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TNF-α inhibits UCP-1 expression in brown adipocytes via ERKs

Opposite effect of p38MAPK

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Abstract Tumor necrosis factor-α (TNF-α) activates extracellular-regulated kinases (ERKs) and p38 mitogen-activated protein kinase (p38MAPK), and inhibits the expression of uncoupling protein-1 (UCP-1) and adipocyte-specific genes in rat fetal brown adipocytes. MEK inhibition with PD98059 abolished the inhibitory effect of TNF-α on UCP-1, but not on adipogenic genes. In contrast, inhibition of p38MAPK with SB203580 potentiated the negative effect of TNF-α on UCP-1 and adipogenic genes. The inhibitory action of TNF-α was partially correlated with changes in C/EBPα and β protein levels and in their DNA binding activity, suggesting a role for these transcription factors. However, other transcription factors might explain the different regulation of UCP-1 and adipogenic genes by ERKs. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Brown adipocyte; Tumor necrosis factor-α; Uncoupling protein-1; p38 mitogen-activated protein kinase; Extracellular-regulated kinase

1. Introduction

Brown adipose tissue (BAT) is specialized in heat production by the mechanism called 'non-shivering thermogenesis'. This process is carried out by the uncoupling protein-1 (UCP-1), present in the mitochondrial inner membrane, which allows dissipation of energy from substrate oxidation as heat [1]. BAT is activated during the perinatal period, after cold exposure or after feeding with a 'cafeteria' diet [1].

Tumor necrosis factor-α (TNF-α) has recently been proposed to be responsible for BAT apoptosis, altered cellular morphology, and reduction in the β3-adrenergic receptors and UCP-1 expression observed in obese (ob/ob) mice, since those BAT abnormalities were lost in mice lacking TNF-α receptors [2]. TNF-α might also be relevant in the control of the number of brown adipocytes during the fetal period and/or in the involution of BAT produced after weaning, since it inhibits cell growth and induces apoptosis in fetal brown adipocytes [3]. In addition, TNF-α could also contribute to the negative regulation of BAT by a direct inhibition of the thermogenic and adipogenic differentiation, not related with the induction of insulin resistance by impairing insulin signaling [4]. TNF-α could do it through activation of different signaling pathways such as extracellular-regulated kinases (ERKs) (p44/p42 mitogen-activated protein kinases (MAPKs)), JNKs, p38MAPKs or NF-κB [5–8].

ERKs are activated by different extracellular signals [7–9], including cytokines, and regulate proliferation, differentiation and apoptosis (review in [9]). On the other hand, the p38 subfamily of MAPKs, which includes p38α, p38β, p38β_2, p38γ (ERK6/SAPK3) and p38δ (review in [9]), was initially associated with stress responses, having been identified p38αMAPK as a stress-induced kinase [10]. However, p38MAPKs can mediate different cellular functions such as differentiation, or developmental processes (review in [11]). Thus, in mice lacking p38αMAPK, placental development is impaired [12]. p38MAPK is also required for the insulin-induced adipocytic differentiation of 3T3-L1 cells [13,14], inducing apoptosis [14].

In rat fetal brown adipocytes, p38MAPK mediates TNF-α-induced growth inhibition and apoptosis, whereas ERKs play the opposite role [15]. ERKs are also essential mediators of the IGF-I/insulin and noradrenaline-induced proliferation, while attenuating the differentiation process [16,17] as they do in the insulin-induced differentiation of 3T3-L1 cells [18]. We analyze here the direct inhibitory effect of TNF-α on the thermogenic and/or adipocytic differentiation of brown adipocytes and the role played by p38 and ERKs.

2. Materials and methods

2.1. Isolation of fetal brown adipocytes and culture

Brown adipocytes from 20 day old rat fetuses were isolated as described [19] and maintained in primary culture. Cells were grown in minimum essential medium supplemented with 10% fetal bovine serum for 24 h. Then, cells were serum-starved overnight and maintained in the absence or presence of TNF-α (10 ng/ml), IGF-I (2.5 nM) or combinations of them for different time periods as indicated. To inhibit ERKs, cells were pretreated with the MEK1 inhibitor PD98059 (Calbiochem #513000) at 20 μM for 1 h. Similarly, to inhibit p38MAPK, cells were pretreated with SB203580 at 5 μM from Calbiochem (#559389) or PD169316 (#513030) at 400 nM.

