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PAPER

Protective effects of noradrenaline against tumor necrosis factor-α-induced apoptosis in cultured rat brown adipocytes: role of nitric oxide-induced heat shock protein 70 expression

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OBJECTIVE: To elucidate the effects and molecular mechanism(s) by means of which noradrenaline (NA) protects against the tumor necrosis factor (TNF)-α-induced apoptosis of brown adipocytes.
DESIGN: Brown fat precursor cells were isolated from young rats; 2.5 million cells were added to each 24-well culture plate and cultured in a defined culture medium. On day 8, the cultured cells were exposed to murine recombinant TNF-α and/or cycloheximide (CHX; 10 μg/ml) and/or NA and/or nitric oxide (NO) donors and/or the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) and/or 10 μM heat shock protein 70 (HSP70) antisense or sense oligomers.
MEASUREMENTS: Analysis of DNA fragmentation on agarose gel as a marker of apoptosis; reverse transcriptase-polymerase chain reaction analysis of mRNA levels; Western blotting analysis of protein levels.
RESULTS: Pretreatment of primary cultures of rat brown fat cells with micromolar concentrations of NA or the NO-donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) induced the expression of HSP70 mRNA and protein, which was associated with cytoprotection against TNF-α plus CHX-induced apoptosis. The L-NAME inhibitor of NO synthase activity inhibited both NA-stimulated HSP70 expression and cytoprotection. Furthermore, pretreatment of brown adipocytes with an antisense oligonucleotide to HSP70 antagonized both SNAP- and NA-induced cytoprotection.
CONCLUSION: These findings demonstrate that the NO produced by NA stimulation can induce resistance to the TNF-α-induced apoptosis of brown adipocytes, possibly by means of the expression of HSP70.
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Keywords: apoptosis; noradrenaline; brown adipocytes; nitric oxide; heat shock protein

Introduction

Obesity in rodents is related to functional atrophy of thermogenically active brown fat and the consequent impairment of adaptive thermogenesis;[1,2] in addition to defects in the molecular mechanisms of adaptive thermogenesis, this atrophy may be due to an increase in the cell death rate. As the white fat of obese subjects is a significant source of endogenous tumor necrosis factor (TNF)-α,[3,4] which induces apoptosis in many cell types,[5–7] this cytokine could be involved in brown adipose tissue (BAT) atrophy. TNF-α induces the apoptosis of cultured rat brown adipocytes[8,9] and a larger number of apoptotic nuclei can be seen in the BAT of obese rats than in that of lean controls;[8] furthermore, a significant reduction in brown adipocyte apoptosis can be obtained in genetically obese mice by partially or completely abolishing TNF-α signaling and function.[10]

Noradrenaline (NA) increases the number of viable brown fat cells, and correspondingly decreases the number of apoptotic cells when added before TNF-α.[8] Moreover, the cold exposure stimulation of sympathetic activity to BAT significantly decreases the number of apoptotic cells in comparison with that observed in obese rats kept at room temperature.[8]

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These results suggest that TNF-α produced by white fat cells can induce the apoptosis of brown adipocytes in obese animals and that the noradrenergic system may counteract this phenomenon. Micromolar NA concentrations markedly increase the Bcl-2/Bax mRNA and protein ratios and protect against the serum deprivation-induced apoptosis of brown adipocytes.[11] Furthermore, NA stimulates the expression of the inducible form of nitric oxide (NO) synthase, an enzyme that ensures NO production, in cultured brown adipocytes and BAT.[12]

Interestingly, it has been found that NO is involved in both cell death[13] and cell survival,[14,15] different behaviours that seem to be related to the cell type. In particular, extensive research has shown that NO can induce resistance to TNF-α-induced hepatotoxicity, possibly as a result of an increased expression of the inducible form of heat shock protein 70 (HSP70).[16]

The aim of this study was to investigate the role of NO in mediating the anti-apoptotic effects of NA in brown fat cells treated with TNF-α plus cycloheximide (CHX), which we found to be partially mediated by the induction of cytoprotective stress proteins such as inducible HSP70.

