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Differential role of PPARγ in the regulation of UCP-1 and adipogenesis by TNF-α in brown adipocytes

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Abstract Extracellular regulated kinases (ERKs) mediate the inhibitory effect of tumor necrosis factor α (TNF-α) on uncoupling protein-1 (UCP-1), but not on lipid accumulation. TNF-α-induced ERK-dependent peroxisome proliferator activator receptor γ (PPARγ) phosphorylation could be responsible for UCP-1 downregulation. Thus, the negative effect of TNF-α on UCP-1 mRNA expression at 4–5 h, under basal conditions or in cells treated with the PPARγ agonist, rosiglitazone, was reversed by the MEK1 inhibitor PD98059. In contrast, fatty acid synthase and malic enzyme mRNA downregulation was not prevented. Moreover, rosiglitazone has no positive effect on adipogenic gene expression or lipid accumulation. Therefore, there is a differential regulation of thermogenic and adipogenic differentiation by PPARγ, which might account for the differences in the TNF-α regulation through ERKs.

Key words: Brown adipocyte; Tumor necrosis factor α; Uncoupling protein-1; Extracellular regulated kinase; Peroxisome proliferator activator receptor γ

1. Introduction

Brown adipose tissue (BAT) is characterized by the presence of the uncoupling protein-1 (UCP-1) in mitochondria, which allows the production of heat by 'non-shivering thermogenesis' [1]. In rats, BAT development occurs at the end of the fetal period and during the first days after birth [2]. Although some of the signals and mechanisms regulating BAT development are known [3–6], there are many missing links. In particular, the negative regulators of BAT are not well characterized, although tumor necrosis factor α (TNF-α) seems to play an important role [7,8]. TNF-α inhibits proliferation [7,9], differentiation [10] and induces apoptosis [7,9] in rat fetal brown adipocytes, suggesting its importance in BAT development regulation. In addition, based on experiments performed in mice lacking TNF-α receptors [11], TNF-α appears to be responsible for BAT apoptosis, and the reduction in UCP-1 expression observed in obese (ob/ob) mice.

We have found that TNF-α inhibits the expression of UCP-1 and adipocyte-specific genes in rat fetal brown adipocytes [10]. Extracellular regulated kinase (ERK) activation by TNF-α seems to mediate the decrease in UCP-1 expression, but not that of adipogenic genes [10], although they are also negative regulators of these genes [5,12]. Although CAAT enhancer binding protein (C/EBP) α and β could be involved in the inhibitory action of TNF-α, other transcription factors might explain the different regulation of UCP-1 and adipogenic genes by ERKs [10]. Here, we present evidence indicating that the TNF-α-induced phosphorylation of peroxisome proliferator activator receptor γ (PPARγ) via ERKs plays an important role in the negative regulation of UCP-1. Moreover, PPARγ seems to have little relevance in adipogenesis regulation in rat fetal brown adipocytes at this stage of differentiation.

2. Materials and methods

2.1. Isolation of fetal brown adipocytes and culture

Brown adipocytes from 20-day-old rat fetuses were isolated [13] and grown in minimum essential medium supplemented with 10% fetal bovine serum for 24 h. The cells were then serum-starved overnight and maintained in the presence or absence of TNF-α (10 ng/ml), rosiglitazone (1 μM) or combinations of both for different time periods. To inhibit ERKs, cells were pretreated with the MEK1 inhibitor, PD98059 (Calbiochem #513000) at 20 μM for 1 h.

2.2. Western blot analysis

UCP-1 and phospho-cAMP-regulatory element (CRE)-binding protein (CREB) were quantified in total cell extracts [9] by Western blot analysis, using a UCP-1 polyclonal antibody generously given by Dr. E. Rial and a phospho-CREB (ser 133) antibody from New England Biolabs (#9191S).

Phosphorylated and unphosphorylated PPARγ were detected by Western blot with a polyclonal antibody against PPARγ from Bio-Mol (#SA-206) using nuclear extracts prepared as described [10].

2.3. Treatment with alkaline phosphatase

Nuclear extracts were incubated for 1 h at 4°C with alkaline phosphatase (100 U/sample) from bovine intestinal mucosa affinity purified type VII-S (Sigma P 5521) previously bound to Affi-Gel 10 active ester agarose (Bio-Rad cat #153-6099).

