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Tumor necrosis factor-α induces apoptosis in rat brown adipocytes

Enzo Nisoli[1,2], Luca Briscini[1], Cristina Tonello[1], Carlo De Giuli-Morghen[1] and Michele O. Carruba[1]

[1] Centro di Studio e Ricerca sull'Obesità, Department of Pharmacology, Chemotherapy and Medical Toxicology, LITA Ospedale L. Sacco, School of Medicine, Milan University, 20157 Milan, Italy

[2] corresponding author: Dr. Enzo Nisoli, Centro di Studioe Ricerca sull'Obesità, Dipartimento di Farmacologia, Chemioterapia e Tossicologia Medica, Via Vanvitelli, 32, 20129 – Milano (Italy). tel: 0039(2)70146370; fax: 0039(2)70146371; E-mail: csro@imiucca.csi.unimi.it

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Abstract

Accumulating evidence demonstrates that adipose tissue is a major site of tumor necrosis factor-α (TNF-α) gene expression, which is markedly high in obese animals and may contribute to obesity-linked insulin resistance. We now report that recombinant murine TNF-α triggers the apoptotic degeneration of brown adipocytes differentiated in culture. Moreover, noradrenaline, which has been described as having trophic effects on brown fat and accelerating the differentiation of brown adipocytes, is capable of dose-dependently preventing the TNF-α-induced apoptosis of brown fat cells. Since obesity is characterized by greatly increased TNF-α production and reduced catecholaminergic activity, apoptosis was studied in the brown fat of genetically obese animals. *In situ* DNA fragmentation analysis revealed a larger number of apoptotic cells in the brown fat of obese (*fa/fa*) than in that of lean (+/+) Zucker rats. The exposure of obese rats to low temperatures for 7 days, which increases the sympathetic activity of brown adipose tissue, significantly reduces the number of apoptotic brown adipocytes. We hypothesize that TNF-α may play a significant role in the control of brown fat homeostasis.

Keywords: apoptosis, tumor necrosis factor-α, brown adipose tissue, noradrenaline, uncoupling protein, polymerase chain reaction

Abbreviations: BAT, brown adipose tissue; TNF-α, tumor necrosis factor-α; UCP, uncoupling protein; PCR, polymerase chain reaction; MTT, 3-[4,5-dimethylthiazol-2-yl-]-2,5-diphenyl tetrazolium bromide; CHX, cycloheximide

Introduction

Recent studies have provided the first indications that tumor necrosis factor-α (TNF-α) is the only cytokine that is overproduced in the white adipose tissue of obese subjects (Hotamisligil *et al*, 1993, 1995; Hofmann *et al*, 1994); several years ago, it was demonstrated to be a potent inhibitor of differentiation, and a potent stimulant of lipid metabolism in 3T3-L1 cells and in white adipocytes in primary culture (Kawakami and Cerami, 1981; Torti *et al*, 1985; Kawakami *et al*, 1989). However no conclusive results have yet been reported about the effects of TNF-α on brown fat cells. Noradrenaline physiologically stimulates brown adipocytes and induces the expression and function of the uncoupling protein (UCP), a mitochondrial protein that is selectively expressed in these cells, and which short circuits the oxidative phosphorylation pathway with energy expenditure as heat (see Lowell, 1996).

Obesity, which is the result of an imbalance between caloric intake and energy expenditure, is associated with the brown fat atrophy and functional deficiency that leads to decreased energy expenditure (Trayhurn, 1986). The critical role of BAT in obesity has recently been conclusively demonstrated. Mice, in which the UCP promoter drives BAT-specific expression of a gene encoding the diphteria toxin A chain are characterized by reduced BAT, and are obese (Lowell *et al*, 1993; Hamann *et al*, 1996).

TNF-α is a pluripotent cytokine that appears to elicit a large number of biological effects in different cell systems, including the induction of apoptosis in non-transformed cells (Beutler and Cerami, 1989). The aim of this study was to investigate whether TNF-α was able to induce apoptosis in brown adipocytes, in an attempt to understand the molecular basis of obesity-linked BAT functional atrophy. Several studies have demonstrated that the cytocidal action of TNF-α is controlled by a balance between two opposing TNF-α effects: the activation of protein-synthesis-independent mechanisms, and the induction of the synthesis of proteins capable of blocking the cytotoxic mechanisms. Protein synthesis blocking agents, such as cycloheximide (CHX), have been described as markedly sensitizing cells to TNF-α cytotoxicity, probably by suppressing the synthesis or activity of protective proteins, and thus 'blocking the blockade' of TNF-α-induced cytotoxic activity (see Wallach, 1997). In order to analyse the potentially apoptotic role of TNF-α in brown fat cells, we studied its effects on cells differentiated in culture and exposed to a concentration of CHX that is insufficient *per se* to induce apoptosis.

Our findings demonstrate that: (i) TNF-α induces the apoptosis of differentiated brown fat cells in a concentration-dependent manner; (ii) this cytotoxic effect is probably mediated mainly by the p55 TNF-α receptor subtype; (iii) noradrenaline protects brown adipocytes against TNF-α-induced apoptosis; (iv) a larger number of apoptotic brown fat cells are present in genetically obese than in lean rats; and (v) long-term exposure of obese animals to low temperature can diminish this number of apoptotic cells.