Materials and methods

Animals

Normal-weight male Zucker rats aged 5–8 weeks were obtained from Charles River (Calco, Como, Italy). 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.[8,10] The BAT fragments were carefully dissected out under sterile conditions and placed in a Hepes-buffered solution (pH 7.4) containing 2 mg/ml 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 allowed to float to the surface (30 min on ice). The infranatant containing the adipocyte precursor cells was then collected, filtered through a 25 μm nylon screen, pelletted by centrifugation for 10 min at 700 *g* in 10 ml of culture medium (see below), and diluted to 20 ml.

Adipose cell culture and treatments

Two-and-a-half million cells were added to each 24-well culture plate (Nunclon Delta, Milan, Italy) and 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, and 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 prewarmed fresh medium on day 1 (when the cultures were first washed with 5 ml of prewarmed DMEM) and every each day (without washing). On day 8, the cultured cells were exposed to murine recombinant TNF-α (freshly diluted in buffers; Genzyme, Cinisello Balsamo, Italy) with a specific activity of 5×10^7 U/mg, and/or CHX 10 μg/ml and/or NA freshly diluted in a buffer containing 0.1% ascorbic acid (in order to prevent NA oxidation for the times indicated in the figure legends), and then harvested. During the long-term experiments (24 h), the cells were exposed to the drugs twice a day.

In order to characterize the effects of NO donors, the brown adipocyte cultures were treated with 100–750 μM *S*-nitroso-*N*-acetylpenicillamine (SNAP; Sigma, Milan, Italy) for 2–24 h. The NOS inhibitor N^G-Nitro-L-arginine methyl ester (L-NAME; Sigma, Milan, Italy) was used at a concentration of 1 mM.

In order to evaluate the involvement of inducible HSP70 in the cytoprotective effects of SNAP and NA, brown fat cells were incubated with 10 μM HSP70 antisense or sense oligomers during 24 h pretreatments with SNAP 500 μM or NA 10 μM, followed by TNF-α plus CHX exposure for a further 4 h in the continued presence of medium containing the oligonucleotides as described by Kim *et al*.[16] The inducible HSP70 antisense (TGTTTTCTTGGCCAT) and sense oligomers (ATGGCCAAGAAAACA) were synthesized from sequences complementary to the initiation and four downstream codons of rat inducible HSP70 mRNA.[17]

Analysis of DNA fragmentation

The confluent brown adipocytes (at 8 days in culture) were lysed in 20 mM Tris–HCl (pH 7.5), 10 mM EDTA and 0.5% Triton X 100 (pH 8), and occasionally shaken while on ice for 30 min. The cytosolic fraction was obtained by centrifugation at maximum speed (13 000 *g*) for 20 min at 4°C, and the cytosolic DNA was extracted with a mixture of phenol and chloroform; one-tenth volume of 5 mM NaCl was added to the solution and DNA was then precipitated overnight by adding an equal volume of isopropanol. After storing overnight at −20°C a DNA pellet was recovered by centrifugation, resuspended in water, and treated with RNase 50 μg/ml. The DNA (3–5 μg) was then loaded on 1.2% agarose gels and stained with ethidium bromide after migration.

RT-PCR analysis

Total RNA was isolated from the cultured cells after drug treatments using RNazol method (TM Cinna Scientific, Friendswood, Texas, USA). For PCR analysis, the RNAs were treated for 1 h at 37°C with 6 U of RNase-free DNaseI per μg of RNA in 100 mM Tris–HCl, pH 7.5, and 50 mM $MgCl_2$ in the presence of 2 U/μl of placenta RNase inhibitor. One microgram of total RNA was reverse transcribed with 200 U of Moloney murine leukemia virus reverse transcriptase (Promega, Milan, Italy) in 20 μl of Promega supplied buffer

