

# Bcl-2 and Bax are involved in the sympathetic protection of brown adipocytes from obesity-linked apoptosis

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**Abstract** Obesity is linked to functional brown adipose tissue (BAT) atrophy, partially due to adipocyte apoptosis. The brown adipocytes of obese rats have lower Bcl-2/Bax mRNA and protein ratios than those of their lean littermates. Exposure to a low temperature for three days markedly increased the Bcl-2/Bax ratio, by increasing the noradrenergic output to BAT, which has previously been shown to reduce apoptotic cell death. This effect could be mimicked *in vitro* by the addition of noradrenaline (NA) to brown adipocytes differentiated in culture. Micromolar NA concentrations increased the Bcl-2/Bax mRNA and protein ratios, and protected against serum deprivation-induced apoptosis. We conclude that NA acts by modulating *bcl-2* and *bax* gene expression.

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**Key words:** Bcl-2; Bax; Obesity; Sympathetic activity; Brown fat

## 1. Introduction

Brown adipose tissue (BAT) is a highly specialised tissue that grows and produces heat in response to the noradrenaline (NA) secreted by sympathetic nerves, particularly during cold exposure (non-shivering thermogenesis) or after eating (diet-induced thermogenesis) [1,2].

In genetically obese animals, brown fat is in an atrophied and thermogenically quiescent state related to a marked decrease in sympathetic activity [3]. We have recently demonstrated that the BAT of obese rats has a larger number of apoptotic brown adipocytes than that of their lean counterparts, and that the prolonged exposure of obese animals to the cold can prevent brown fat cells apoptosis [4]. In *in vitro* experiments, we have found that a combination of pmol concentrations of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and a protein synthesis inhibitor, such as cycloheximide, induced the apoptosis of brown adipocytes differentiated in culture, and that this was prevented by NA in a concentration- and time-dependent manner [4]. These findings suggest that obesity-linked TNF- $\alpha$  overexpression in white fat [5,6] and the impairment of sympathetic nervous activity may contribute to the brown fat functional atrophy typical of this condition [3]. Like many other growth factors in different cell systems NA may thus act as a survival factor, by preventing the onset of brown adipocyte apoptosis [4].

In the present study, we further analysed the mechanism(s) underlying the protective effect of NA on brown adipocyte

apoptosis. Several lines of evidence indicate that NA is able to modulate the balance between the survival and death of brown adipocytes [7,8], and one such molecular mechanism might be the modulation of *bcl-2* gene family proteins, which contain both antiapoptotic (such as Bcl-2) and proapoptotic members (such as Bax) [9]. The members of the Bcl-2 family interact with each other to form a dynamic equilibrium between homo- and heterodimers, and it is known that the ratio between Bcl-2 and Bax is one of the critical points in the prevention or induction of cell death [10,11]. We here describe: (i) *bcl-2* and *bax* gene expression in the brown fat of obese and lean rats; (ii) the effect of cold exposure on the Bcl-2/Bax gene product ratio in obese and lean animals; (iii) the *in vitro* effect of NA and TNF- $\alpha$  on the Bcl-2/Bax ratio in cultured brown adipocytes; (iv) NA protection against serum-starved-induced apoptosis of brown adipocytes; and (v) the NA-induced modifications of Bcl-2 and Bax in serum-starved cells.

## 2. Materials and methods

### 2.1. Animals

The 4- and 8-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 22°C with a 19:00–7:00 h dark cycle, and allowed free access to food and water for at least 10 days before being killed by decapitation between 9:00 and 11:00 h. All of the animal experiments were conducted in accordance with the highest standards of humane animal care.