We suggest that these mechanisms may be involved in the obesity-linked atrophy of brown fat that leads to the decreased energy expenditure typical of this clinical condition.

Results

TNF-α induces apoptosis in mature brown adipocytes

10–100 pM TNF-α applied for 5–7 days to differentiated brown adipocytes (fresh TNF-α was added every other day with replacement medium) reduced survival by 55±4% in comparison with untreated cells, as determined by means of the 3-[4,5-dimethylthiazol-2-yl-]-2,5-diphenyl tetrazolium bromide (MTT) dye-reduction assay. Furthermore, the treatment induced intranucleosomal DNA fragmentation in the differentiated brown fat cells (350±32% more than in the untreated cells), as measured by means of the photometric enzyme immunoassay for the *in vitro* determination of cytoplasmic histone-associated DNA fragments (Boehringer, Mannheim, Germany). We thus concluded that the prolonged exposure of brown adipocytes to TNF-α can induce features reminiscent of apoptosis.

The differentiated brown fat cells simultaneously treated with TNF-α and CHX died within a few hours; CHX has previously been shown to enhance TNF-α-mediated cytotoxicity in other cultured cells (Kull and Cuatrecasas, 1981; Reid *et al*, 1989, 1991).

In order to determine the time required for this cytotoxicity, confluent brown adipocytes were placed in 96-well plates and treated with 10 pM TNF-α alone or in combination with 10 μg/ml CHX for 0.5–6 h. The results measured by means of MTT staining indicated that significant cell loss occurred 4–6 h after incubation with both of the agents together, and appreciable cytotoxicity was obtained after 0.5–2.0 h of treatment (Figure 1). TNF-α alone was not toxic to these cells in this time range, and the concentration of CHX used led to a minimal reduction in cell number over the 6 h pulse. Even after 24 h of treatment with CHX, the survival of the brown adipocytes was 60–70% that of controls. On the basis of these results, TNF-α (0, 0.1, 1, 10 and 100 pM) and CHX (0, 1, 10 and 100 μg/ml) were titered in order to determine the optimal concentration for TNF-α-mediated brown adipocyte killing in the presence of CHX. After 2 h of treatment, the optimal concentration of CHX was 10 μg/ml. At this dose, the cytotoxicity due to CHX alone was minimal, whereas the addition of as little as 1 pM TNF-α led to nearly 50% cytotoxicity (data not shown).

Under these experimental conditions, the confluent brown fat cells degenerated asynchronously, and had a small, condensed and irregularly-shaped appearance at phase-contrast microscopy (Figure 2C). The presence of typical apoptotic nuclei was demonstrated by using Hoechst 33258, as previously described (Oberhammer *et al*, 1992); the nuclei appeared to be slightly smaller, fragmented and had a brighter fluorescence than the nuclei of the untreated cells (F in Figure 2C and D).

In order to confirm that TNF-α plus CHX induced degeneration through an apoptotic pathway, transmission

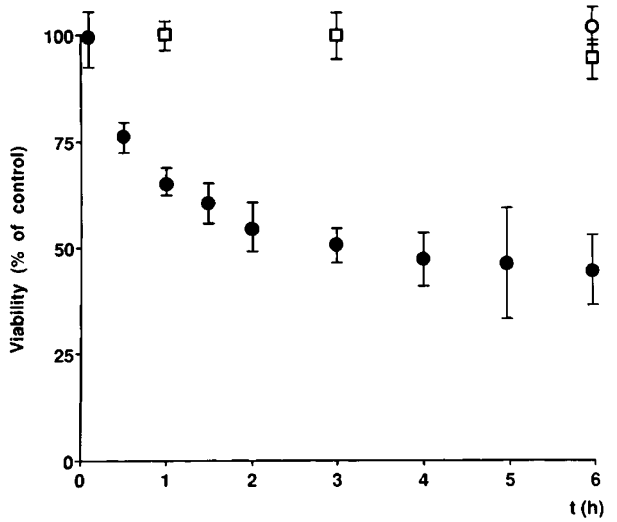

Figure 1 Percent survival of brown adipocytes differentiated in culture after exposure to TNF-α (10 pM) plus CHX (10 μg/ml). Viability was quantified using an MTT dye-reduction assay and is expressed as a percentage of control values. The error bars represent standard deviation (●), TNF-α+CHX; (○), TNF-α alone; (□), CHX, alone