containing 0.4 mM dNTP, 2 U/μl RNase inhibitor, and 0.8 μg oligo(dT)$_{15}$ primer (Promega). A control experiment without reverse transcriptase was performed for each sample in order to verify that the amplification did not originate from residual genomic DNA. The PCR was performed using truncated *Thermophilus acquaticus* DNA polymerase (Biotaq™, Bioprobe Systems, Milan, Italy) in 50 μl of standard buffer (20 mM Tris–HCl, pH 8.55, 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 150 μg/ml bovine serum albumin, and 200 μM dNTP) containing 10 ng of cDNA from the various preparations, and 1 μM of the sense and antisense oligonucleotide primers specific for inducible HSP70 (5′-ACATCAGCCAGAACAAGCG-3′, amino acids 932–950, and 5′-TGATGCTCTTGTTCAGGTCG-3′, amino acids 1248–1267),[18] Bcl-2 (5′-AGAGGGGCTACGAGTGGGAT-3′, amino acids 1895–1914, and 5′-CTCAGTCATCCACAGGGCG-A-3′, amino acids 2330–2349),[19] and Bax (5′-GGTTTCATCCAGGATCGAGACGG-3′, amino acids 84–106, and 5′-ACAAAGATGGTCACGGTCTGCC-3′, amino acids 509–530).[20] The mRNA for constitutive β-actin was examined as the reference cell transcript. β-actin mRNA amplification products were present at equivalent levels in the lysates of treated and untreated cells at the different time points. The reaction was performed using specific primers described elsewhere.[12] β-Actin was separately co-amplified with HSP70, Bcl-2 and Bax cDNA, with the β-Actin primers being added after 10 amplification cycles in order to avoid saturation. The PCR conditions were as follows: denaturation at 94°C for 30 s, annealing at 61°C (63°C for HSP70) for 30 s, and polymerisation at 72°C for 30 s. After 28 cycles, 5 μl of the reaction mixture were separated by electrophoresis (1.2% agarose gel in Tris-Borate-EDTA buffer) and made visible by means of ethidium bromide staining. The number of cycles and the reaction temperature of the semiquantitative RT-PCR assay were previously established in our laboratory.[11] Indeed, we observed a linear relationship between the amount of input template and the amount of PCR product generated over a wide concentration range (0.5–10 μg of total RNA) also after 30 cycles of amplification. For this reason we judged as an optimal experimental condition for linearity to perform 28 cycles of amplification.

The RT-PCR products were semiquantitatively analysed using a QuickImage densitometer (Canberra, Packard, Milan, Italy) and a Phoretix 1D (version 3.0.1) software image analyser. In order to reduce the detection limits of the densitometric analysis, the measurements were made within the linear response range of the image capturing device (optical density 0–3.0); for this purpose, a standard image was acquired at the same time as the experimental images. Each experiment was densitometrically analysed at least four times; the coefficient of analytical variation between the different analyses was less than 5%. If the density of the β-actin band of a given sample differed by > 10% from the mean density of the samples in a gel, it was removed from the analysis. The HSP70 mRNA levels were expressed relative to the levels of the co-amplified β-actin mRNA. 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. In order to normalize between-experiment densitometer differences, the data were corrected for background and expressed as a percentage of a specific within-experiment reference value of one (see figure legends).

Western blot analysis

Western blot analysis was performed as previously described.[12] Briefly, 70 μg total protein extracts were resolved by 8% SDS–PAGE and transferred to nitrocellulose filter papers. The primary antibody (monoclonal antibody directed against the mouse inducible HSP70 at a dilution of 1 : 1000; StressGen, Lexington, KY, Canada) was diluted in Tris-buffered saline, pH 7.6, and incubated with membrane for 2 h at room temperature. The secondary antibody (horseradish peroxidase-conjugated anti-mouse, Pierce, Rockford, IL, USA) was incubated with membrane for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence according to the specifications of the manufacturer (Super Signal Substrate, Pierce) and then densitometrically analysed.

Statistical methods

The data are presented as the mean values ± s.d. of at least three separate experiments. The comparisons between two values were made by means of one-way ANOVA followed by the Student–Newman–Kauls *post-hoc* comparison. A *P*-value of 0.05 was considered significant.

Results

SNAP pretreatment protects brown adipocytes against TNF-α plus CHX-induced apoptosis

We have previously demonstrated that the pretreatment of brown adipocytes with NA provides protection against TNF-α plus CHX-induced apoptosis.[8] Since NA increases NO production by stimulating the inducible form of NO synthase in brown adipocytes,[12] we investigated whether NO pretreatment may offer similar protection against TNF-α-induced toxicity. For this aim, brown adipocytes differentiated in culture were treated with 100 μM SNAP for 24 h, washed twice with fresh medium, and then added with TNF-α (1 nM) plus CHX (10 μg/ml) for 4–6 h.

