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TNF-α induces apoptosis in rat fetal brown adipocytes in primary culture

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Abstract The effect of TNF-α on cell death in rat fetal brown adipocytes maintained in primary culture was determined. TNF-α inhibited proliferation and induced apoptosis in these cells. Most of the cells undergoing apoptosis after TNF-α treatment did not express PCNA, suggesting an induction of apoptosis by TNF-α in non-proliferative cells. IGF-I but not EGF prevented TNF-α-induced apoptosis.

Key words: TNF-α; Apoptosis; Brown adipocyte

1. Introduction

Apoptosis is a specific process of cell elimination in normal tissues, considered as an important physiological mechanism for regulation of proliferation, development and oncogenesis (see [1] for review).

Little is known, for adipose tissue, about the process of adipocyte loss. A regression of adipose tissue in diabetic rats [2] and processes of 'dedifferentiation' in vitro [3] have been described. Moreover, a dramatic loss of adipose tissue is observed in some diseases such as cancer and severe infection, that can render cachexia. Adipocyte apoptosis was demonstrated to occur in patients with malignancy [4] and in vitro, in human adipocytes after growth factor deprivation or mild heat injury [5]. Therefore, the loss of adipocytes in certain diseases might be a consequence of the induction of apoptosis.

TNF-α induces apoptosis in different cell types [6–10]. High levels of TNF-α and other cytokines are produced in obesity and in malignancy. TNF-α has different effects on adipose tissue including inhibition of differentiation and induction of dedifferentiation in mature adipocytes [11–13]. Therefore, it induces a decrease in the number and/or in the size of mature adipocytes by these mechanisms. However, a direct role for TNF-α inducing apoptosis in white adipose tissue, as a mechanism to eliminate adipocytes, remains unexplored.

In brown adipose tissue, no process of apoptosis has been described. Brown adipose tissue produces heat by a mechanism called 'non-shivering thermogenesis'. This tissue is active in newborn mammals, in animals exposed to cold, in arousal from hibernation and after diet-induction [14]. Noradrenaline [15], T3 [16,17], IGF-I [18–20] and insulin [20] are positive regulators of this tissue. IGF-I [18–20] and insulin [20] induce its differentiation in fetal brown adipocytes in culture as in white adipocytes [21,22]. However, the signals involved in the negative regulation of the tissue and in the inactivation or involution of this tissue are not yet clear. For example, after the neonatal period it is not known which signals determine the inactivation and/or disappearance of the tissue. Since the adipocytic differentiation is essentially regulated as in white adipose tissue, it is likely to think about similar mechanisms for loss of both adipose tissues. Thus, TNF-α might be a negative regulator of this tissue by different mechanisms such as inhibition of differentiation as in white adipocytes [11–13] and/or induction of apoptosis.

In this paper, we present evidences demonstrating that TNF-α induces apoptosis and inhibits cell growth in brown adipocytes. IGF-I, but not EGF, rescues from TNF-α-induced apoptosis.

2. Materials and methods

2.1. Isolation of fetal brown adipocytes and culture

Fetal brown adipocytes from 20 day old rat fetuses were isolated as described (15) and maintained in primary cultures. Cells were grown in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) for 24 h. Then, cells were serum starved o/n and maintained in the absence or in the presence of TNF-α (1–10 ng/ml), IGF-I (2.5 nM), EGF (20 ng/ml), IGF-I plus TNF-α or EGF plus TNF-α for 24 h, 32 h, 48 h.

2.2. Flow cytometric analysis

Analysis of DNA content, cell cycle and PCNA content of the cells in the different phases of the cell cycle was performed in a FACScan flow cytometer (Becton-Dickinson, San José, CA). DNA was stained with propidium iodide (PI) using the Bio-Rad reagent kit (Kinesis 50, #470-0023), following the manufacturer's protocol. When PCNA content was analyzed in parallel to the cellular DNA content, the Bio-Rad reagent kit containing PI and an anti-PCNA antibody (PCNA/Kinesis 50, #470-0043) was used. Measurements were carried out using a Double Discriminator Module in order to discriminate doublets. 10000 cells were acquired per sample. Then, the percentage of cells with DNA content lower than 2C was calculated as well as the percentage of cells in the G0/G1, S and G2/M phases of the cell cycle, using Multicycle software (Phoenix software). When PCNA and cellular DNA content were simultaneously analyzed, the percentage of cells expressing PCNA with different DNA contents was also calculated using CellQuest software (Becton-Dickinson).