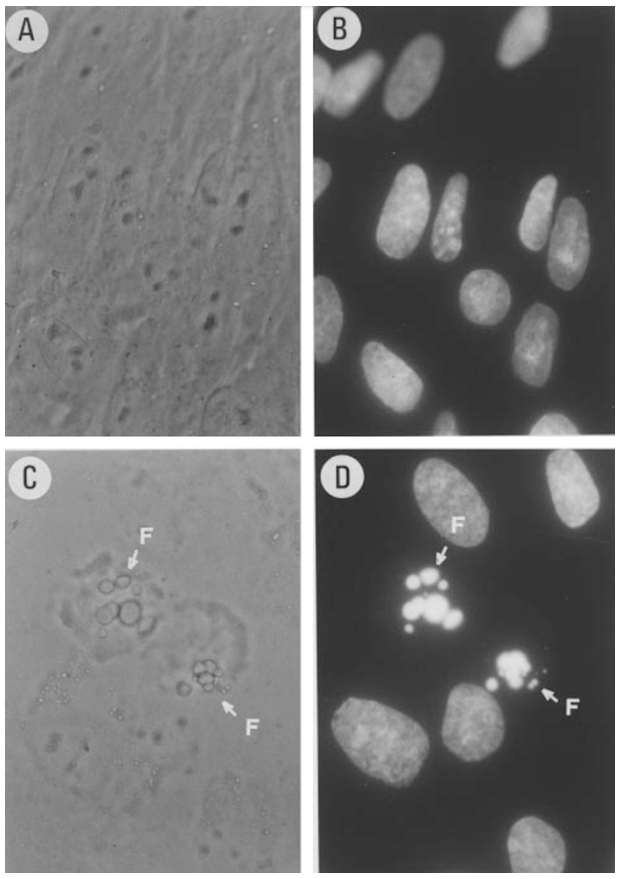

Figure 2 Treatment with TNF-α plus CHX induces the death of cultured brown adipocytes as shown by phase-contrast microscopy (**A** and **C**) and Hoechst 33258 apoptotic dye staining (**B** and **D**). **A** and **B**: untreated cells; **C** and **D**: TNF-α (10 pM) plus CHX (10 μg/ml) for 2 h. Chromatin condensation was visualized by Hoechst 33258. F, fragmented apoptotic nucleus (white arrow). Magnification, ×900

electron microscopy was used to determine whether the treated adipocytes also showed ultrastructural changes consistent with apoptosis. Transmission electron microscopy of TNF-α (10 pM) plus CHX (10 μg/ml) treated cells revealed compact patches of condensed nuclear chromatin (Figure 3B). The cells were observed to be in various stages of degeneration, which is consistent with the asynchronism shown by phase-contrast microscopy. The cytoplasm was generally depleted of lipid droplets, and showed mitochondria and organelle breakdown. The adipocytes in untreated cultures has a normal ultrastructure (Figure 3A). Treatment with TNF-α plus CHX therefore induces ultrastructural changes in cultured brown adipocytes that are characteristic of apoptosis.

A definitive biochemical feature of many cell types undergoing apoptosis is fragmentation of DNA into oligonucleosome-length fragments (Wyllie *et al*, 1980). Figure 4A shows the results obtained using the Boehringer photometric enzyme immunoassay after the cells were exposed to various doses of TNF-α plus CHX (10 μg/ml) for 2 h; furthermore, DNA was extracted from these treated cells and analyzed by means of agarose gel electrophoresis. Two hours of incubation with TNF-α plus CHX induced a typical DNA fragmentation in a dose-dependent manner (Figure 4B), which paralleled the dose-response curve of the nucleosome enrichment in brown adipocyte cytoplasm obtained using the Boehringer assay (Figure 4A). Neither TNF-α nor CHX alone induced DNA fragmentation.

Two distinct types of TNF-α receptors have been identified and molecularly cloned in both humans and rodents: p55 TNF-α receptor (also referred to as TNFR1, TNFRβ and CD 120a) and p75 TNF-α receptor (also called TNFR2, TNFRα and CD 120b), whose respective molecular mass is approximately 55 KDa and 75 KDa (see Lewis *et al*, 1991; Vandenabelle *et al*, 1995). Remarkably, human TNF-α only interacts with the p55 and not with the p75 rodent TNF-α receptor subtype (Lewis *et al*, 1991). Lane 2 of Figure 4B shows that, under our experimental conditions, human TNF-α (10 pM) plux CHX (10 μg/ml) induced DNA fragmentation in brown adipocytes, thus suggesting that the TNF-α-induced apoptosis of brown adipocytes is mediated through the p55 TNF-α receptor subtype.

Noradrenaline inhibits the TNF-α-induced apoptosis of brown adipocytes

It is well known that noradrenaline physiologically accelerates the differentiation of brown adipocytes in culture (Néchad *et al*, 1987), and that it plays a trophic role in *in situ* BAT (Girardier and Seydoux, 1986). Our results suggest that these effects of noradrenaline may also be due to a counteraction against the TNF-α-induced programmed cell death of brown adipocytes. Figure 5 shows that the exposure of mature cells to different doses of noradrenaline for 24 h, before a challenge dose of 2 h TNF-α (10 pM) plus CHX (10 μg/ml), increased the number of viable cells and correspondingly decreased the number of cells undergoing apoptosis.

Taken together, the present findings thus demonstrate for the first time that TNF-α stimulates apoptotic processes in brown adipocytes differentiated in culture, and that noradrenaline antagonizes them.