Apoptosis was determined by measuring DNA fragmentation on agarose gels. Figure 1A shows that 4 h treatment with TNF-α plus CHX (but neither TNF-α nor CHX alone) induced a marked DNA fragmentation, thus confirming our previous results.[8] Twenty-four-hour pretreatment with 100 μM SNAP protected the brown adipocytes from TNF-α plus CHX toxicity to the same extent as 10 μM NA (Figure 1B). In addition, the anti-apoptotic effects of NA were antagonized by 1 mM

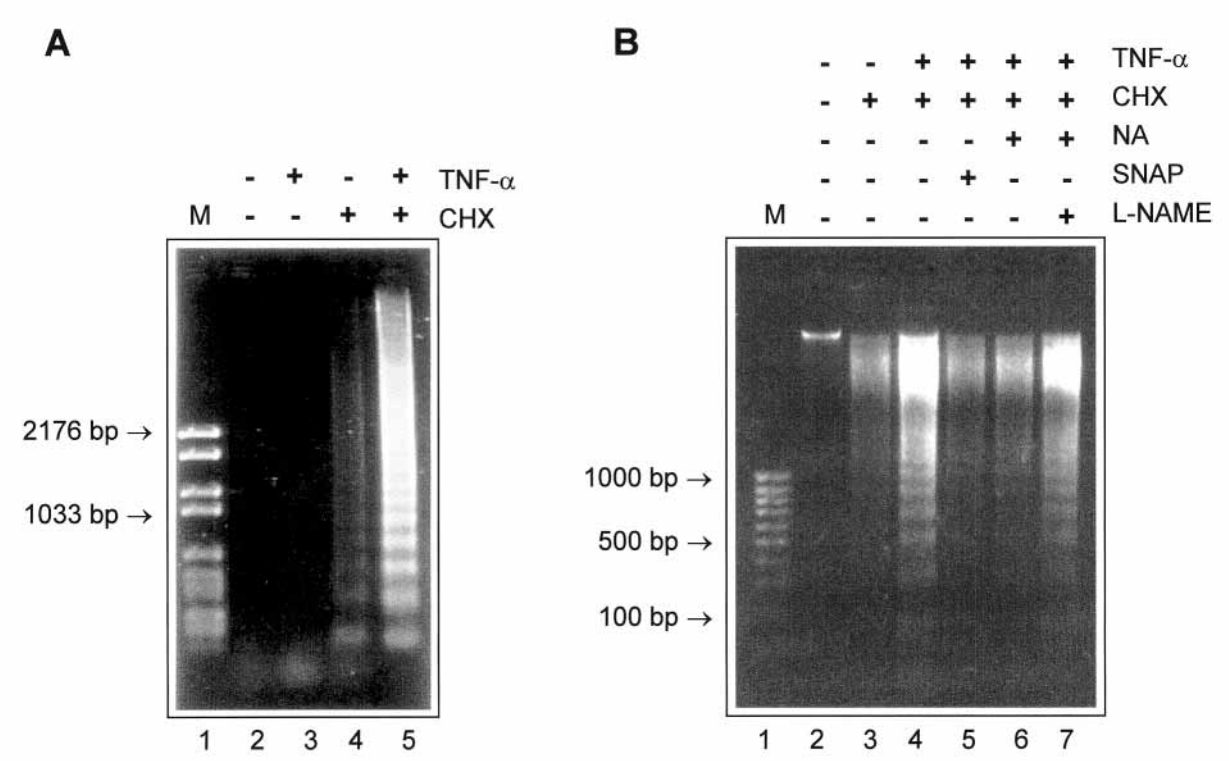

Figure 1 NA and SNAP pretreatments antagonize TNF-α plus CHX-induced apoptosis in cultured brown adipocytes. (A) Four-hour treatment with 1 nM TNF-α plus 10 μg/ml CHX induced apoptosis of brown fat cells. (B) Brown fat cells were pretreated with either NA 10 μM or SNAP 100 μM or L-NAME 1 mM for 24 h and incubated with TNF-α (1 nM) plus CHX (10 μg/ml) for 4 h. Cytosolic DNA was isolated as described in Materials and methods and electrophoresed on agarose gel after normalizing the amount of DNA by protein concentration. The DNA was visualized using UV light and photographed. M, molecular weight markers (A = Standard 6, Boehringer, Mannheim, Germany; B = Gene Ruler 100 bp DNA ladder, Genenco, Milan, Italy). The gels shown are representative of the data obtained from six independent experiments.