### 2.2. Adipose cell isolation, culture and treatment

Brown fat precursor cells and mature adipocytes were isolated from the control rats (150–180 g body weight) as previously described [12]. Precursor adipocytes (2.5 million) were added to each 24-well culture plate (Nunc, Delta, Milan, Italy). The cells were grown in a culture medium consisting of Dulbecco's modified Eagle medium (DMEM), supplemented with 4 nM glutamine, 10% newborn calf serum, 4 nM insulin, 10 mM HEPES, 50 IU of penicillin, 50  $\mu$ g of streptomycin, and 25  $\mu$ g of sodium ascorbate per ml (all from Flow Laboratories, Milan, Italy), at 37°C in a water-saturated atmosphere of 8% CO<sub>2</sub> in air. The medium was completely exchanged with fresh pre-warmed medium on day 1 (when the cultures were first washed with 2 ml pre-warmed DMEM), and on days 3 and 7 (without wash). The confluent cells reminiscent of brown adipocytes were exposed to NA (freshly diluted in a buffer containing 0.1% ascorbic acid to prevent NA oxidation) or TNF- $\alpha$  for the reported times and then harvested. For the experiments performed in the absence of serum, the cells were serum starved for 8 h (serum concentration = 0), and then maintained in the absence or presence of different concentrations of NA for a further 6 h.

### 2.3. Reverse transcriptase-polymerase chain reaction assay

Total cytoplasmic RNA was isolated from  $1 \times 10^6$  cultured cells using the RNeasy method (TM Cinna Scientific, Friendswood, TX, USA). For PCR analysis, the RNA was treated for 1 h at 37°C with 6 U ribonuclease (RNase)-free deoxyribonuclease I/ $\mu$ g RNA in 100 mM Tris-HCl, pH 7.5, and 50 mM MgCl<sub>2</sub> in the presence of 2 U/

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**Abbreviations:** BAT, brown adipose tissue; NA, noradrenaline; TNF- $\alpha$ , tumour necrosis factor- $\alpha$

placental RNase inhibitor. The concentration of RNA was determined by absorbance at 260 nm, and its integrity was confirmed by means of electrophoresis on 1% agarose gels, and then stained with 0.1 µg/ml ethidium bromide. One microgram of total RNA was converted to complementary DNA (cDNA) using 200 U Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) in 20 µl of the buffer supplied by Promega, which contained 0.4 mM deoxy-NTP, 2 U/ml RNase inhibitor, and 0.8 µg oligo(deoxythymidine)<sub>15</sub> primer (Promega). A control experiment without reverse transcriptase was performed for each sample in order to verify that the amplification did not come from residual genomic DNA. An aliquot (5%) of the cDNA was amplified using the reverse (5'-AGAGGGGCTACGAGTGGGAT-3') and forward primer (5'-CTCAGTCATCCACAGGGCGA-3') to yield a 450 bp PCR product for Bcl-2 [13], and the reverse (5'-GGTTTCATCCAGGATC-GAGACGG-3') and forward primer (5'-ACAAAGATGGTCA-CGGTCTGCC-3') to yield a 429 bp PCR product for Bax [14]. In order to verify that equal amounts of cDNA were present in the PCR reactions, β-actin was separately coamplified with both the Bcl-2 and Bax cDNA, with the β-actin primers being added after ten amplification reaction cycles to avoid saturation [12]. For the Bcl-2 and Bax mRNA, the PCR conditions were as follows: denaturation at 94°C for 30 s, annealing at 61°C for 30 s and polymerisation at 72°C for 30 s with truncated *Thermus aquaticus* DNA polymerase (Promega). After 28 cycles, 5 µl of the PCR products were separated by electrophoresis and revealed by ethidium bromide staining.

The number of cycles for the semiquantitative RT-PCR assay and the reaction temperature conditions were estimated to be optimal to provide a linear relationship between the amount of input template and the amount of PCR product generated over a wide concentration range: from 0.5 to 10 µg of total RNA.