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Abbreviations: BAT, brown adipose tissue; C/EBP, CAAT enhancer binding protein; CRE, cAMP-regulatory elements; CREB, CRE-binding protein; ERKs, extracellular regulated kinases; FAS, fatty acid synthase; PPRE, peroxisome proliferator activator response element; PPARγ, peroxisome proliferator activator receptor γ; TNF-α, tumor necrosis factor α; UCP-1, uncoupling protein-1

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2.4. Flow cytometric analysis of lipid accumulation after staining with Nile red

Lipid accumulation was measured in a FACScan flow cytometer (Becton-Dickinson) following lipid staining with Nile red (4 μg/ml). Total lipid content per cell and lipid content versus cell size were quantified, determining the percentages of cells with different lipid content.

2.5. RNA extraction and Northern blot analysis

Northern blot analysis and total RNA extraction was performed [7]. Blots were hybridized with a UCP-1 [14], fatty acid synthase (FAS) [15], malic enzyme [16] or c-jun probe [9] and with a 18S ribosomal probe to normalize.

2.6. Gel mobility shift assays

UCP-1 CRE mobility shift assays were performed as described [9], using nuclear extracts. A specific double-stranded oligonucleotide, 5′-GGGAGTGACGCGCGTCTG-3′, which corresponds to positions −139 to −122 of the rat UCP-1 gene [17], was used.

2.7. Statistical analysis

Statistical analysis of the results was performed using paired Student's *t*-test.

3. Results and discussion

3.1. ERKs are responsible for the inhibitory effect of TNF-α on UCP-1 protein content but not on lipid accumulation in brown adipocytes

TNF-α is a potent inhibitor of insulin-induced adipocytic differentiation in white and brown adipocytes [18] acting through the impairment of insulin signaling [8,18] or by direct inhibition of adipogenic gene expression [18]. In rat fetal brown adipocytes, we also found a direct inhibitory effect of TNF-α on thermogenic and adipogenic differentiation, where ERKs are the mediators of the negative action of TNF-α on UCP-1 mRNA, but not on FAS and malic enzyme mRNAs [10]. Additional experiments to establish the relevance of this pathway and the mechanisms involved have been done.

As shown in Fig. 1A, TNF-α diminished UCP-1 protein content after 48 h of treatment, which was prevented by inhibition of ERKs with PD98059. UCP-1 level was slightly increased by PD98059 under control conditions. In contrast, the decrease in the lipid content induced by TNF-α after 48 h (Fig. 1B) was not abolished by PD98059. Therefore, ERKs mediate the inhibition of thermogenic but not adipogenic differentiation induced by TNF-α.

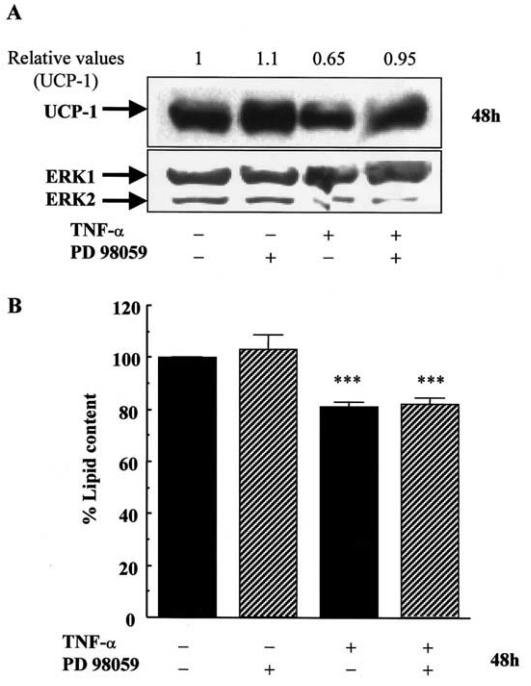

Fig. 1. Effect of PD98059 on the inhibitory effect of TNF-α on UCP-1 protein content and lipid accumulation in brown adipocytes. Serum-starved cells were triggered with TNF-α (10 ng/ml) for 48 h and pretreated or not with 20 μM PD98059 for 1 h. A: Representative UCP-1 Western blot analysis and relative values from the densitometric analysis expressed as fold increase, upper panel; Western blot reblotted with anti-ERK1/2 to normalize, lower panel. Results are means of three independent experiments, in which the variation within the experimental group is less than 15%. B: Flow cytometric analysis of total lipid content per cell after staining with Nile red. Results are expressed as percentages compared with the control value, which is considered 100%, and are means ± S.E.M. of three independent experiments. Statistical analysis was carried out by Student's *t*-test: $^{***}P < 0.001$ as compared with control.