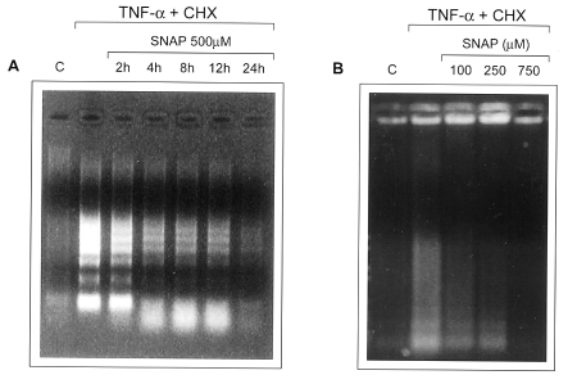

Figure 2 SNAP pretreatment protects brown adipocytes from TNF-α plus CHX-mediated apoptosis in a time- (A) and dose-dependent (B) manner. The brown fat cells were pretreated with 500 μM (A) or different concentrations (B) of SNAP for various time periods (A) or 24 h (B), washed twice with fresh medium, and then incubated with TNF-α (1 nM) plus CHX (10 μg/ml) for 4 h. DNA fragmentation was measured as described in Figure 1.

of the NOS inhibitor L-NAME (Figure 1B, lane 7). Both SNAP and L-NAME alone did not induce any DNA fragmentation.

Pretreatment with SNAP 2 h before the addition of TNF-α plus CHX had no effect; however, if SNAP was added 4 – 12 h before TNF-α plus CHX treatment, the degree of DNA fragmentation was markedly reduced, and 24 h before apoptosis was almost completely inhibited (Figure 2A). When SNAP concentrations of 100 – 750 μM were added to the brown adipocytes 24 h before TNF-α plus CHX, protection against apoptosis was evident even at the lowest dose (Figure 2B).

Effects of 8 Br-cGMP on TNF-α plus CHX-induced apoptosis of brown adipocytes

It is known that the inhibitory or stimulatory effects of NO on the apoptosis of different cell types take place through cyclic GMP (cGMP)-dependent or -independent mechanisms.[21–23] In order to investigate the role of this cyclic nucleotide in inhibiting the TNF-α plus CHX-induced

apoptosis in brown adipocytes, we studied the effects of the membrane-permeable cyclic GMP analogue, 8 Br-cGMP. Figure 3 shows that the pretreament of brown adipocytes with either 250 (lane 4) or 500 μM (lane 5) 8 Br-cGMP for 24 h did not affect TNF-α plus CHX-induced DNA fragmentation, as well as 500 μM 8 Br-cGMP alone (lane 6) did not induce DNA fragmentation, thus suggesting that the anti-apoptotic effects of NO on brown fat cells are cGMP-independent.

SNAP pretreatments do not increase the Bcl-2/Bax mRNA ratio

Given that NA increases in a concentration-dependent manner the Bcl-2 mRNA and protein levels, leaving unchanged Bax mRNA and protein levels in brown fat cells (ie the Bcl-2/Bax mRNA and protein ratios are increased),[11] which suggests that its cytoprotective effects may be partly due to a modulation of the balance between survival (Bcl-2) and death factors (Bax), we investigated the effects of SNAP on Bcl-2 and Bax gene expression. Figure 4 shows that SNAP treatment (100 – 750 μM for 24 h) did not change the levels of either Bcl-2 or Bax mRNA, and so the Bcl-2/Bax mRNA ratio also remained unchanged. This suggests that the anti-apoptotic effects of the NO donor do not seem to depend on the modulation of bcl-2 gene family proteins.