The presence of phosphatidylserine in the outer layer of the plasma membrane (an early feature of apoptosis) from control and TNF-α treated brown adipocytes, was detected by specific binding to annexin-V using the Annexin-V-Fluos kit from Boehringer Mannheim (cat. no. 1828681). Cells were incubated with propidium iodide and/or annexin-V-Fluorescence and analyzed by flow cytometry. The percentage of cells positive for annexin-V, considered as apoptotic, and those positive for annexin-V and propidium iodide, considered as necrotic, was determined.

2.3. Analysis of DNA fragmentation

DNA fragmentation was determined by a modified version [23] of the method of Lyons [24]. After scrapping, brown adipocytes were resuspended in a buffer containing 10 mM EDTA, 0.25% Triton X-100, 2.5 mM Tris-HCl, pH 8.0 and maintained at 4°C for 15 min. Intact nuclei were pelleted and eliminated by centrifugation at 500×*g* for 10 min and the supernatant centrifuged at 25000×*g* at 4°C for 30 min. DNA from the supernatant was precipitated with ethanol at −80°C, pelleted by microcentrifugation at 4°C for 15 min, dried and resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE buffer). Then, it was incubated with 0.1 mg/ml RNase A for 30 min

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at 37°C and with 0.25 mg/ml proteinase K for 2–3 h at 37°C to eliminate RNA and proteins. DNA was purified by phenol-chloroform extraction, precipitated with ethanol at −80°C, and resuspended in TE buffer. Then, DNA was electrophoresed in a 1.5% agarose gel.

2.4. Confocal microscopy analysis

Morphology of chromatin from apoptotic and intact nuclei was detected by confocal microscopy analysis of brown adipocytes attached to plates, incubated for 10 min with propidium iodide (0.005%) (PI) and SYTO 13 in PBS. Only necrotic cells and lately apoptotic cells can be labeled by PI, since PI only goes into cells that loose their plasma membrane integrity. SYTO 13 labels all nuclei. Thus, using this method, we can distinguish between intact nuclei and necrotic/apoptotic nuclei. MRC 1000 confocal microscopy (Bio-Rad, Hempstead, UK) was used and digital images were printed using Lasergraphics.

3. Results and discussion

3.1. TNF-α inhibits proliferation in rat fetal brown adipocytes

Proliferation, differentiation and apoptosis are necessary processes for development [1,25]. Sometimes, signals that inhibit proliferation in a certain cell type, are also capable to induce apoptosis in order to remove the excess of cells [25] under certain circumstances.

To study the effect of TNF-α on the cell cycle, the flow cytometric analysis of cellular DNA stained with propidium iodide was used. The percentage of cells in the different phases of the cell cycle was calculated and it is shown in Table 1. Brown adipocytes untreated or treated for 24 h with TNF-α, IGF-I (the best mitogen for these cells) or IGF-I plus TNF-α were studied to assess the role of TNF-α on basal (control) and IGF-I-induced cell growth. TNF-α induced a significant decrease ($P < 0.05$) in the percentage of cells in the S phase of the cell cycle as compared with untreated cells (control). IGF-I, which is a potent mitogen for these cells [18], produced a significant ($P < 0.05$) increase in the percentage of cells in the S phase as well as in the percentage of cells in the G2/M phases ($P < 0.01$) of the cell cycle. When cells were treated with IGF-I plus TNF-α, the IGF-I-induced mitogenic effect was completely abolished. Thus, a significant ($P < 0.01$) decrease in the percentage of cells in the S phase was observed as well as in the percentage of cells in the G2/M phases ($P < 0.05$) of the cell cycle when compared with IGF-I treated cells. Therefore, TNF-α was able to inhibit the endogenous and the IGF-I-induced cell growth in fetal brown adipocytes. So, the next step was to determine whether TNF-α, besides inhibiting proliferation, would be able to induce apoptosis in brown adipocytes.