#### 2.4. Western blotting

The Bcl-2 and Bax proteins were detected by means of immunoblotting. The cells were scraped in a lysis buffer containing 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 5 µg/ml aprotinin. The suspension was then sonicated for 30 s at full power. The adipose tissue samples (100 mg) were cut into small pieces with a razor blade and resuspended in 3 vol lysis buffer, and then sonicated as described above. Protein content was determined by means of BCA protein assay (Pierce, Rockford, IL, USA). Equal amounts of protein (80–100 µg), diluted with an equal volume of 2-fold concentrated Laemli SDS sample buffer (50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) and boiled for 5 min, were run on 10% SDS-PAGE under reducing conditions. The separated proteins were then electrophoretically transferred to a nitrocellulose membrane (Protran, Schleicher and Schuell, Dassel, Germany). The amounts of the Bcl-2 and Bax proteins were determined by means of immunoblotting using specific polyclonal anti-Bcl-2 and anti-Bax antibodies (clone N-19 and clone N-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:1000 dilution for 2 h at room temperature. The immunostaining was detected using horseradish peroxidase-conjugated anti-rabbit immunoglobulin (1:5000) for 1 h at room temperature with a Super-Signal Substrate (Pierce, Rockford, IL, USA), and then densitometrically analysed.

#### 2.5. DNA fragmentation analysis

The 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 then incubated for 1 h at 55°C. The DNA was subsequently precipitated overnight with ethanol, recovered by means of centrifugation, resuspended in water, and treated with RNase (50 µg/ml). The DNA (3–5 µg) was loaded onto 1.2% agarose gels, which were stained with ethidium bromide after migration.

#### 2.6. Densitometric and data analysis

The RT-PCR products were electrophoresed on agarose gel, transferred to a nylon membrane and analysed by means of Southern blotting. The radiolabelled probes specific for each molecule were hybridised to the membrane, revealed, and semiquantitatively analysed by using QuickImage-D densitometer (Canberra Packard, Milan, Italy) and Phoretix 1D (version 3.0.1) software image analyser. To lower the detection limits of densitometric analysis measurements

were performed inside the linear response region of the image capture device (optical density range: 0 to 3.0). For this purpose, a standard was acquired contemporarily to the experimental images. Each experiment was densitometrically analysed at least four different times, the coefficient of analytical variation between the different analyses was lower than 5%. If the density of the β-actin band of a given sample deviated by >10% from the mean density of the samples in a gel then it was removed from the analysis. The Bcl-2 and Bax mRNA levels were expressed as the ratio of signal intensity for the target genes in relation to that for the coamplified β-actin, and the Bcl-2/Bax mRNA ratio was calculated. To normalise densitometer differences among experiments, data were corrected for background and expressed as a percentage of a specific reference value taken as one within each experiment as reported in the legend of the figures.

The data are reported as the mean values ± S.E.M. of three independent determinations. The comparisons were made using one-way ANOVA followed by Student-Newman-Keuls post-hoc comparisons; a *P* value of <0.05 vs. controls was considered significant.

### 3. Results

#### 3.1. Detection of Bcl-2 and Bax in different BAT preparations

The cultured rat brown fat precursor cells grew and divided rapidly. At confluence (8–10 days), they had a differentiated appearance reminiscent of mature brown fat cells. The expression of Bcl-2 and Bax mRNA was revealed by RT-PCR in both the preconfluent and confluent cells (Fig. 1A). Appropriate Bcl-2 and Bax internal standard and Southern hybridisation with specific probes, obtained by PCR amplification of cloned Bcl-2 and Bax gene with the specific primers, of nitrocellulose filters to which PCR gels were blotted, were used as control for the PCR assay (data not shown). The presence of the members of the Bcl-2 proto-oncogene family was confirmed by Western blot analysis: both the preconfluent and confluent cells contained protein species of approximately 29 kDa (Bcl-2) and 21 kDa (Bax) which respectively cross-re-