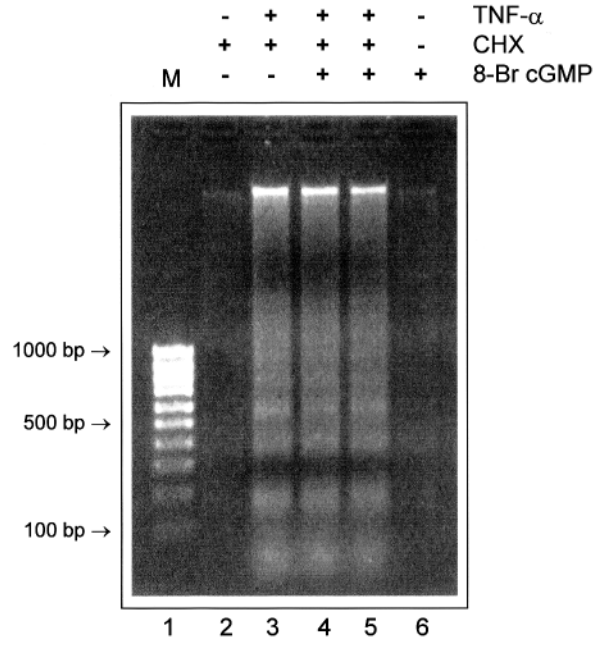

Figure 3 8 Br-cGMP does not affect TNF-α plus CHX-induced apoptosis of cultured brown adipocytes. The brown fat cells were pretreated with 250 (lane 4) or 500 μM of 8 Br-cGMP (lane 5) for 24 h, washed twice with fresh medium, and incubated with TNF-α (1 nM) plus CHX (10 μg/ml) for 4 h. Lane 6, no apoptotic effect was evident after treatment with 500 μM 8 Br-cGMP alone. DNA fragmentation was measured as described in Figure 1. M, molecular weight marker (Gene Ruler 100 bp DNA ladder, Genenco, Milan, Italy). The gel shown is representative of the data obtained from three independent experiments.

NA and SNAP pretreatments induce HSP70 expression

The cytotoxic effects of TNF-α are antagonized by the inducible heat shock protein HSP70 in various cell types.[21,24–26] In particular, inducible (but not constitutive) HSP70 is involved in the SNAP-induced protection of hepatocytes from TNF-α-mediated cytotoxicity.[21] In order to determine whether SNAP or NA increase inducible HSP70 expression in brown adipocytes, we exposed cultured brown fat cells to increasing concentrations for different times and examined HSP70 mRNA levels by means of RT-PCR analysis. HSP70 mRNA expression increased at SNAP concentrations as low as 100 μM and was maximal at 500 μM, and at NA concentrations as low as 1 μM (maximal at 100 μM; Figure 5A). Figure 5B shows that, after exposure to SNAP 500 μM and NA 10 μM, HSP70 mRNA levels progressively increased for 4 h and then declined. All of these results were confirmed at protein level by Western blot analysis by means of a monoclonal antibody specific for the inducible HSP70 isoform. In particular, Figure 5C shows that SNAP and NA increased HSP70 protein levels in a dose-dependent manner after 8 h treatment, and that these increases were blunted after 24 h treatment. In addition, Figure 5A and B show that exposure to increasing concentrations of 8 Br-cGMP for different times did not change HSP70 expression.

Interestingly, even if the time-dependent NA-induced increases in HSP70 mRNA levels were markedly antagonized by 1 mM of L-NAME, this anatgonism was not complete (Figure 6). These findings may suggest an additional protective mechanism induced by NA, since it is unlikely that there is a concentration effect due to insufficient amounts of L-NAME being used. Indeed, L-NAME concentrations up to 5 mM did not antagonize fully the NA effects.

Antisense oligomer to HSP70 blocks HSP70 expression and its protection against TNF-α plus CHX-induced apoptosis

In order to investigate the role of HSP70 in the SNAP- and NA-induced inhibition of apoptosis mediated by TNF-α plus CHX, its expression was suppressed using an antisense HSP70 oligomer. Treatment with antisense did not influence cell morphology. The brown fat cells were incubated for 24 h with 10 μM of HSP70 antisense or sense oligonucleotides during 500 μM SNAP or 10 μM NA pretreatment, followed by TNF-α plus CHX exposure for a further 4 h in the continued presence of the oligonucleotides. Figure 7A shows that HSP70 antisense, differently from the sense oligomer, inhibited either SNAP- or NA-induced protection against TNF-α plus CHX-mediated apoptosis. In addition, neither HSP70 antisense or sense alone induced apoptosis. These