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Abbreviations: BAT, brown adipose tissue; C/EBP, CAAT enhancer binding protein; CRE, cAMP regulatory element; CREB, CRE binding protein; ERK, extracellular-regulated kinase; FAS, fatty acid synthase; p38MAPK, p38 mitogen-activated protein kinase; PPARγ, peroxisome proliferator activator receptor γ; TNF-α, tumor necrosis factor-α; UCP-1, uncoupling protein-1

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2.2. Western blot analysis

Active ERK (ERK1/2) levels were quantified in total cell extracts by Western blot analysis using an anti-phospho-ERKs antibody from New England Biolabs (#9101S) and total ERK levels with the anti-ERK1/2 antibody from UBI (06-182) as described [17]. Similarly, active p38MAPK was quantified using an anti-phospho-p38MAPK antibody from New England Biolabs (#9211S), and total p38MAPK levels with an anti-p38MAPKα antibody from Santa Cruz (#sc-535) [15].

CAAT enhancer binding protein (C/EBP) α and β were also quantified in total cell extracts by Western blot analysis, using C/EBPα- (sc-61) and C/EBPβ- (sc-150) specific antibodies from Santa Cruz.

Blots were developed using the ECL system (Amersham).

2.3. MAPKAPK2 kinase assay

MAPKAPK2 kinase activity was assayed in immunocomplexes, prepared with an anti-MAPKAPK2 antibody from StressGen Biotechnologies Corp. (#KAP-MA015E) as described [15], and human recombinant Hsp27 protein (StressGen Biotechnologies Corp. #SPP-715) was used as substrate. Phosphorylated Hsp27 was visualized by autoradiography.

2.4. RNA extraction and Northern blot analysis

Northern blot analysis and total RNA extraction were performed as previously described [17]. Blots were hybridized with probes for UCP-1 [20], fatty acid synthase (FAS) [21] and malic enzyme [22] and, then, reproved with 18S ribosomal probe to normalize. Radioactivity was quantified in a Fuji-Film BAS-1000 apparatus.

2.5. Gel mobility shift assays

C/EBP mobility shift assays were performed using nuclear extracts as described [15], using a specific double-stranded oligonucleotide corresponding to the NF-IL6 site of the COX2 promoter: 5′-GGGTATTATGCAATTGGAAG-3′.

2.6. Statistical analysis

Statistical analysis of the results was done using the Student's *t* test.

3. Results

3.1. TNF-α activates ERKs and p38MAPK. Inhibition by PD98059 and SB203580, respectively

TNF-α activated ERKs, in the absence or presence of IGF-I (Fig. 1A). IGF-I, as previously described [17], also activated ERKs. This TNF-α-induced activation was totally prevented by pretreatment with the MEK inhibitor, PD98059 (Fig. 1A).

p38MAPK was also activated by TNF-α, in the absence or presence of IGF-I, but not by IGF-I (Fig. 1B). MAPKAPK2, one of the p38MAPK downstream targets, was also activated by TNF-α (Fig. 1C), and this activation was blocked by pretreatment with the specific p38MAPK inhibitor SB203580 (Fig. 1C).