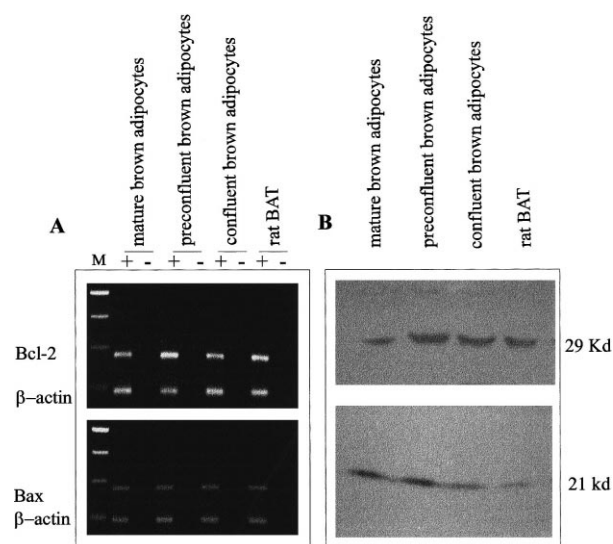


Fig. 1. RT-PCR (A) and Western blot (B) of RNAs and proteins isolated from mature brown adipocytes, preconfluent and confluent brown adipocytes, and rat BAT. A: Ethidium bromide agarose gel electrophoresis of Bcl-2 (upper) and Bax (lower) cDNA fragments amplified by RT-PCR from the various preparations. Each horizontal bracket delimits two lanes containing PCR samples obtained from RNA treated (+) or untreated (–) with reverse transcriptase; M, molecular weight markers. B: Immunoblot obtained by separating 100 µg of protein on 10% SDS-polyacrylamide gel under reducing conditions.

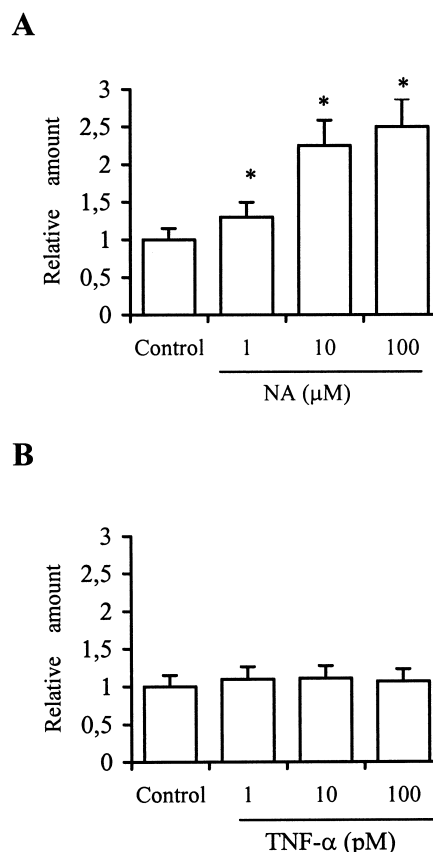


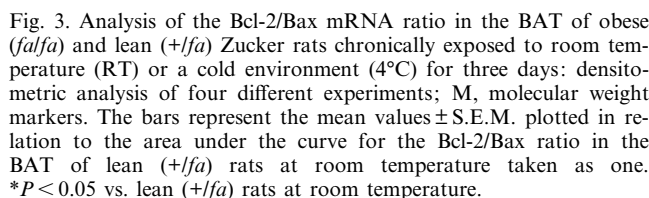
Fig. 4. Effects on the Bcl-2/Bax mRNA ratio of 24-h treatment with different concentrations of NA (A) and TNF- $\alpha$  (B) in confluent cultured brown fat cells (day 8). The bars represent the mean values  $\pm$  S.E.M. plotted in relation to the area under the curve for the Bcl-2/Bax ratio of the untreated cells taken as one. \* $P < 0.05$  vs. untreated cells.

### 3.2. *Bcl-2 and Bax expression in the BAT of obese and lean animals in room- or low-temperature environments*

Fig. 2A shows that the levels of Bcl-2 mRNA were markedly lower in the 8-week-old Zucker-*fa/fa* than in the 8-week-old *+/fa* control rats, but no differences were observed between the 4-week-old rats in which obesity was much less evident in terms of body weight (*+/fa*:  $160 \pm 10$  g b.w.; *fa/fa*:  $175 \pm 15$  g b.w.). On the contrary Bax mRNA moderately increased with age and the development of obesity (Fig. 2A). These results were confirmed at protein level (Fig. 2B). In the 8-week-old control and obese rats acclimatised at 4°C for three days, the levels of Bcl-2 and Bax mRNA were respectively higher and lower than those found in the BAT of the control and obese rats housed at 22°C: consequently, the Bcl-2/Bax mRNA ratios were also higher in the former than in the latter (Fig. 3). These results were also confirmed at protein level (data not shown).