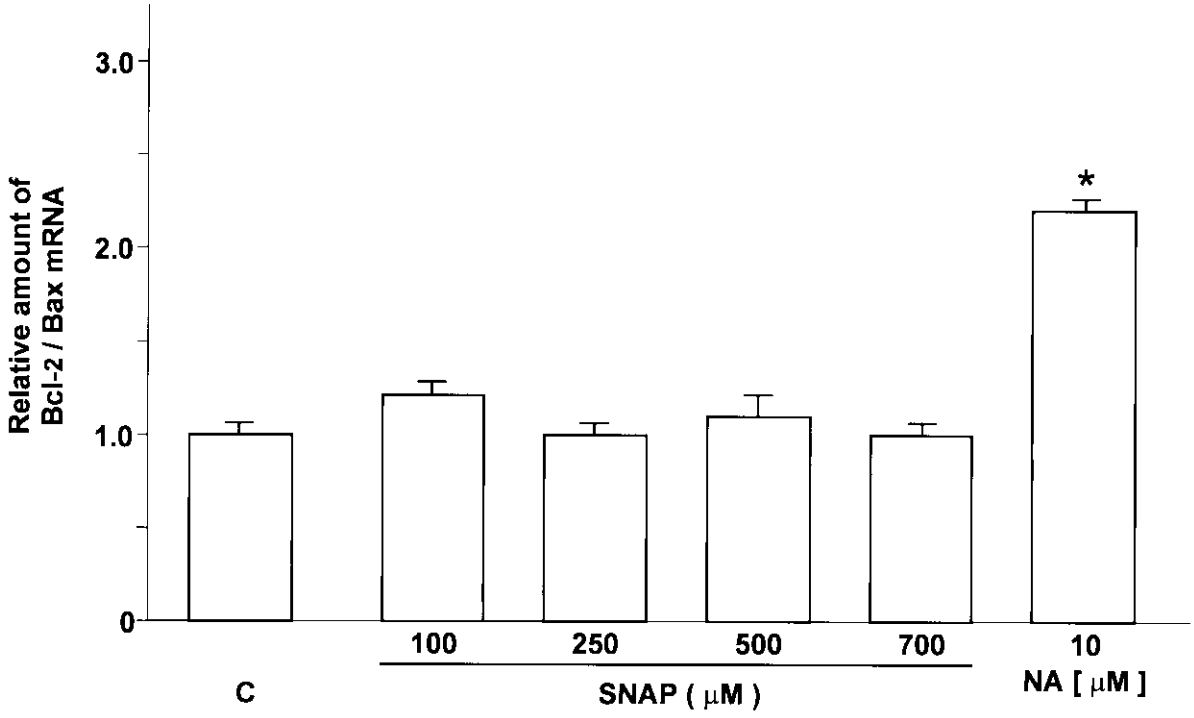

Figure 4 Effects of NA and SNAP on the Bcl-2/Bax mRNA ratio. The brown adipocytes were exposed to NA 10 μM or different concentrations of SNAP for 24 h. The bars represent the mean values ± s.d. of three separate experiments plotted in relation to the area under the curve for the Bcl-2/Bax ratio of the untreated cells (C) taken as one. $*P < 0.05$ *vs* untreated cells.

findings clearly suggest that NO-induced HSP70 expression is involved in the NO and NA cytoprotection of brown fat cells. The efficacy and selectivity of the antisense strategy was proved by Western blot analysis. Indeed, Figure 7B shows that the antisense oligomer blocked the SNAP-induced HSP70 expression at protein level, while the sense oligomer had no effect, as expected. Since NA has been found to increase inducible NO synthase expression and NO production in brown fat cells,[12] the present findings suggest that the HSP70-mediated cytoprotective effects of NA may be partly due to NA-stimulated NO production. As described above 1 mM of L-NAME counteracted the anti-apoptotic effects of NA (Figure 1B), by confirming this hypothesis.

Discussion

The aim of this study was to elucidate the role of NO and HSP70 in the NA protection of brown adipocytes from TNF-α-induced apoptosis.[8] As an experimental model, rat brown fat precursor cells were grown in culture because they divide rapidly and their differentiation at confluence (8–10 days) makes them reminiscent of mature brown fat cells. Various lines of evidence indicate that a complex array of different cell signaling pathways are involved in the balance between cell survival and death, one of which is the modulation of the Bcl-2 protein family.[27] Pro- and anti-apoptotic family members such as Bcl-2 and Bax can heterodimerize and apparently titrate each other's function, thus suggesting that their relative concentrations may act as a rheostat for the suicide program.[28,29] We have previously demonstrated that NA can stimulate Bcl-2 gene expression in cultured brown adipocytes in such a way as to increase the Bcl-2/Bax mRNA and protein ratios in a dose-dependent manner,[11] which suggested that it may counteract TNF-α plus CHX-induced apoptosis through this signaling pathway; on the contrary, TNF-α treatment did not lead to any change in the Bcl-2/Bax ratio,[11] thus indicating that its cytotoxic action on brown fat cells may be associated with the activation of alternative pathways.