3.2. TNF-α inhibits brown adipocytes differentiation. Regulation by ERKs and p38MAPK

Next, we studied whether TNF-α played a direct role inhibiting the expression of UCP-1 and adipogenic genes in brown adipocytes, and the involvement of ERKs and p38MAPK, using the specific inhibitors, PD98059 and SB203580, respectively. TNF-α decreased basal and IGF-I induced UCP-1 mRNA expression (Fig. 2A) as well as FAS and malic enzyme mRNA levels (Fig. 2B). Pretreatment with PD98059 totally reverted the inhibitory effect of TNF-α on UCP-1 and enhanced the expression induced by IGF-I (Fig. 2A). However, the TNF-α-induced downregulation of adipocyte-specific mRNAs, FAS and malic enzyme, was not prevented, although their levels were always higher in cells pretreated with PD98059 (Fig. 2B). So, the upregulation of adipocyte-specific genes by MEK inhibition was only significant when IGF-I was present. Different from this, pretreatment with SB203580 enhanced the inhibitory effect of TNF-α on UCP-1, FAS and malic enzyme mRNAs expression, either in control or IGF-I-treated cells (Fig. 3). These results were confirmed using PD169316, another p38 inhibitor (not shown). Furthermore, data from FAS and malic enzyme mRNA measurements were confirmed by cytometric analysis of lipid content after staining with Nile red under all the conditions studied (not shown), and data from UCP-1 mRNA quantifications by Western blot analysis of UCP-1 (not shown).

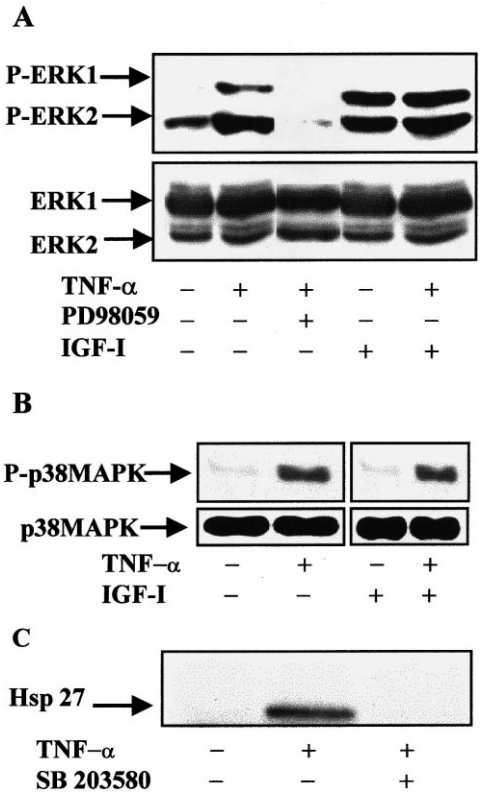

Fig. 1. TNF-α, either in the presence or absence of IGF-I, activates ERKs and p38MAPK pathways in rat fetal brown adipocytes. Inhibition by PD98059 and SB203580, respectively. Serum-starved cells were triggered with IGF-I, TNF-α or both for 10 min. When indicated, they were pretreated with PD98059, or with SB203580. (A) ERKs activity and inhibition by PD98059. Upper panel and lower panel, representative phospho-ERK1/2 and total ERK1/2 Western blots, respectively. (B) p38MAPK activity. Upper panel and lower panel, representative phospho-p38MAPK and total p38MAPK Western blots, respectively. (C) MAPKAPK2 activity in immunocomplexes and inhibition by SB203580. Representative autoradiogram showing phospho-Hsp27.

3.3. TNF-α decreases C/EBPs protein expression and DNA binding activity. Regulation by ERKs and p38MAPK

C/EBP transcription factors are known positive regulators of the adipocytic (review in [23,24]) and thermogenic differentiation (review in [24]). Thus, we studied the effect of TNF-α on C/EBP DNA binding ability, and on the levels of C/EBP proteins, and the role played by ERKs and p38.

Treatment with TNF-α for 24 h highly reduced the binding of nuclear proteins to an oligonucleotide probe corresponding to the C/EBP consensus site (Fig. 4A). Inhibition of p38 with