### 3.3. Effect of NA and TNF- $\alpha$ on Bcl-2/Bax mRNA and protein ratios in brown adipocytes differentiated in culture

As TNF- $\alpha$  is overexpressed in obesity and causes the apoptosis of cultured brown adipocytes [4–6], and NA antagonises this effect [4], the brown adipocytes differentiated in culture were treated with increasing concentrations of NA or TNF- $\alpha$ . The TNF- $\alpha$  treatments (0.1–100 pM for up to 48 h) did not lead to any change in either Bcl-2 or Bax mRNA levels (Fig. 4B), but the effects of NA treatment mimicked those of cold



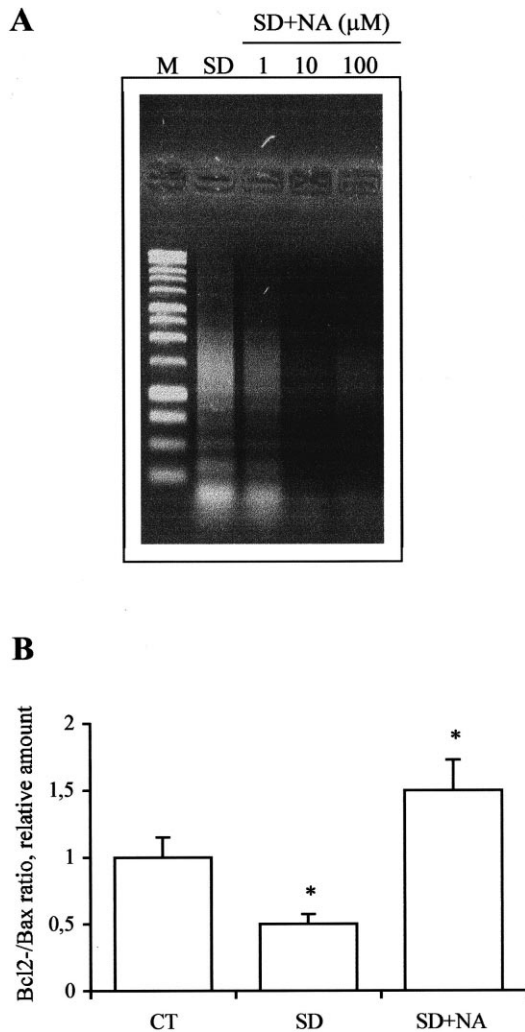


Fig. 5. Effects on DNA fragmentation (A) and the Bcl-2/Bax mRNA ratio (B) of treatment with NA of confluent serum-starved culture of brown fat cells (day 8). A: The confluent cells were exposed for 6 h to various concentration of NA after 6–8 h of serum deprivation (SD); M, molecular weight markers. B: The confluent cells were exposed to 10 μM NA after 6–8 h of serum deprivation (SD). The bars represent the mean values  $\pm$  S.E.M. plotted in relation to the area under the curve for the Bcl-2/Bax ratio of the untreated cells (CT) taken as one. \* $P < 0.05$  vs. untreated cells.

exposure. Fig. 4A shows that the Bcl-2/Bax mRNA ratios increased after NA treatment in a concentration-dependent manner and that this effect was maximal at a concentration of 100 μM NA. These data were also confirmed at protein level (data not shown). Furthermore, NA treatment also increased the Bcl-2/Bax mRNA and protein ratios in a time-dependent manner, with the maximum effect being observed after 24 h (data not shown).