In situ DNA fragmentation in the BAT of obese rats

To verify that our *in vitro* results also have some relevance *in vivo*, TUNEL staining was used for the *in situ* detection of apoptotic cells in BAT sections obtained from 5-week-old obese Zucker (*fa/fa*) rats and their lean littermates (+/+). It is known that obesity is characterized by increased TNF-α production in both white and brown fat (Nisoli *et al*, unpublished results), and by decreased sympathetic nervous system activity (Himms-Hagen, 1985). Figure 6 shows that

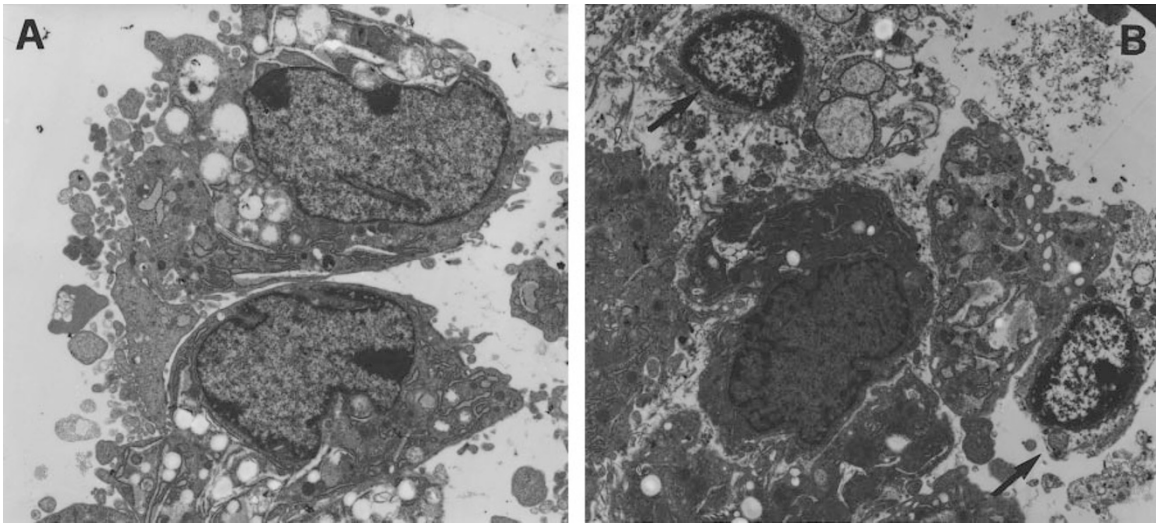

Figure 3 Transmission electron microscopy of brown adipocytes differentiated in culture. (**A**) Untreated brown fat cells showing well-preserved organelles, abundant cytoplasm, and lipid droplets; the nuclei contain dispersed chromatin. (**B**) Brown adipocyte culture after 6 h of treatment with TNF-α (10 pM) plus CHX (10 μg/ml), showing nuclear changes (arrows). Patches of condensed chromatin lie against the nuclear membrane. The lipid droplets have disappeared and the cytoplasm is condensed. Magnification, ×3900

there were more positive nuclei for DNA fragmentation in the BAT of the obese (D) than in that of the lean control rats (B). It is worth noting that the nuclei of the brown adipocytes taken from the obese animals were, as expected, less abundant and more flattened to the cell membrane than those taken from the lean animals (C, A). These characteristics markedly increased with age (data not shown), thus demonstrating that, as obesity develops, brown adipocytes undergo morphological changes that are more reminiscent of mature white adipocytes, and some of them undergo apoptotic degeneration. It is also worth noting that exposure of the obese rats to cold for 7 days, which increases BAT sympathetic activity (Girardier and Seydoux, 1986) without significantly affecting tissue TNF-α levels (data not shown), significantly decreased the number of apoptotic brown adipocytes in comparison with that observed in obese rats kept in an environment at room temperature (Figure 6E and F). Furthermore, BAT weight increased in the animals exposed to the cold in comparison with that of the animals acclimatized to room temperature, in which the tissue was atrophied and replaced by a tissue that was morphologically similar to white fat.