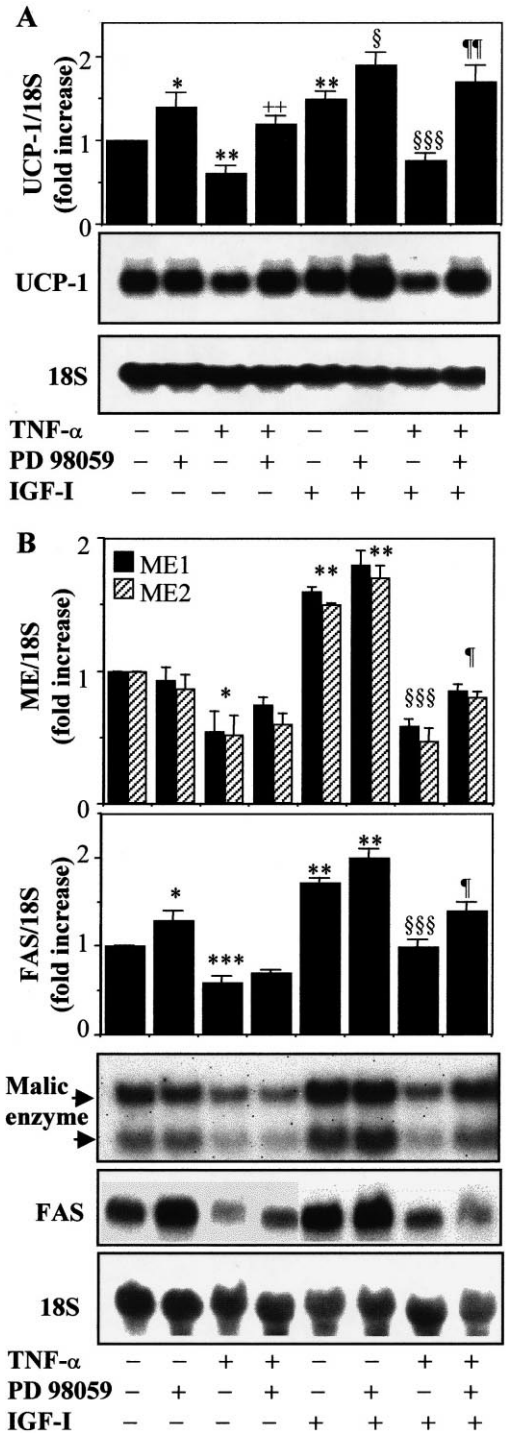

Fig. 2. Role played by ERKs in the TNF-α-induced decrease in UCP-1, malic enzyme and FAS mRNA levels in rat fetal brown adipocytes. Serum-starved cells, pretreated with PD98059 or non-pretreated as indicated, were triggered with IGF-I, TNF-α or both for 48 h, or maintained untreated. Representative Northern blot analysis of UCP-1 (A), malic enzyme (ME) and FAS mRNAs (B), and fold increase of these mRNAs normalized with 18S ribosomal probe. Results are means ± S.E.M. of four independent experiments. Statistical analysis was carried out by Student's *t* test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ as compared with control; $^{++}P<0.01$ as compared with TNF-α-treated cells; $^{\S}P<0.05$, $^{\S\S\S}P<0.001$ as compared with IGF-I-treated cells; $^{\P}P<0.05$, $^{\P\P}P<0.01$ as compared with cells treated with hIGF-I plus TNF-α.

