Rapid Communication

Thiazolidinediones (PPAR γ ligands) increase IRS-1, UCP-2 and C/EBP α expression, but not transdifferentiation, in L6 muscle cells

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Abstract

Aims/hypothesis. Several effects of thiazolidinediones (TZD) have been shown in adipose tissue but very little is known about the effects in skeletal muscle. We examined the effects of TZD and PPAR α ligands on the expression of different genes in L6 muscle cells.

Methods. L6 muscle cells were exposed to PPAR α and PPAR γ ligands for different times. The gene expression of the signalling molecules *IRS-1*, *IRS-2*, *PKB/Akt*, the transcription factor *C/EBP α* , the uncoupling protein *UCP-2* and the adipocyte marker *aP2* were measured with real-time PCR. To directly examine the effect of *C/EBP α* on gene expression, we also transfected L6 cells with this gene.

Results. L6 muscle cells showed a low expression of PPAR γ 1, no expression of PPAR γ 2, and this was not

changed by TZD. PPAR γ , but not PPAR α , ligands rapidly increased the expression of *C/EBP α* while *UCP-2* and in particular the *IRS-1* gene was activated with a slow onset (12–24 h). In contrast, neither *PKB/Akt* nor *IRS-2* expression were changed. Transfection with *C/EBP α* did not increase *IRS-1* expression. There was no evidence of transdifferentiation of the muscle to an adipocyte phenotype by TZD since no *aP2* expression was found.

Conclusion/interpretation. TZD increase *IRS-1*, *UCP2* and *C/EBP α* expression in L6 muscle cells. Activation of *C/EBP α* by TZD could be necessary but it is not sufficient to account for the increased *IRS-1* expression. [Diabetologia (2003) 46:48–52]

Keywords Insulin signalling, IRS, thiazolidinediones, gene transcription, PPAR, C/EBP α , UCP2.

Thiazolidinediones (TZD) are a group of recently identified antidiabetic agents that have been shown to improve insulin sensitivity in humans and in animal models of obesity and diabetes [1, 2]. These drugs are high-affinity ligands for the nuclear receptors PPAR γ but they also have effects that apparently are indepen-

dent of PPAR γ activation [3]. However, it is still not clear how TZD improve insulin sensitivity.

Activation of PPAR γ and its heterodimer partner, RXR leads to the binding of specific DNA sequences; the PPAR response element (PPRE). Very few genes directly involved in insulin signalling and action are known to have PPRE in their promoter region. Using human fat cells and 3T3-L1 adipocytes we recently found [4] that TZD rapidly activated the *IRS-2* gene; an important intracellular docking protein for insulin-stimulated PI3-kinase activation as well as for beta-cell growth. However, it has been difficult to clone the entire *IRS-2* promoter with the putative PPRE and, thus, we still do not know if the effect of TZD is direct or indirect.

Another important family of transcription factors which is activated by TZD are the CCAAT-box/enhancer binding proteins (C/EBP). C/EBP α has been

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Abbreviations: TZD Thiazolidinediones; PPAR peroxisome proliferator activated receptor; C/EBP CCAAT-box/enhancer binding protein; PKB protein kinase B; UCP uncoupling protein.

shown to be important for the activation of genes and proteins involved in insulin-stimulated glucose transport and GLUT4 translocation [5]. Thus, activation of C/EBP α seems to be a key mechanism through which TZD can enhance insulin action.

It is well-established that the skeletal muscle is the major organ for insulin-stimulated glucose disposal and, thus, it would be logical to believe that the insulin-sensitizing effect of TZD is mainly exerted in the muscles. However, recent studies with lipodystrophic mice have shown that the adipose tissue seems to be critical for this effect of TZD.

Only a few studies have addressed the effect of TZD in vitro in muscle cells. A metabolite of troglitazone was found to enhance total GLUT1 and GLUT4 protein expression in the L6 muscle cell line and also to increase insulin-stimulated glucose uptake [6]. A substantial increase in glucose uptake and GLUT4 translocation in L6 muscle cells after troglitazone exposure has also been reported [7]. Furthermore, using cultured satellite cells from human skeletal muscle it has been found that TZD increases insulin-stimulated PI3-kinase activity associated with IRS-1 [8].

We examined the effect of PPAR α - and PPAR γ ligands on the expression of several different genes including some key molecules for insulin's intracellular signalling cascade: PKB/Akt, IRS-1 and IRS-2.