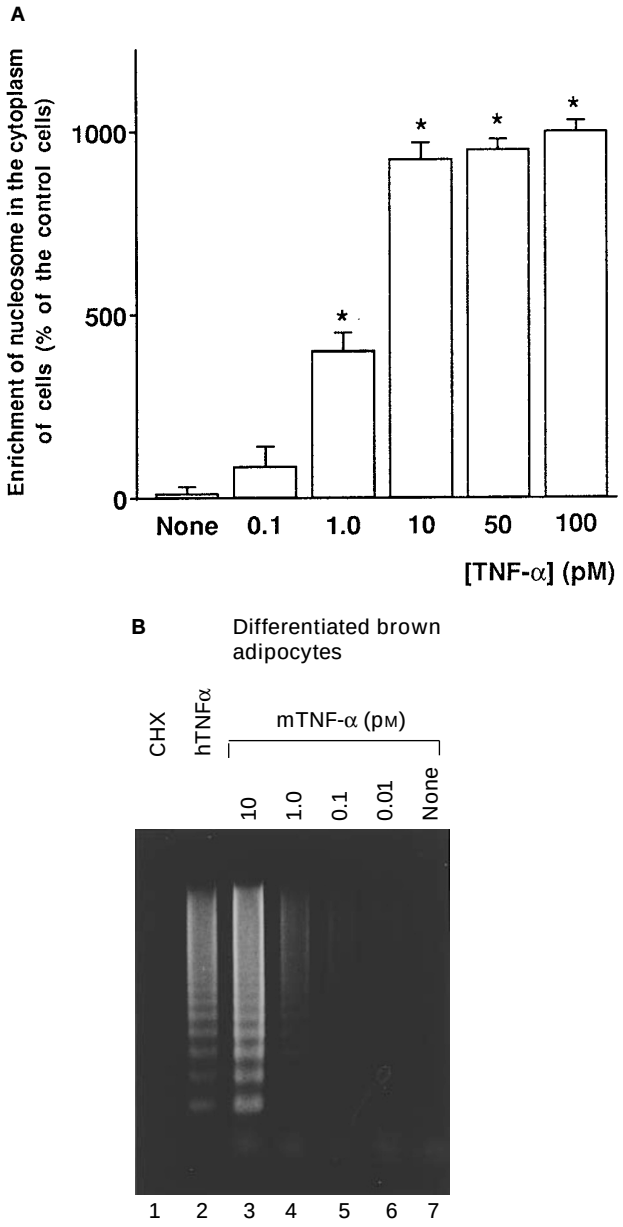

Figure 4 Treatment with TNF-α plus CHX induces internucleosomal fragmentation of genomic DNA in cells exposed to various doses of TNF-α plus CHX (10 μg/ml) for 2 h. (**A**) Photometric enzymatic determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) (Boehringer, Mannheim, Germany). (**B**) Agarose gel electrophoresis of DNA fragmentation. Lane 1, CHX alone; lane 2, recombinant human TNF-α (10 pM) plus CHX; lanes 3–6, various doses of TNF-α plus CHX; lane 7, control. The gel shown is representative of the data obtained in six independent experiments. *$P<0.01$ *vs* control cells (analysis of variance using Newman-Keuls multiple-comparions posthoc test)

Discussion

It has been shown that various stimuli can lead to an increase in the number of brown adipocytes, but very little is known about the processes that lead to a decrease in brown fat cell mass. In other tissues, stimuli such as changes in the hormonal or cytokine milieu cause a rapid involution as a result of apoptosis or reduced cell renewal. We investigated whether apoptotic processes may be involved in the pathophysiological involution of brown adipocyte mass that occurs in obesity. Using different experimental approaches, we found that TNF-α concentrations (10 pM=170 pg/ml) comparable with those present in the plasma of obese animals (ranging from 34–165.6 pg/ml, with a mean ± S.E. of 85.6 ± 10.0 pg/ml) (Hotamisligil *et al*, 1993) trigger the degeneration of brown adipocytes differentiated in culture by means of apoptosis. As has previously been described for other cell lines (Kull and Cuatrecasas, 1981; Reid *et al*, 1989, 1991), the apoptotic effect of TNF-α on brown adipocytes was enhanced by concurrent treatment with CHX, although why this should potentiate TNF-α cytotoxicity in non-transformed cells is unclear. TNF-α is a pleiotrophic cytokine that may induce more than one response in a single cell line, and the presence of CHX may inhibit the synthesis of certain signaling molecules and thus potentiate TNF-α killing activity; alternatively, it may simply inhibit the synthesis of one or more general survival factors and thus make cells more sensitive to TNF-α cytotoxicity. Since brown fat would not be exposed to CHX in intact animals, any extrapolation of *in vitro* findings obtained using CHX to the *in vivo* situation must obviously be made very cautiously. Nevertheless, under our experimental conditions, we found that noradrenaline dose-dependently prevents the TNF-α/CHX-induced apoptosis of brown fat cells, and this result was paralleled by two relevant findings obtained *in vivo*. Since obesity is characterized by a marked increase in TNF-α production and a reduction in catecholaminergic activity, the described results suggested that the brown fat of genetically obese animals should be more apoptotic than the BAT of their lean counterparts, and our *in situ* DNA fragmentation analysis confirmed that there is indeed a larger number of apoptotic cells in the brown fat of obese (*fa/fa*) than in that of lean (+/+) Zucker rats. Furthermore, the chronic exposure of obese rats to the cold, which increases BAT sympathetic activity, significantly reduced the number of apoptotic cells. Our suggestion is that, in pathophysiological