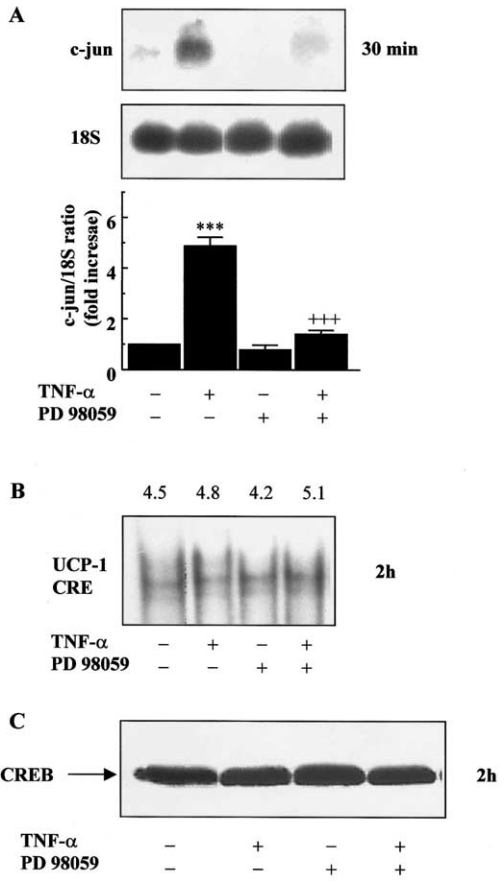

Fig. 2. Effect of TNF-α on the binding activity to the AP-1/CRE site in the UCP-1 promoter. Regulation by ERKs. Serum-starved cells were triggered with TNF-α (10 ng/ml) for 30 min (A) or 2 h (B, C) and pretreated or not with 20 μM PD98059 for 1 h. A: Northern blot analysis of c-jun mRNA. A representative experiment of three independent experiments is shown in the upper panel and the c-jun/18S ratio in the lower panel. Results are means ± S.E.M. of three independent experiments. Statistical analysis was carried out by Student's *t*-test: $^{***}P < 0.001$ as compared with control and $^{+++}P < 0.001$ as compared with TNF-α-treated cells. B: Representative UCP-1-CRE gel shift assay, lower panel; relative values from the densitometric analysis, upper panel. Results are means of three independent experiments, in which the variation within the experimental group is less than 15%. C: Representative phospho-CREB Western blot.

3.2. c-Jun is not responsible for the inhibition of the UCP-1 expression induced by TNF-α

The inhibitory action of TNF-α was partially correlated with changes in C/EBPα and β proteins and in their DNA binding activity, mediated through ERKs [10]. However, these changes did not explain the different regulation exerted by ERKs on UCP-1 and adipogenic genes expression. Thus, other transcription factors might be involved.

Searching for other transcription factors, we investigated c-Jun, which can be upregulated through the ERK pathway [19]. c-Jun can inhibit UCP-1 gene expression through binding to the proximal CRE region of its promoter [17]. Hence, we checked whether changes in c-Jun levels could regulate UCP-1 expression in TNF-α-treated cells. TNF-α increased c-jun mRNA levels, whereas MEK inhibition by PD98059 abolished this effect (Fig. 2A). However, gel shift assays using an UCP-1-CRE oligonucleotide did not reveal any significant change in the DNA binding activity inducible by TNF-α, either in the presence or absence of PD98059, at 2 h (Fig. 2B) or at other time periods (not shown). As CREB/ATF family members can also bind this site, leading to activation of the UCP-1 promoter [17], we quantified phospho-CREB levels. We found very high levels of phospho-CREB in control cells, which remained constant from 5 min up to 48 h with different treatments (Fig. 2C). Hence, it is very unlikely that c-Jun can regulate UCP-1 through the CRE site. On the contrary, because of the high levels of CREB, this transcription factor would preferentially bind to this site avoiding any possible regulation by c-Jun.