Material and methods

Cell culture. Rat L6 myoblasts, stably transfected with the human insulin receptor (kindly provided by Dr. F. Beguinot, University of Naples) were cultured, differentiated and incubated with various agents during different time periods as indicated in the Results. Except as specified, the cells used for incubations had been differentiated for at least 8 days and had assumed a myotube phenotype. During the incubations, medium and agents were replaced every 24 h.

Protein separation and immunoblotting. Cell lysates and immunoblotting were carried out as described [9]. Antibodies against IRS-1 and IRS-2 (Upstate Biotechnology, Lake Placid, N.Y., USA), PKB/Akt (Biolab, Boston, Mass., USA) and C/EBP α (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) were used for detection of proteins.

Quantification of the proteins was done by using a densitometer (Molecular Dynamics, Sunnyvale, Calif., USA).

RNA extraction and northern blots. Total cellular RNA was isolated from cells with guanidinium thiocyanate. Northern blot analyses were done on total cellular RNA (25–40 μ g) with labelled cDNA probes made against the ribosomal protein 18S as a housekeeping gene and IRS-1, IRS-2 and PKB as previously described [4].

Nuclear extraction. Briefly, pelleted cells were resuspended in hypotonic buffer containing protease inhibitors and homogenized. Nuclei were collected after centrifugation and resuspended in KCl buffer. The nuclei were allowed to extract under continuous mixing and pelleted by centrifugation. The nuclear

extract was dialyzed, precipitated protein and nucleic acids were removed by centrifugation.

Cell transfections. Transient overexpression experiments were done by using a pMEX expression plasmid containing C/EBP α (kind gift from Dr. J. Auwerx, Strasbourg).

Transfection was carried out with the Lipofectamine plus reagent (Life Technologies, Paisley, Scotland) according to the manufacturers instructions.

Quantification using real-time RT-PCR. cDNA was synthesized using Taq Man Reverse Transcription Reagents (Applied Biosystems, Foster City, Calif., USA) according to the manufacturer's instructions. Relative quantification was done by using the ABI Prism 7700 Sequence Detection System with Taq Man sets available upon request. Primers and probes were designed using Primer Express and optimised according to the recommendations in the Taq Man Universal PCR Master Mix protocol (Applied Biosystems). A *p* value of less than 0.05 was considered statistically significant.

Results

Also in marked contrast to our recent findings in adipocytes [4], IRS-1 showed a consistent increase in gene expression by both pioglitazone (Fig. 1A) and rosiglitazone at 1 to 10 μ mol/l but not with the PPAR α ligand.

Time-course experiments with northern blots showed no effect on IRS-1 expression after 4, 8 or 12 h of exposure to pioglitazone, but a gradual increase was seen after 24 h, and this was not changed by adding insulin (Fig. 1A).

The time-course for the increased IRS-1 gene expression in response to pioglitazone using the sensitive Taq Man real-time RT-PCR analysis is shown (Fig. 1B). A consistent effect of pioglitazone was seen at 24 h and this remained throughout the 72 h observation time while no effect of the PPAR α ligand WY was found (Fig. 1B).

The finding that PPAR γ 1 is expressed in L6 cells was also supported by the increased expression of two PPAR γ -responsive genes, C/EBP α and UCP-2, following pioglitazone. A more rapid effect was seen for C/EBP α than for IRS-1 or UCP-2 mRNA (Fig. 2A). The increased C/EBP α expression was sustained throughout the observation time. Since C/EBP α previously has been shown to play a key role for inducing genes associated with an insulin-sensitive cellular phenotype [5, 10], we transfected the cells with C/EBP α to address the possibility of C/EBP α being a mediator of the TZD effect on IRS-1.

C/EBP α was clearly overexpressed in both cell lysates and nuclear extracts (Fig. 2B). However, IRS-1 protein expression and mRNA levels were essentially unchanged and the stimulatory effect of pioglitazone was still maintained (Fig. 2C).

A mRNA: IRS-1/18s **B** **C**

IB: IRS-1

Fig. 1A–C. **A** Northern blots of *IRS-1* mRNA levels relative to *18s* expression. Differentiated L6 muscle cells were cultured for the indicated times with or without 10 μ mol/l pioglitazone (pio) alone or with 100 nmol/l insulin (pio+ins). All data are relative to the basal state (=1) and bars show means $\pm$ SEM of the scanned data ($n=4$). ** $p<0.01$. **B** Time course for *IRS-1* mRNA expression relative to *18s* measured by Real-Time