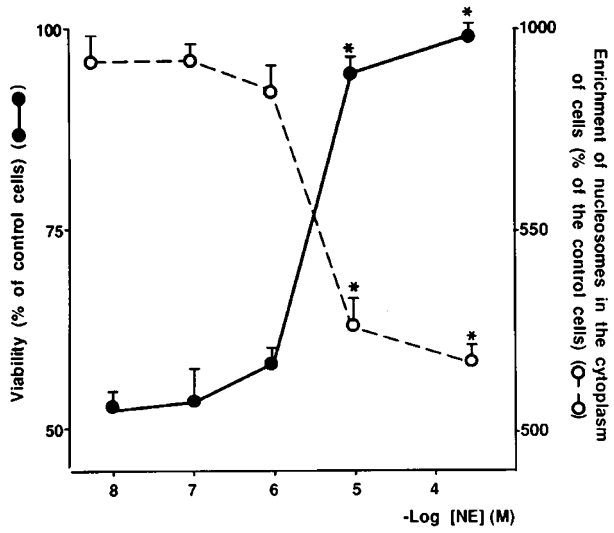

Figure 5 Noradrenaline pretreatment protects differentiated brown adipocytes from TNF-α plus CHX-induced apoptosis. Brown fat cells were pretreated with different concentrations of noradrenaline for 24 h, and incubated with TNF-α (10 pM) plus CHX (10 μg/ml) for 2 h. The results are expressed in terms of cell viability (quantified by MTT dye-reduction assay and expressed as percentage of control values) and as cell cytoplasm nucleosome enrichment (quantified by photometric enzyme immunoassay). The error bars represent standard deviation. *$P<0.001$ *vs* TNF-α plus CHX-treated cells (analysis of variance using Newman-Keuls multiple-comparisons posthoc test)

conditions such as obesity, the balance between the cytotoxic and protective mechanisms of TNF-α is shifted towards changes that are capable of inducing apoptosis *in vivo* even in the absence of a protein-synthesis inhibitor, such as CHX.

Taken together, our results provide evidence that TNF-α is able to control BAT homeostasis by inducing the programmed cell death of brown adipocytes, which suggests a putative mechanism for the occurrence of BAT atrophy in obese rats. It is known that the genetic ablation of brown fat in transgenic mice markedly enhances susceptibility to obesity and diabetes (Lowell *et al*, 1993; Hamann *et al*, 1996), which conclusively supports the critical role of BAT in fat accumulation and glucose homeostasis. Furthermore, it has recently been reported that the amount of TNF-α mRNA, which is expressed in the white adipose tissues of uninfected animals, is 5–10 times greater in certain obese strains than in lean controls (Hotamisligil *et al*, 1993). The local and systemic level of TNF-α protein are also high, although circulating levels are low or undetectable in humans (Hotamisligil *et al*, 1993). Analysis of a number of animal models with a variety of metabolic disturbances indicates that TNF-α overexpression in white fat best correlates with massive obesity and insulin resistance; TNF-β, IL-1α, IL-1β, and IL-6 are not expressed in fat tissue nor regulated in any other organ in obesity (Hotamisligil *et al*, 1993). In addition to the data reported by Spiegelman's group (Hotamisligil *et al*, 1993, 1995, 1996; Hofmann *et al*, 1994), several other indirect lines of evidence, support the hypothesis that TNF-α may play a potentially important pathophysiological role in animal and human obesity and insulin resistance. Recent genetic studies using sibling pair analysis have shown a statistically significant link between a polymorphism in the TNF-α locus and body fat content and body mass index in Pima Indians (Bouchard and Pérusse, 1996), and so the apoptotic effect of TNF-α on brown adipocytes described in this study could represent an important pathophysiological mechanism in obesity and its morbid complications, such as insulin resistance, which is believed to be important in various pathological consequences of obesity, including hypertension, hyperlipidemia and, especially, non-insulin-dependent diabetes mellitus (Moller and Flier, 1992).

It is reasonable to speculate that the TNF-α produced by white and brown adipose cells may represent a self-limiting mechanism designed to prevent further accumulation of fat stores at different molecular levels. TNF-α has been reported to stimulate lipolysis (Green *et al*, 1994) and decrease the activity of adipose tissue lipoprotein lipase (Kawakami and Cerami, 1981), thus decreasing the ability of white fat to accumulate fatty acids. In addition, it may contribute towards limiting the increase in fat stores by means of a centrally-mediated reduction of food intake and increased BAT activity (Coombes *et al*, 1987). However, TNF-α is also known to reduce the expression of the glucose transporter (GLUT-4) in insulin-sensitive tissues (Hotamisligil *et al*, 1993), including BAT (Nisoli and Carruba, unpublised observation); and it can interfere with insulin action most probably through its ability to induce the serine-phosphorylation of insulin receptor substrate-1 (IRS-1) and the conversion of IRS-1 into an inhibitor of insulin receptor tyrosine kinase activity (Hotamisligil *et al*, 1996). As obesity advances, persistently high levels of TNF-α may therefore impair homeostatic control of BAT function by impairing glucose metabolism in brown adipocytes (Himms-Hagen, 1992). Concomitantly, the sympathetic activity that has trophic activity on BAT decreases (Bukowiecki *et al*, 1982; Girardier and Seydoux, 1986; Trayhurn, 1986), and this would blunt the noradrenaline counteraction of TNF-α-induced apoptosis and decrease the percentage of viable brown adipocytes, as described in the present study.