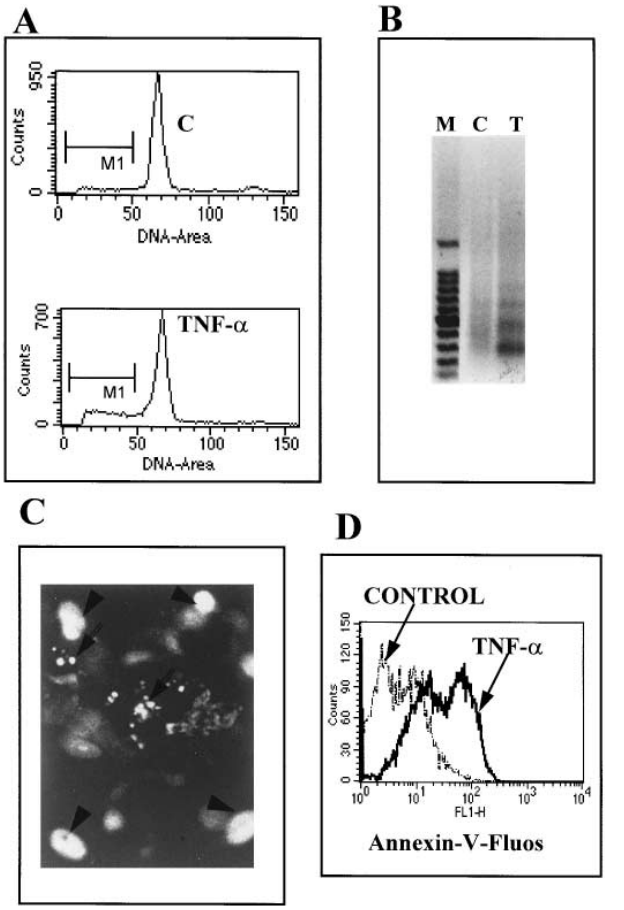

Fig. 1. Effect of TNF-α on cellular DNA content, DNA fragmentation, nuclei morphology and plasma membrane annexin-V-FITC labeling. A: DNA content histograms from flow cytometric measurements in brown adipocytes untreated (C) or treated with 10 ng/ml TNF-α for 48 h. M1 bar represents cells with a DNA content lower than 2C, being 7.09 ± 1.03 for control cells and 22 ± 0.80 for TNF-α treated cells. B: Representative DNA ladder. Cytoplasmic DNA from brown adipocytes untreated (C) or treated with 10 ng/ml TNF-α (T) for 48 h was extracted and analyzed as described under Section 2; M, molecular weight markers (100 bp ladder DNA). C: Confocal microscopy analysis of nuclear morphology in brown adipocytes treated with 10 ng/ml TNF-α for 48 h after staining with propidium iodide (PI) and SYTO 13. Head arrows show intact nuclei stained by SYTO 13 and arrows show apoptotic bodies and fragmented nuclei stained by PI. D: Flow cytometric analysis in brown adipocytes untreated (Control) and treated with 10 ng/ml TNF-α (TNF-α) for 48 h after labeling with annexin-V.

3.2. Characterization of the TNF-α-induced apoptotic cell death in rat fetal brown adipocytes. Relation between PCNA expression and TNF-α-induced apoptosis

Rat fetal brown adipocytes were maintained in primary culture in the absence of serum, untreated or treated with TNF-α for 24 h, 32 h or 48 h, and the effect of TNF-α on cell death was studied. TNF-α began to induce certain morphological changes after 24–32 h, being more visible after 48 h.

Table 1
TNF-α inhibits endogenous and IGF-I-induced proliferation in brown adipocytes

Treatment	G0+G1 phases	S phase	G2/M phases
Control	92.06 ± 0.45	5.30 ± 0.41	2.50 ± 0.19
TNF-α	94.75 ± 0.41[a]	3.14 ± 0.20[a]	1.99 ± 0.33
IGF-I	88.43 ± 1.25[a]	7.33 ± 0.90[a]	3.57 ± 0.33[b]
IGF-I+TNF-α	92.83 ± 0.58[c]	4.53 ± 0.45[d]	2.63 ± 0.13[c]

Cell cycle study was performed by flow cytometry using propidium iodide for DNA staining as described under Section 2. Cells were serum starved o/n and maintained for 24 h in the absence of serum untreated (control) or treated either with TNF-α, IGF-I or IGF-I+TNF-α, before the analysis. Results are expressed as percentages of cells in the G0+G1, S and G2/M phases of the cell cycle. Statistical analysis was carried out by the Student's *t*-test by comparing with control values [a]$P \leq 0.05$ and [b]$P \leq 0.01$ or with values after IGF-I treatment [c]$P \leq 0.05$ and [d]$P \leq 0.01$.