RT-PCR. L6 muscle cells were cultured without addition (bas), with 10 μ mol/l pioglitazone (pio) or 10 μ mol/l WY14643 (WY) for the times indicated. Basal state is set to one for each time point. Data shown are representative of two-four experiments. **C** Immunoblots of IRS-1 protein. Differentiated L6 cells were cultured as described. Results shown are means $\pm$ SEM of scanned data from four experiments

Fig. 2. **A** Time course for C/EBP α and UCP-2 mRNA expression relative to *18s* using Real-Time RT-PCR. Differentiated L6 muscle cells were cultured without or with 10 μ mol/l pioglitazone (pio) for the indicated times. Results shown are representative of two to four experiments. **B** C/EBP α in nuclear extracts (top) or cell lysates (below) in L6 cells, either wildtype (control) or transfected (transfect) with C/EBP α . After transfection the cells were cultured for 24 or 48 h without or with 10 μ mol/l pioglitazone (pio) as indicated. IRS-1 protein expression (bottom) and mRNA levels (**C**), measured with Real-Time RT-PCR, from the same experiments are also shown. Data are representative of four experiments

Discussion

We have shown that TZD, both pioglitazone and rosiglitazone, increase the gene and protein expression of the key signalling molecule *IRS-1*, as well as C/EBP α and UCP-2, in L6 myocytes. The effect on *IRS-1* gene and protein expression was clearly related to PPAR γ and could not be seen with the specific PPAR α ligand, WY14643.

The slow time-course for *IRS-1* gene activation also suggests that it could be due to an indirect effect on the *IRS-1* promoter. This is consistent with the reported sequence of the murine *IRS-1* promoter which does not show any typical PPRE (GeneBank accession No. S80679).

One possibility for such a putative effect is that the activation of the *IRS-1* gene is not related to PPAR γ activation at all, albeit elicited by TZD. It has been shown that some TZD effects are not related to PPAR γ activation, such as the anti-inflammatory effects including inhibition of cytokine release [3].

However, most of our findings are consistent with the concept that the effect of TZD on *IRS-1* gene activation is mediated through PPAR γ activation. Firstly, we have observed with the sensitive real-time RT-PCR technique that PPAR γ 1 is expressed in L6 cells. Secondly, the fact that both pioglitazone and the PPAR γ -specific ligand, rosiglitazone but not the selective PPAR α ligand, induced *IRS-1* gene activation strongly supports the role of PPAR γ activation. Thirdly, we found that TZD did increase other PPAR γ -responsive

genes like *UCP-2* and *C/EBP α* . This effect of pioglitazone to increase *C/EBP α* expression in L6 myocytes is similar to previous findings in 3T3-L1 adipocytes, although the time-course was more rapid in 3T3-L1 cells [11].

It is well established that PPAR γ activation leads to an increased *C/EBP α* expression and usually also vice versa since these transcription factors augment the expression of each other. Our data also show that TZD activate *C/EBP α* expression in L6 myocytes. However, we did not find that *C/EBP α* activation also increased PPAR γ 1 expression. This finding is consistent with the recent observation [12] that only the PPAR γ 2, and not PPAR γ 1, promoter contains functional *C/EBP* sites increasing the gene expression. An increased PPAR γ 2 expression would also lead to an adipocyte phenotype, defined as an increased lipid accumulation or aP2 expression, in the muscle cells and this was not seen in the wildtype L6 cells after TZD.

Taken together, our data support the conclusion that TZD increase IRS-1 gene and protein expression through PPAR γ activation, possibly as an indirect effect. A likely candidate for such an indirect effect would be *C/EBP α* which has previously been shown to be important for IRS-1 gene expression. For instance, *C/EBP α -/-* fibroblasts have low IRS-1 expression which became increased after the cells had been transfected with *C/EBP α* [10]. The mouse IRS-1 promoter contains several *C/EBP α* sites and, in addition, *C/EBP α* has been shown to play a key role for the expression of other important proteins related to insulin action such as GLUT4 [5].

However, transfecting the cells with *C/EBP α* , under conditions where *C/EBP α* was markedly increased in the nuclei, did not increase IRS-1 expression. These data suggest that *C/EBP α* could be necessary but it is not sufficient to account for the activation of the IRS-1 promoter by TZD. Thus, it is likely that other transcription factors and/or PPAR γ co-activators or repressors are necessary for the activation of the IRS-1 promoter by TZD.

In conclusion, we show that TZD increase IRS-1 mRNA and protein in L6 myocytes. This finding might be an important reason for the ability of TZD to increase insulin sensitivity in skeletal muscle. Furthermore, the data clearly show tissue or cell-specific effects of TZD, emphasizing the critical role of tissue or cell-specific transcriptional regulation.

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