

In contrast to PPARγ activators, the expression of p85αPI-3K was significantly increased by Wy-14643 and 9-*cis*-RA in myotubes. These results indicated that p85αPI-3K gene is preferentially under the transcriptional control of the PPARα/RXR rather than the PPARγ/RXR heterodimer in human muscle cells. In rat skeletal muscle, FATP-1 mRNA expression is also up-regulated by activation of PPARα with fenofibrate [26]. Because PPARα is expressed at higher level than PPARγ in muscle cells, one could assume that the tissue-specific regulation of these genes may depend on the relative abundance of the PPARs. However, in human myotubes, PPARβ is more expressed than PPARα and its activation did not change the mRNA expression of p85αPI-3K. Furthermore, we have recently shown that UCP-2 gene expression is increased by activation of PPARβ and not by activation of PPARα in human myotubes [25]. Therefore, all these results suggest that, in addition to the relative abundance of the different PPARs, specific regulatory mechanisms are involved

in the transcriptional regulation of these genes. Studies of their promoters are required to verify whether the same PPREs mediate the tissue-specific effects of the different PPARs or whether different regions of the promoters are involved in a tissue-dependent manner.

The product of the p85αPI-3K gene is an adapter protein that links the catalytic subunit of PI-3K to upstream signaling molecules (such as the IRSs), playing thus a major role in the intracellular action of insulin [21]. We found that insulin-induced PI3-kinase activity is markedly induced in cells treated with the PPARα/RXR agonists. Activation of the PI-3K pathway is required for a variety of insulin effects, including the regulation of glucose uptake and glycogen synthesis in skeletal muscle [21]. These metabolic pathways are altered in pathologies characterized by insulin resistance [22], and defective expression and activation of PI-3K have been reported in subjects with type-2 diabetes or obesity [28,29]. Activation of PPARα with fibrates is mainly known to lower plasma lipid concentrations [1,2]. In addition to this effect, PPARα activators can also improve insulin sensitivity both in type 2 diabetic patients [30] and in rodent models of insulin resistance [31]. Therefore, the identification of p85αPI-3K as a possible target gene of PPARα in muscle could explain, at least in part, the beneficial effect of fibrates on insulin action.

Another interesting results of this study is the demonstration that 9-*cis*-RA was able to increase p85αPI-3K mRNA and protein expression in human muscle cells. It appeared therefore that activation of RXR can transactivate p85αPI-3K gene, either through a PPARα/RXR heterodimer or through a complex involving another partner of RXR. This result suggests that treatment with RXR activators may affect insulin sensitivity. In agreement with this hypothesis, a specific agonist of RXR has been recently shown to improve insulin action in a model of insulin-resistant mice [32].

In conclusion, the present study and our previous work [20] indicate that p85αPI-3K gene expression is regulated in a tissue-specific manner by PPARα activation in muscle and by PPARγ activation in adipose tissue. Similar complementary actions of PPARα and γ activators have been previously observed in rodents concerning genes involved in lipid metabolism [26,27,33]. We found that these dual effects can also occur in human tissues for a key gene of insulin action. If this tissue-selective regulation of p85αPI-3K also results in an improved insulin sensitivity in vivo, our observations suggest that the development of drugs with a dual action on both PPARγ and PPARα may be of interest for the treatment of pathologies characterized by insulin resistance like type 2 diabetes mellitus.

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