Cells lost cellular contacts, presented cellular blebbing and finally, they detached from the plate (data not shown). All these changes in the cellular morphology resembled those occurring in an apoptotic cell death process. To assess whether TNF-α treated brown adipocytes were dying by a process of apoptosis, their DNA content by flow cytometry after staining with propidium iodide was first analyzed. Peaks of cells presenting a DNA content lower than 2C after 48 h are shown in Fig. 1A, labeled by the M1 bars. Cells treated with TNF-α (10 ng/ml) for 48 h showed 20–23% of cells with DNA content lower than 2C, while untreated cells only showed 5–9%. These data pointed out that a percentage of TNF-α treated cells, higher than in control cells, could be dying by a process of apoptosis. Previously, a complete dose-response study was carried out using this cytometric technique. TNF-α induced a dose-dependent (1–10 ng/ml) increase in the percentage of cells with DNA content lower than 2C, reaching maximal levels with 5–10 ng/ml (data not shown). Thus, we used 10 ng/ml for further analysis. A complete time-course study was also done and a gradual increase in the number of cells with DNA content lower than 2C was produced in a time-dependent manner, being 15% at 24 h and 22% at 48 h.

To confirm that TNF-α was inducing a process of apoptosis, we used some other methods: analysis of the presence of fragmented DNA in the cytoplasmic fraction by electrophoresis on agarose gels, the presence of apoptotic nuclei by confocal microscopy and the presence of phosphatidylserine residues in the outer plasma membrane by flow cytometry after binding to annexin-V and staining with propidium iodide [26,27].

The study of the extranuclear DNA in cells untreated or treated with TNF-α for 48 h revealed an induction of DNA fragmentation by the TNF-α treatment as shown in Fig. 1B. DNA from TNF-α treated cells presented different low molecular weight DNA fragments like a 'ladder' that were not present in control cells. The presence of these DNA fragments is usually associated with the cleavage of genomic DNA occurring in cells undergoing late apoptosis.

The analysis of nuclei by confocal microscopy after staining with propidium iodide and SYTO 13 showed the presence of a higher number of condensed and/or fragmented nuclei in cells treated with TNF-α for 48 h than in control cells as it occurs in apoptotic cells. In Fig. 1C, a confocal image of nuclei from TNF-α treated cells is shown, where apoptotic bodies and fragmented nuclei, generated by DNA cleavage, are clearly visible.

Phosphatidylserine is only present in the inner layer of the plasma membrane in normal living cells, while in necrotic and apoptotic cells it is also present in the outer layer and can be bound to annexin-V. Necrotic cells have a plasma membrane totally permeable to propidium iodide, meanwhile apoptotic cells are less permeable, even negative. Thus, using this flow cytometry multiparameter assay, we can distinguish between the necrotic cells that are positive for annexin-V and propidium iodide and the apoptotic cells that are positive for annexin-V and negative or low positive for propidium iodide. TNF-α treated brown adipocytes showed a higher percentage of annexin-V positive cells than control brown adipocytes (Fig. 1D). All these evidences listed before demonstrated a TNF-α-induced apoptosis occurring in brown adipocytes. TNF-α is also known to induce apoptosis in other cell types [6–10], though in most of the cases, an inhibition of protein synthesis is required. In our cell system, this was not necessary.

Since we have already demonstrated that TNF-α induces apoptosis and inhibits cell proliferation (basal and IGF-I-induced) in rat fetal brown adipocytes in primary culture, TNF-α might be blocking the progression through the cell cycle of a percentage of cells and as a consequence these cells would be committed to apoptosis. Thus, cells treated with TNF-α would be arrested in G0/G1, would not be able to enter into the cell cycle and would undergo apoptosis. To prove this hypothesis, we measured simultaneously by flow cytometry DNA content and expression of PCNA, the δ subunit of DNA polymerase, that is maximally expressed during the S phase. This could allow us to know whether PCNA is expressed in cells which DNA content was lower than 2C, that were demonstrated to be apoptotic.