3.3. ERKs mediate TNF-α-induced phosphorylation of PPARγ

PPARγ is another transcription factor that activates the expression of certain adipogenic genes [20–22] and the UCP-1 gene [21,23–25]. It can be regulated by ligand binding, phosphorylation, coactivators, transcriptional mechanisms and ligand-dependent proteolysis [10,22,25,26]. We analyzed the possible role played by PPARγ on TNF-α effects through an ERK-dependent mechanism.

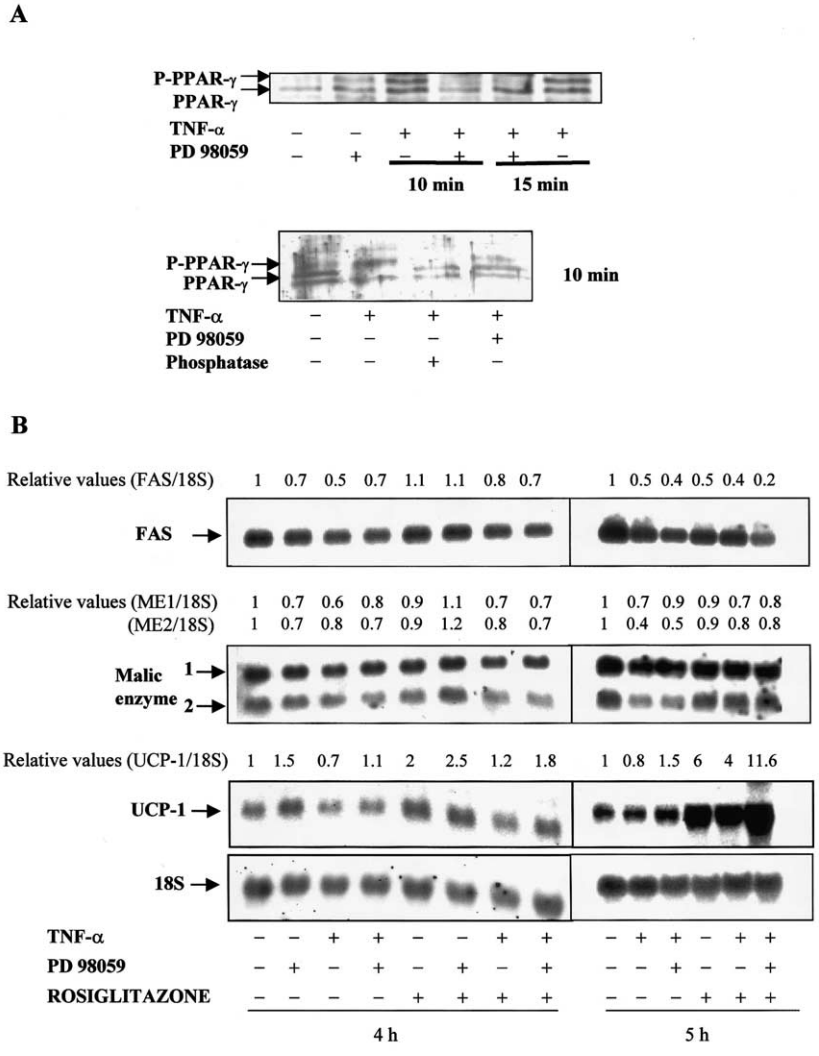

Fig. 3. Effect of TNF-α and the specific PPARγ ligand, rosiglitazone, on UCP-1 and lipogenic gene mRNA expression and its regulation by ERKs. Possible role played by TNF-α-induced PPARγ phosphorylation. Serum-starved cells were triggered with TNF-α (10 ng/ml) for 10–15 min (A) or 4–5 h (B) and/or with rosiglitazone (1 μM) for 4–5 h. When indicated, they were pretreated with 20 μM PD98059 for 1 h. A: Representative PPARγ Western blots performed with nuclear extracts pretreated or not with alkaline phosphatase. B: Representative Northern blot analysis of UCP-1, malic enzyme (ME) and FAS mRNAs and fold increase of these mRNAs normalized with 18S ribosomal probe. Values are means of three independent experiments, in which the variation within the experimental group is less than 15%.