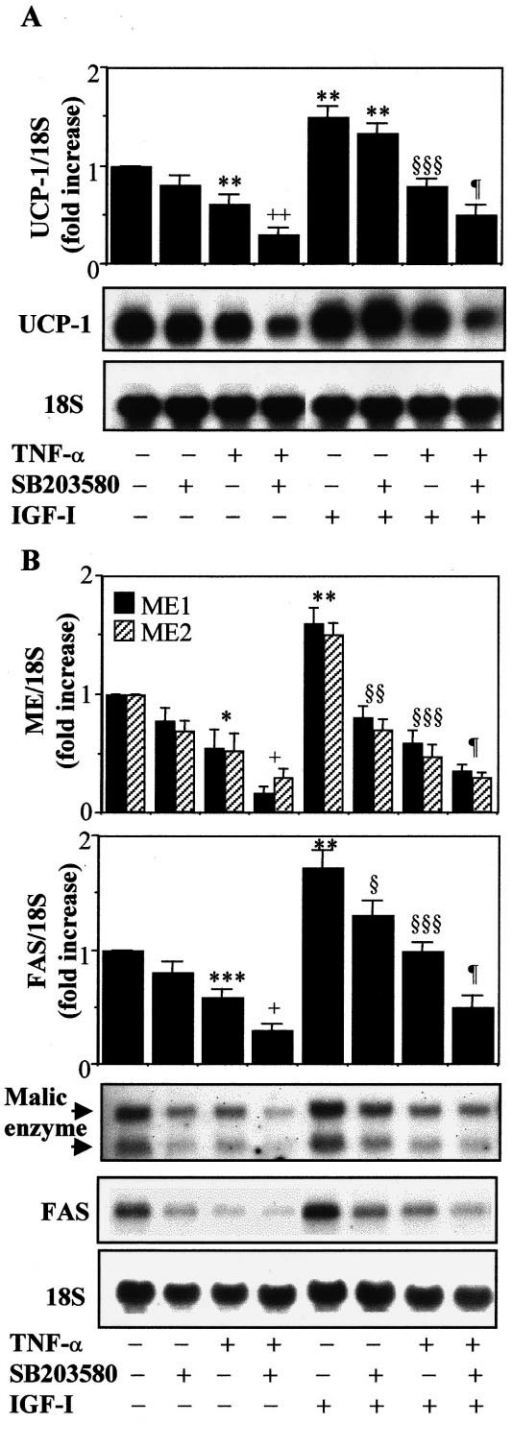

Fig. 3. p38MAPK plays a positive role in the regulation of UCP-1, malic enzyme and FAS mRNA levels by TNF-α in rat fetal brown adipocytes. Serum-starved cells, pretreated with SB203580 or non-pretreated as indicated, were triggered with IGF-I, TNF-α or both for 48 h, or maintained untreated. Representative Northern blot analysis of UCP-1 (A), and malic enzyme (ME) and FAS mRNAs (B), and fold increase of these mRNAs normalized with 18S ribosomal probe. Results are means ± S.E.M. of four independent experiments. Statistical analysis was carried out by Student's *t* test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ as compared with control; $^{+}P<0.05$, $^{++}P<0.01$ as compared with TNF-α-treated cells; $^{\S}P<0.05$, $^{\S\S}P<0.01$, $^{\S\S\S}P<0.001$ as compared with IGF-I-treated cells; $^{\P}P<0.05$ as compared with cells treated with IGF-I plus TNF-α.