#### 3.4. Effect of NA on the Bcl-2/Bax mRNA ratio in serum-starved brown adipocytes

It is known that serum starvation induces apoptosis in cultured human adipocytes [15]. When our culture-differentiated rat brown adipocytes were deprived of serum for 6–8 h, they underwent DNA fragmentation (a hallmark of cell apoptosis), but NA (1–100 μM) treatment for 6 h was able to counteract this effect (Fig. 5A). We therefore used this experimental model to analyse the pattern of *bcl-2* and *bax* gene expression. The

differentiated brown adipocytes were kept in a serum-free primary culture for 8 h and then treated with NA 10 μM for a further 6 h. Fig. 5B shows that prolonged serum deprivation down-regulated the Bcl-2/Bax mRNA ratio, and that this effect was counteracted by NA: this implies that serum starvation decreases Bcl-2 and increases Bax mRNA levels, and that NA respectively stimulates and inhibits the expression of the two genes.

#### 4. Discussion

We have recently shown that programmed cell death (apoptosis) can occur in brown adipocytes and contribute to the functional atrophy of brown fat in genetically obese Zucker-falga rats [4]. With the aim of improving our understanding of the molecular mechanisms involved in obesity-linked brown fat apoptosis, we investigated the in vivo and in vitro expression of Bcl-2 and Bax, two proto-oncogenes known to modulate cell survival or death [9–11]. Given that Bcl-2 is considered a cell death repressor and Bax a cell death promoter [11], the ratio between them is determinant for the cell fate [9–11]. We found that Bcl-2 and Bax mRNA and protein are present in BAT in toto and in enzymatically dispersed or in vitro cultured brown adipocytes. Furthermore, their gene expression is markedly modified in obese *falga* rats with decreased Bcl-2 and increased Bax mRNA and protein levels; this reduced Bcl-2/Bax ratio is compatible with an increase in the apoptotic death of these adipocytes in obesity, and further confirms the possibility that brown fat cell apoptosis may contribute to the development and/or maintenance of obesity-linked BAT atrophy [4]. Since it has been hypothesised that brown fat cell apoptosis may be a consequence of an imbalance between the apoptotic stimuli mediated by TNF- $\alpha$  (which is overexpressed in the white fat of genetically obese rats [5,6]), and the antiapoptotic stimuli brought about by NA-induced sympathetic activity (that is decreased in obesity [3,8]), we investigated the relative importance of these two factors in determining brown adipocyte death. TNF- $\alpha$  was unable to modify the Bcl-2/Bax ratio in in vitro experiments, whereas NA markedly increased the Bcl-2/Bax ratio in brown adipocytes by increasing *bcl-2* and decreasing *bax* gene expression. This suggests that TNF- $\alpha$  induces brown adipocyte apoptosis by means of a mechanism that does not directly involve *bcl-2* and *bax* gene expression, whereas this expression seems to be relevant to the antiapoptotic effect of NA. This hypothesis is supported by the observation that NA protection from the brown adipocyte apoptosis induced by serum deprivation is paralleled by an increase in the Bcl-2/Bax ratio.

That this conclusion drawn from in vitro experiments is representative of the in vivo situation is shown by the fact that, by increasing the noradrenergic output to BAT, acclimatisation at 4°C for three days of both lean and obese rats leads to statistically higher Bcl-2/Bax mRNA and protein ratios than those observed in rats acclimatised at room temperature. These increases in the Bcl-2/Bax ratios were particularly evident in the obese animals, in which they were basically low. Our results not only confirm, but also better clarify our previous observations of a reduction in the number of apoptotic brown adipocytes measured by means of terminal deoxynucleotidyl-transferase-dUTP-end-labelling staining in obese rats exposed to a cold environment in comparison with those housed at room temperature [4].

In conclusion, we have shown for the first time that Bcl-2 and Bax are expressed in brown adipocytes, at both mRNA and protein level, and that their expression is modulated in vivo and in vitro by NA, which can thus influence the balance between cell death and survival. Furthermore, in addition to clarifying one of the mechanisms involved in the programmed death of a particular cell type, the present findings indicate the involvement of this process in the function and trophism of a tissue that is particular for the development of insulin-resistance and obesity.

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