Table 2 shows the total percentage of cells with DNA content lower than 2C (apoptotic cells) and the percentage of these cells detected either as positive (PCNA positive) or negative (PCNA negative) for PCNA expression based on their ability to bind to an anti-PCNA antibody. These data were derived from the cytometric dual parameter analysis of DNA and PCNA contents carried out as shown in Fig. 2 for control and TNF-α treated cells. Under control conditions, the percentages of apoptotic cells PCNA positive and PCNA negative were similar (Table 2). IGF-I treated cells showed no changes in relation to control values. However, TNF-α treated cells presented an increase in the percentage of apoptotic cells, the percentage of these cells that were PCNA negative being much higher. Thus, these results show that the majority of TNF-α treated cells committed to programmed cell death do not express PCNA, and, therefore, they are

Table 2
TNF-α induces apoptosis in PCNA negative brown adipocytes

	Percentage of cells with DNA content lower than 2C		
Treatment	PCNA negative	PCNA positive	Total
Control	3.64 ± 1.09	3.43 ± 0.26	7.07 ± 1.07
TNF-α	13.49 ± 3.49[a]	7.41 ± 2.40	20.14 ± 2.7[b]
IGF-I	3.86 ± 0.47	2.78 ± 0.44	6.64 ± 0.55
IGF-I+TNF-α	8.10 ± 1.60[c]	5.41 ± 1.86	13.51 ± 3.4[d]

Flow cytometric DNA and PCNA content quantitations analyzed as shown in Fig. 2. Serum starved brown adipocytes were maintained for 48 h untreated (control) or treated either with TNF-α, IGF-I or IGF-I+TNF-α, before the analysis. Results are expressed as the percentage of cells with DNA content lower than 2C (apoptotic cells) with no detectable PCNA expression (PCNA negative) or detectable PCNA expression (PCNA positive) as estimated by their binding to an anti-PCNA antibody. Results are means ± S.E.M. of three separate experiments. Statistical analysis was carried out by the Student's *t*-test by comparing with control values [a]$P \leq 0.05$, [b]$P \leq 0.01$ or with values after TNF-α treatment [c]$P \leq 0.05$, [d]$P \leq 0.01$.

not proliferating. This effect of TNF-α was partially abolished when IGF-I was also present. Therefore, these results indicate that TNF-α inhibits proliferation of brown adipocytes and as a consequence, the progression through the cell cycle of a certain number of cells is blocked and these cells undergo apoptosis.

3.3. *IGF-I but not EGF prevents the TNF-α-induced apoptosis in rat fetal brown adipocytes maintained in primary culture*

IGF-I is a potent mitogen for brown adipocytes that also induces their differentiation [18]. EGF has also a mitogenic effect on brown adipocytes. Since in many cell types, apoptosis is prevented by the presence of mitogens that allow cells to progress through the cell cycle, we checked for this possibility in brown adipocytes.

Once we have demonstrated by several techniques that brown adipocytes suffered TNF-α-induced apoptosis and that cells with DNA content lower than 2C were actually dying by a process of apoptosis, we used the flow cytometric analysis of the cell cycle to quantitate apoptotic percentages and to assess the effect of IGF-I and EGF on TNF-α-induced apoptosis in rat fetal brown adipocytes.

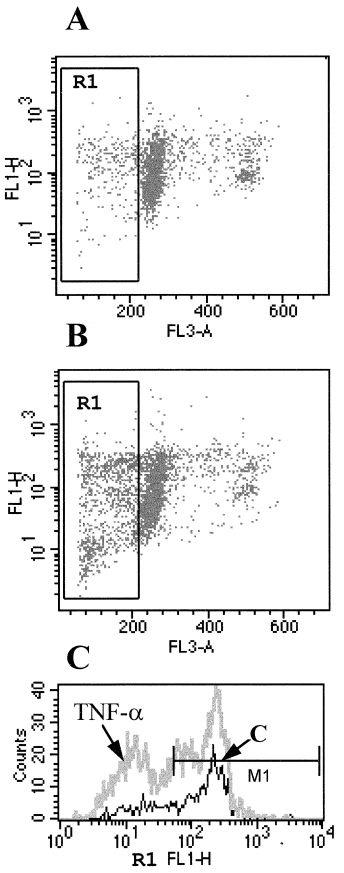

Fig. 2. TNF-α induces apoptosis in PCNA negative brown adipocytes. A and B: Representative flow cytometric dual parameter analysis of DNA and PCNA contents in brown adipocytes untreated (A) and treated with 10 ng/ml TNF-α (B) for 48 h. DNA (FL3-A, abcises) and PCNA (FL1-H, ordinates) contents were simoultaneously analyzed by flow cytometry after cells were stained with propidium iodide and labeled with an anti-PCNA antibody as described under Section 2. C: Representative PCNA histograms from cells inside R1 area, from A and B histograms. These cells have a DNA content lower than 2C. M1 bar represents PCNA positive cells.