Although these data do not represent a direct demonstration that the increased production of TNF-α in obese rats is actually responsible for brown adipocyte apoptosis, and thus for BAT atrophy, they are at least suggestive of this hypothesis. Conclusive *in vivo* demonstration that obesity-linked BAT atrophy is actually due to the brown fat cell apoptosis induced by the overexpression of TNF-α in white and brown fat will require further studies. Nevertheless, our findings seem to suggest that an imbalance between apoptotic (i.e., TNF-α) and anti-apoptotic (i.e., noradrenaline) stimuli in BAT may contribute to BAT atrophy and obesity, and this has an impact on devising alternative pharmacological approaches to the treatment of obesity and related disorders.

Materials and Methods

Animals

For the animal experiments, 5-week-old male Zucker-*fa/fa*/Ola obese rats (*fa/fa*) and Zucker-+/+/Ola controls (+/+) were obtained from Charles River (Calco, Como, Italy). The animals were housed at 24°C

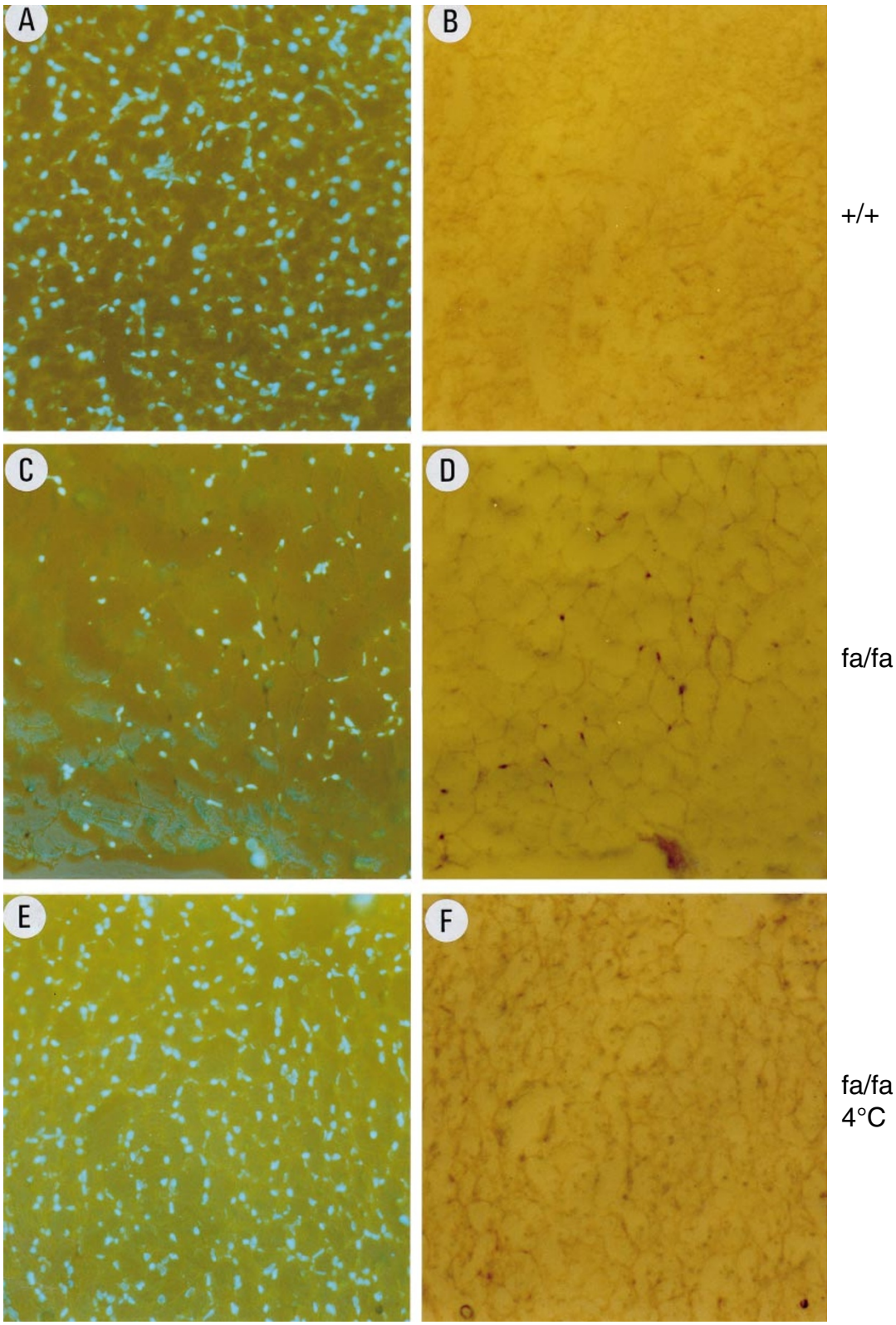

Figure 6 Hoechst 33258 (**A**, **C** and **E**) and TUNEL staining (**B**, **D** and **F**) of BAT slices from 5-week-old male Zucker-*fa/fa*/Ola obese rats (*fa/fa*) and Zucker-+/+/Ola controls (+/+) acclimatized to room or low (*fa/fa*, 4°C) temperatures. Hoechst 33258 shows the respectively round or flattened nuclei of the BAT cells of the lean and obese rats. Magnification, ×100

with a 7:00 p.m. – 7:00 a.m. dark cycle and killed by decapitation between 9:00 and 11:00 a.m. All of the animal experiments were conducted in accordance with the highest standards of humane animal care.