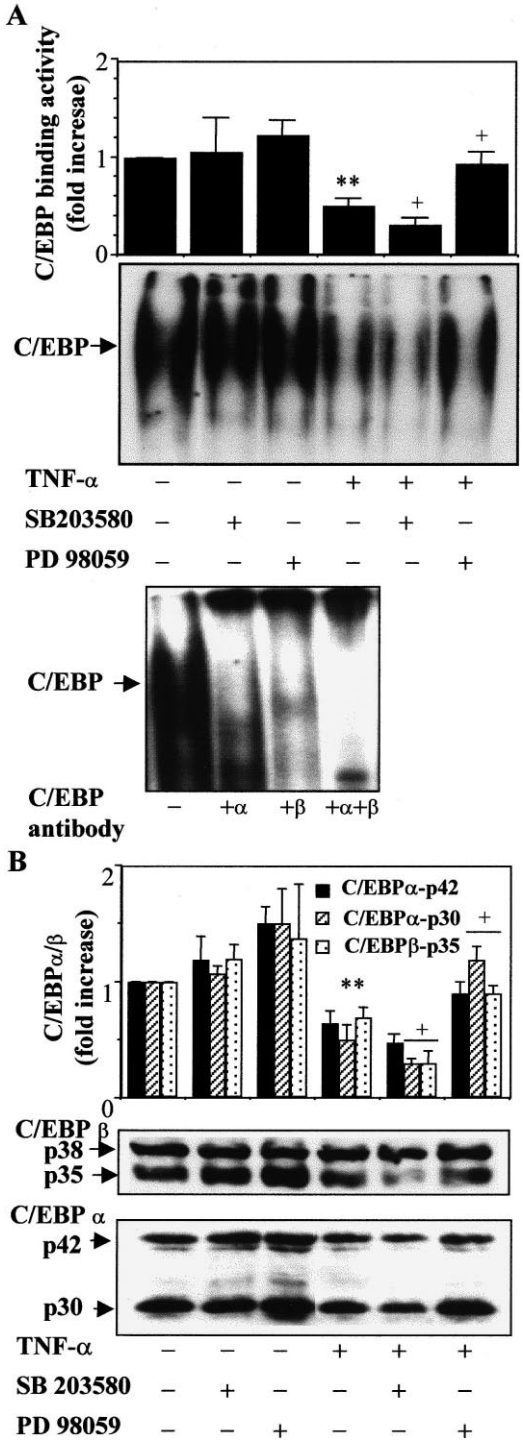

Fig. 4. Regulation of C/EBPα/β protein levels and their DNA binding activity by TNF-α in rat fetal brown adipocytes. Role played by ERKs and p38MAPK. Serum-starved cells, pretreated with PD98059, SB203580 or non-pretreated as indicated, were triggered with TNF-α for 24 h or maintained untreated. (A) C/EBP DNA binding activity. Middle panel, representative gel-shift assay; lower panel, representative supershift assay of control cells, in the absence of C/EBP antibodies, or with either C/EBPα and/or β antibodies; upper panel, fold increase of C/EBP binding activity. (B) C/EBPα and β protein levels. Lower panel, C/EBPα Western blot analysis showing p42 and p30 C/EBPα proteins; middle panel, C/EBPβ Western blot analysis showing p38 and p35 C/EBPβ; upper panel, fold increase of p35 C/EBPβ, and p42 and p30 C/EBPα protein levels. Results are means ± S.E.M. of three independent experiments. Statistical analysis was carried out by Student's t test. $^{**}P < 0.01$ as compared with control; $^{+}P < 0.05$ as compared with TNF-α-treated cells.
←

SB203580 enhanced this effect, while ERKs inhibition with PD98059 partially reversed it (Fig. 4A). In supershift assays, incubation with anti-C/EBPβ eliminated most of the binding to the C/EBP site (Fig. 4A), and incubation with an α-specific antibody decreased this binding, too, showing that C/EBPα and β proteins bound this C/EBP sequence. C/EBPδ did not bind (not shown).

Changes in the binding of nuclear proteins to a C/EBP site can reflect changes in the levels of C/EBP proteins. So, we measured C/EBPα, β and δ protein levels by Western blot. Fig. 4B shows a reduction in the levels of p35 C/EBPβ, and p42 and p30 C/EBPα after 24 h of TNF-α treatment. This effect was potentiated by SB203580, whereas it was prevented by PD98059 (Fig. 4B). C/EBPδ protein levels did not change under these conditions (not shown).

4. Discussion

TNF-α has been shown to be a potent inhibitor of the insulin-induced adipocyte-specific gene expression in white and brown adipocytes (review in [25]). Some of these TNF-α effects are due to the impairment of the insulin signaling, which leads to insulin resistance in white [25] and brown adipocytes [4,25]. A direct effect of TNF-α inhibiting the expression of some adipogenic genes has also been described in white adipocytes (review in [25]). However, a direct effect of TNF-α inhibiting thermogenic and adipogenic differentiation of brown adipocytes had not been found until now.

p38MAPK was shown to be required for the insulin-induced adipocytic differentiation of 3T3-L1 cells [13,14]. We have now seen that in brown adipocytes p38 also mediates this action in response to TNF-α, an inhibitor of adipogenesis. In addition, we have found a new role for p38 as a positive regulator of UCP-1 expression, upon TNF-α stimulation. This positive regulation of brown adipocytes differentiation by p38 appears to be mediated through the upregulation of C/EBPα and β. This might be related to the mechanism described for 3T3-L1 cells induced to differentiate by insulin, where p38MAPK mediated phosphorylation of C/EBPβ, leading to an increase of its transcriptional activity, that could be responsible for the p38-dependent expression of peroxisome proliferator activator receptor γ (PPARγ) [13,14]. However, our results are different from those suggesting that p38 might be a negative regulator of 3T3-L1 differentiation [26]. These suggestions were based on the ability of p38MAPK to phosphorylate CHOP (GADD153), whose overexpression blocked 3T3-L1 differentiation but not that of a mutant unable to be phosphorylated by p38 [26]. This apparent contradiction could be originated by the fact that under our conditions, CHOP protein levels are very low [15], while C/EBPα and β are much more abundant, and, therefore, p38 might preferentially phosphorylate C/EBPβ instead of CHOP, leading to adipocytic differentiation.