In 3T3 L1 cells, TNF-α can decrease PPARγ mRNA and protein levels depending on the dose and time of treatment [27]. Therefore, we first examined the possible regulation of PPARγ mRNA and protein levels by TNF-α through ERKs, but no changes were found (not shown) upon TNF-α treatment for 24 up to 48 h. Therefore, other mechanisms might be operating, e.g. phosphorylation through ERKs.

PPARγ serine residues are phosphorylated by ERKs upon insulin treatment in 3T3 L1 cells [28], but it has never been shown that TNF-α can induce this phosphorylation. We investigated this possibility by looking for the retardation of the PPARγ band in a Western blot, as is detected in insulin-induced phosphorylation. We analyzed PPARγ in nuclear extracts from cells treated with TNF-α for 10–15 min (pretreated or not with PD98059). TNF-α induced a shift in the PPARγ band that was abolished by pretreatment with PD98059 (Fig. 3A). Treatment of nuclear proteins with alkaline phosphatase also suppressed this shift, which indicates that PPARγ band shift is due to its phosphorylation. Therefore, these results indicate that TNF-α induces the phosphorylation of PPARγ through ERKs.

3.4. Relevance of PPARγ in the inhibitory effect of TNF-α on thermogenesis, but not on adipogenesis

ERK-mediated phosphorylation of PPARγ upon insulin treatment has been associated with the inhibition of adipogenic differentiation [28], but it is unknown whether it can play any role in the inhibitory effect of TNF-α on adipogenic and thermogenic differentiation in brown adipocytes. We explored this by measuring the effect of ERK inhibition on the TNF-α actions, which regulate mRNA expression for UCP-1 and for some adipogenic genes. We also studied whether TNF-α could modulate the effect of the selective PPARγ agonist, rosiglitazone, on the levels of these mRNAs, in either the absence or presence of PD98059. Since PPARγ agonists are able to induce the expression of the UCP-1 mRNA in brown adipocytes at 4 h by a mechanism dependent on ligands but not protein synthesis [29], all these studies were done with short incubations (1–5 h). In this way, it is very likely that we can detect the effect of PPARγ phosphorylation on the mRNAs expression.

We did not observe any change in the expression of UCP-1, FAS and malic enzyme mRNAs before 4 h (data not shown). However, at 4 h, UCP-1 mRNA expression was decreased by TNF-α, and pretreatment with PD98059 abolished this effect (Fig. 3B, left panel). Rosiglitazone induced an increase in UCP-1 mRNA level, that was enhanced by ERKs inhibition and blocked by TNF-α. Pretreatment with PD98059 restored the positive effect of rosiglitazone (Fig. 3B, left panel). These results were confirmed by studies carried out at 5 h (Fig. 3B, right panel), with the only difference that the positive effect of rosiglitazone was much higher at this time and, consequently, it was not totally inhibited by TNF-α.

In contrast, FAS and malic enzyme mRNAs levels were decreased by TNF-α at 4 and 5 h (Fig. 3B), but this effect was not prevented by PD98059, although a partial reversion was observed in the case of the malic enzyme mRNAs. Surprisingly, rosiglitazone did not increase FAS and malic enzyme mRNAs expression, in either the presence or absence of PD98059. Moreover, rosiglitazone induced a significant decrease of FAS mRNA at 5 h either alone or in combination with TNF-α or TNF-α plus PD98059. Therefore, there does