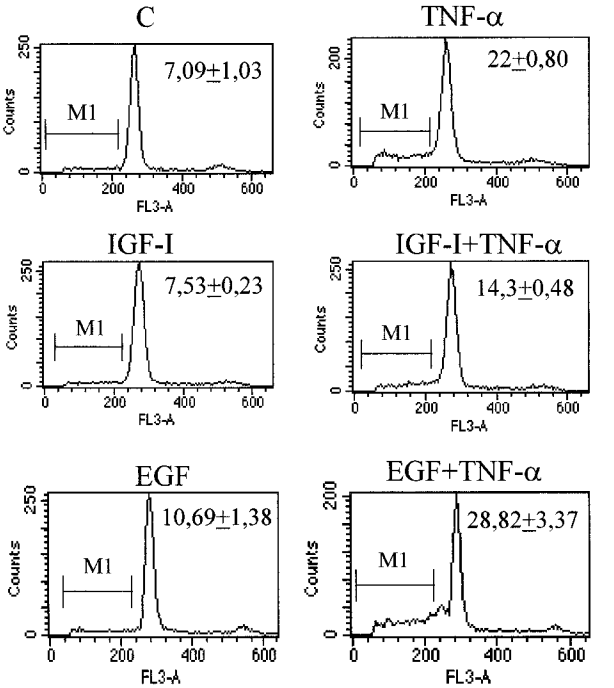

Fig. 3. IGF-I but not EGF prevents TNF-α-induced decrease in DNA content in brown adipocytes. DNA content histograms from flow cytometric measurements in brown adipocytes untreated (C) or treated for 48 h with TNF-α, IGF-I, IGF-I+TNF-α, EGF or EGF+TNF-α carried out as described under Section 2. M1 bar represents the percentage of cells with a DNA content lower than 2C. This percentage was determined and mean values ± S.E.M. of three separate experiments are represented in each histogram.

As it is shown in Fig. 3, brown adipocytes treated with TNF-α for 48 h presented 20–23% of cells with DNA content lower than 2C, in contrast to the 5–9% observed in control cells. IGF-I treated cells presented similar values to those from control cells (6–8%). When cells were treated with IGF-I and TNF-α, the percentage of cells with DNA content lower than 2C was significantly diminished to 12–15% when compared to TNF-α treated cells ($P < 0.01$). In contrast, EGF was not able to decrease the percentage of cells with DNA content lower than 2C or even increased it up to 22–36% (EGF+TNF-α). EGF treated cells also presented a higher value of apoptotic cells than control cells (8–14%).

Based on these results, IGF-I prevented the apoptotic cell death induced by TNF-α in fetal brown adipocytes in a specific way, since EGF was not able to do it or even enhanced it. Similarly, IGF-I prevented the TNF-α-induced apoptosis in p6 and BALB/c 3T3 cells [9], while PDGF or EGF did not. IGF-I also maintained the viability of different cell types [28–31], where the process of apoptosis was induced by other means. However, IGF-I did not block the apoptosis process in all the cells at 48 h as it has been observed for other survival factors in other cell types [23,29].

The different effects of IGF-I and EGF on the TNF-α-induced apoptosis in brown adipocytes could be explained by the activation of different signaling pathways by these two growth factors. Thus, it is likely to think that though some of the signaling pathways may be common to both signals,

some others may be different [29] and the overall balance of them might determine the putative antiapoptotic effect.

In summary, we have described here for the first time, a process of apoptosis occurring in brown adipocytes maintained in primary culture. This process is induced by TNF-α and is prevented by IGF-I, but not by EGF. Thus, TNF-α might be a mechanism to eliminate brown adipocytes during development by the induction of apoptosis. IGF-I, which induces cell growth and differentiation in rat fetal brown adipocytes [18], would maintain cell viability under these circumstances.

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