ERKs appear to mediate the inhibitory effect of TNF-α on the thermogenic marker, UCP-1, but not on lipogenic markers

as seen in 3T3-L1 cells [27], although ERKs are also negative regulators in this case. This effect might be related with the decrease in the levels of C/EBPα and β, in their binding to DNA or even with PPARγ phosphorylation. Thus, PPARγ, which is another transcription factor that activates the expression of adipogenic genes [23,28,29] and the UCP-1 gene [28–30], seemed to be phosphorylated in response to TNF-α (data not shown) through ERKs as it occurs in 3T3-L1 cells treated with insulin [31]. However, all these transcription factors bind to the regulatory sequences of UCP-1 and those of many adipogenic genes, inducing their expression. Hence, these changes in C/EBPs and PPARγ appeared not to be sufficient by themselves to explain why TNF-α-induced downregulation of UCP-1 was reverted by inhibition of ERKs, while that of adipogenic genes was not. So, we analyzed the possibility that c-Jun, whose mRNA is upregulated via ERKs [15], would be inhibiting UCP-1 expression through binding to the proximal cAMP regulatory element (CRE) region of its promoter [32]. However, no change was found at this level (not shown), probably because of the high levels of phospho-CRE binding protein (CREB) present in the cells [15]. Thus, CREB, instead of c-Jun, would bind to this CRE site in the UCP-1 promoter [32]. Hence, the different regulation of UCP-1 by TNF-α through ERKs might be due to other causes and it could be related with the action of coactivators and/or other transcription factors. Thus, in the case of PPARγ, that is essential for the function of the UCP-1 enhancer [30,33], its effect is strongly increased by binding of the coactivator, PGC-1 (PPARγ coactivator-1), through a ligand-independent way [30,34,35]. As it binds to the region of PPARγ phosphorylated by ERKs [34], this binding could be impaired, leading to inhibition of UCP-1 expression. PGC-1 is expected to be highly expressed under our experimental conditions, for it is transcriptionally upregulated by CREB [30], which is present in high levels [15]. In contrast, regarding adipogenic genes, although ERKs-mediated PPARγ phosphorylation can play a negative role, as suggested by the effect of overexpression of a PPARγ mutant not phosphorylated by ERKs [31], the interaction between PPARγ and the adipogenic cofactor PGC-2 is not affected by this phosphorylation [36]. In addition, other transcription factors that activate adipogenesis might be regulated by TNF-α through ERKs-independent pathways. Hence, a decrease in the levels of ADD1/SREBP-1 (adipocyte differentiation and determination factor-1/sterol regulatory element binding protein 1) in response to TNF-α might be possible, impairing its positive effect on lipogenic genes expression [23] through a PPARγ-independent pathway. Another possibility is that TNF-α could increase Id (inhibitor of DNA binding) 2 and Id 3 protein levels [37], which would inhibit ADD1 binding to FAS promoter [38].

Based on these results, we have found opposite roles for ERKs and p38MAPK in the inhibitory effect of TNF-α on thermogenic and adipogenic differentiation in fetal brown adipocytes. We have also previously found [15] that ERKs were positive regulators of proliferation and survival, whereas p38MAPK mediated the TNF-α-induced apoptosis and cell cycle arrest. Thus, it appears that TNF-α activates different signaling pathways with opposite effects on the regulation of proliferation, differentiation and apoptosis in these cells. This might have a physiological meaning, related with a tight regulation of the number of brown adipocytes during development, and its differentiation state.

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