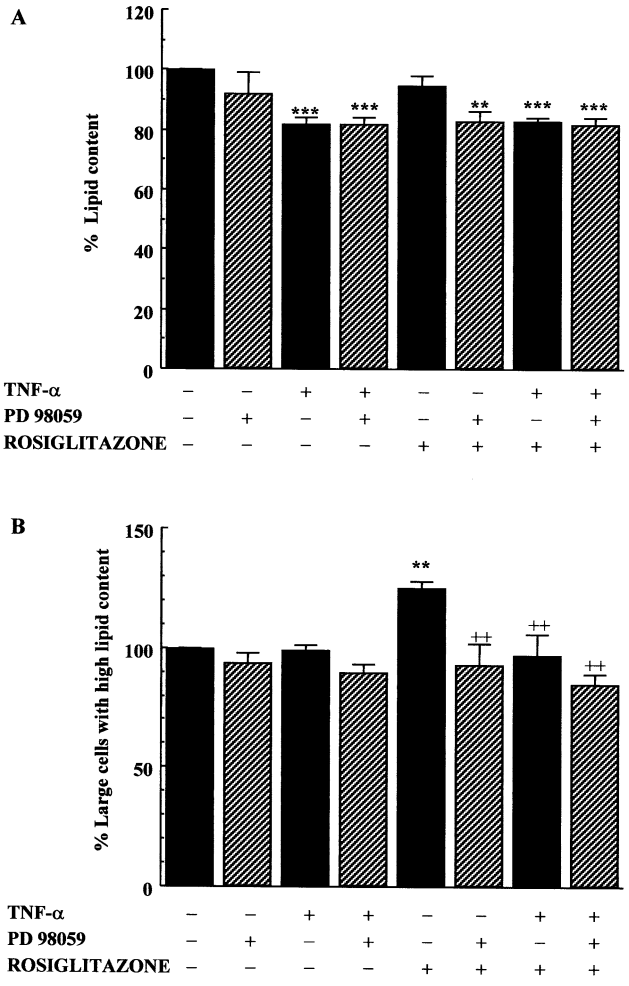

Fig. 4. Effect of TNF-α and the specific PPARγ ligand, rosiglitazone, on lipid accumulation and its regulation by ERKs. Flow cytometric analysis of cellular lipid content after staining with Nile red. Serum-starved cells were triggered with TNF-α (10 ng/ml) and/or with rosiglitazone (1 μM) for 64 h and pretreated or not with 20 μM PD98059 for 1 h. A: Total lipid content per cell. B: Percentage of large cells with high lipid content (28% in controls). A and B: Results are expressed as percentages compared with the control value, which is considered 100%, and are means ± S.E.M. of four independent experiments. Statistical analysis was carried out by Student's *t*-test: $^{***}P < 0.001$; $^{**}P < 0.01$ as compared with control; $^{++}P < 0.01$ as compared with rosiglitazone treatment.

not seem to be a clear regulation of adipogenesis by PPARγ and/or its phosphorylation at the level of these lipogenic genes. However, based on the fact that there are no clear peroxisome proliferator activator response element (PPRE) sites in the FAS promoter [30] and those PPRE sites in the malic enzyme promoter are more likely to be activated by PPARα [31], it would be possible that PPARγ-dependent activation of adipogenesis could be exerted by modulation of other adipogenic-related genes [20,30]. We quantified possible changes in lipid accumulation. As shown in Fig. 4A, treatment with TNF-α for 64 h decreased cellular lipid content and inhibition of ERKs did not prevent it. Moreover, treatment with rosiglitazone had no significant effect on lipid content and the presence of PD98059 induced a decrease. Therefore, these results confirmed data from FAS and malic enzyme mRNA analysis.

It has been described that some of the actions of rosiglita-

zone and other thiazolidinediones in white adipocytes are due to the induction of an increase in the number of small white adipocytes, while decreasing the number of large white adipocytes [21]. Based on this, we analyzed changes in the lipid content in the different subpopulations of brown adipocytes by flow cytometry. We detected a significant increase in the percentage of large cells with a high lipid content (Fig. 4B), that was abolished by PD98059 pretreatment.

In conclusion, our present results indicate that PPARγ is important for the thermogenic differentiation of rat fetal brown adipocytes, but seems to have little relevance for adipogenesis at this stage of differentiation, regulating only the maturation of a small subpopulation of cells (large cells with high lipid content). This would explain the differences in the TNF-α regulation of thermogenic and adipogenic differentiation through ERKs. The relevance of PPARγ in the regulation of UCP-1 expression agrees with previous results obtained in cells maintained in culture [23,24,32,33] and with the absence of BAT in PPARγ knock-out mice after the rescue of the placenta defects [21].

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