Brown adipocyte isolation

Brown fat precursor cells were isolated from young rats as previously described (Nisoli *et al*, 1996). The BAT fragments (12 – 15% w/v) were carefully dissected out under sterile conditions, and placed in a Hepes-buffered solution (pH 7.4), containing 0.2% (w/v) type II collagenase. After 30 min of enzyme treatment at 37°C, the tissue remnants were removed by filtration through a 250-μm nylon screen, and the mature adipocytes were then allowed to float to the surface (30 min on ice). The infranatant containing adipocyte precursor cells was then collected, filtered through a 25-μm nylon screen, pelleted by centrifugation for 10 min at $700 \times g$ in 10 ml of culture medium, and diluted to 20 ml.

Adipose cell culture and treatment

2.5 million cells were added to each 24-well culture plate (Nunclon Delta, Milan, Italy). The cells were cultured in 2.0 ml of a culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM glutamine, 10% newborn calf serum, 4 nM insulin, 10 mM Hepes, 50 IU of penicillin, 50 μg of streptomycin, and 25 μg of sodium ascorbate per ml (all from Flow Laboratories, Milan, Italy), at 37°C in a water-saturated atmosphere of 6% CO_2 in air. The medium was completely exchanged with fresh prewarmed medium on day 1 (when the cultures were first washed with 5 ml of prewarmed DMEM), and on days 3 and 9 (without washing). In the experiments analysing the effects of TNF-α on differentiated adipocytes on day 8, the cultured cells were exposed to murine or human recombinant TNF-α (freshly diluted in buffers; Genzyme, Cinisello Balsamo, Italy) with specific activities of 5×10^7 U/mg, and/or cycloheximide (CHX) (10 μg/ml) and/or NE for the times indicated in the figure legends and then harvested. In the long-term experiments, the cells were exposed to the drugs twice a day for different days. The cells were morphologically controlled every day.

Cytotoxicity assays

Cell viability was assessed using morphological criteria. In addition, 10^4 cells were seeded in flat-bottom 96-well plates and incubated in 100 μl of DMEM with 10% FCS to allow the cells to adhere. They were then incubated in medium with or without various concentrations of CHX and/or serial dilutions of murine recombinant TNF-α. After the reported times, 20 μl of tetrazolium bromide MTT (Sigma, Milan, Italy, 2.5 mg/ml in PBS) were added to the cultures and incubation was continued for a further 2 h to allow the metabolization of MTT to 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylformazan, which was solubilized with isopropanol-HCl (24 : 1) and colorimetrically determined at 540 nm in a microplate reader (TECAN SLT-Spectra, Cologno Monzese, Italy). Each condition was represented in three or four wells per experiment and repeated three or four times in independent experiments.

Electron microscopy

Brown adipocytes grown in a 25 cm^2 tissue culture flask and treated with 0.1 nM TNF-α plus 10 μg/ml CHX or vehicle for 6 h, were examined by means of transmission electron microscopy. The cells were postfixed in 1% buffered OsO_4, dehydrated in graded alcohols, and embedded in Epson (PolyBed 812). The sections were stained with uranyl acetate and lead citrate, and then examined using a CM 10 Philips microscope.

Analysis of DNA fragmentation

Confluent brown adipocytes were lysed in 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 0.5% lauryl sarcosyl containing 200 μg/ml of proteinase K, and incubated for 1 h at 55°C. The DNA was then precipitated overnight by means of ethanol precipitation, recovered by centrifugation, resuspended in water, and treated with RNase (50 μg/ml). The DNA (3 – 5 μg) was loaded on 1.2% agarose gels, which were stained with ethidium bromide after migration.

In vitro determinants of cytoplasmic histone-associated DNA fragments were also made using a photometric enzyme immunoassay from Boehringer (Mannheim, Germany), according to the manufacturer's protocol.

Measurement of apoptosis by Hoechst 33258 staining and TUNEL

Confluent brown fat cells were cultured and treated as described above. After removal of the medium, they were rinsed once with cold PBS (pH 7.2) and fixed for 10 min with 4% formaldehyde in PBS at 4°C, washed with distilled water and dried at room temperature. The nuclei were stained with Hoechst 33258 (5 μg/ml) for 5 min, washed and dried.

In situ fragmentation of DNA was investigated in BAT slices derived from control (+/+) or obese (*fa/fa*) Zucker rats using a kit (Boehringer, Mannheim, Germany) based on the direct alkaline phosphatase detection of digoxigenin-labeled 3′-OH ends of genomic DNA fragments. The kit was used according to the manufacturer's instructions. Photomicrographs were randomly obtained by a blinded observer using a Leika DMRB microscope.

Statistical methods

The raw data from each experiment was normalized, combined and analyzed using analysis of variance with Newman – Keuls multiple-comparisons posthoc test or the *t* test.

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