In another work NA was demonstrated to stimulate expression of inducible NO synthase, ensuring NO production in brown adipocytes.[12] NO was implied as an anti-apoptotic signal in several cell models.[14,15] In the present work, exposure of the differentiated cells to the NO-generating compound clearly showed that SNAP offers protection against TNF-α plus CHX-induced apoptosis. We therefore investigated whether the protective effects of NO are due to the modulation of Bcl-2 and Bax gene expression. As pretreatment with NO donors does not change the Bcl-2/Bax mRNA and protein ratios in cultured brown

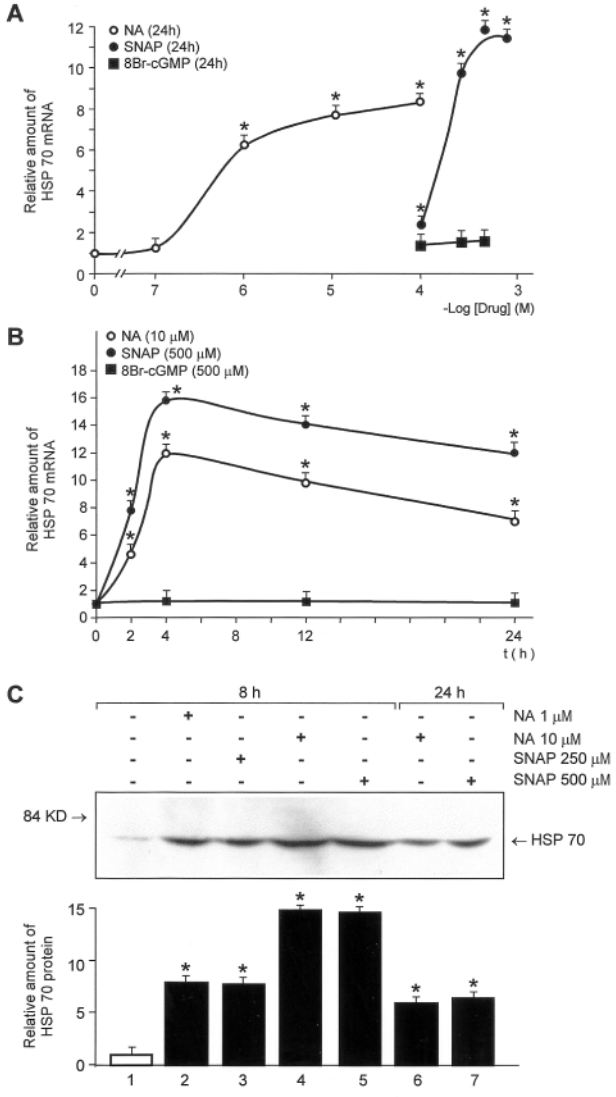

Figure 5 RT-PCR and Western blot analyses of NA- and SNAP-mediated HSP70 induction. (A, B) RT-PCR analysis: the brown adipocytes were treated with increasing concentrations of SNAP (closed circles) or NA (open circles) for 24 h (A) or different time periods with 500 μM SNAP (closed circles) or 10 μM NA (open circles) (B) and HSP70 mRNA levels were measured. The effects of different concentrations (A) or 500 μM of 8 Br-cGMP (B) for 24 h (A) or for different time periods (B) are shown as closed squares. (C) Immunoblot obtained by separating 70 μg of protein on 8% SDS–polyacrylamide gel under reducing conditions (*top*) and densitometric analysis (*bottom*). The points and bars represent the mean values ± s.d. of three separate experiments plotted in relation to the area under the curve for the HSP70 mRNA and protein levels in the untreated cells taken as one. *$P < 0.001$ *vs* untreated cells.

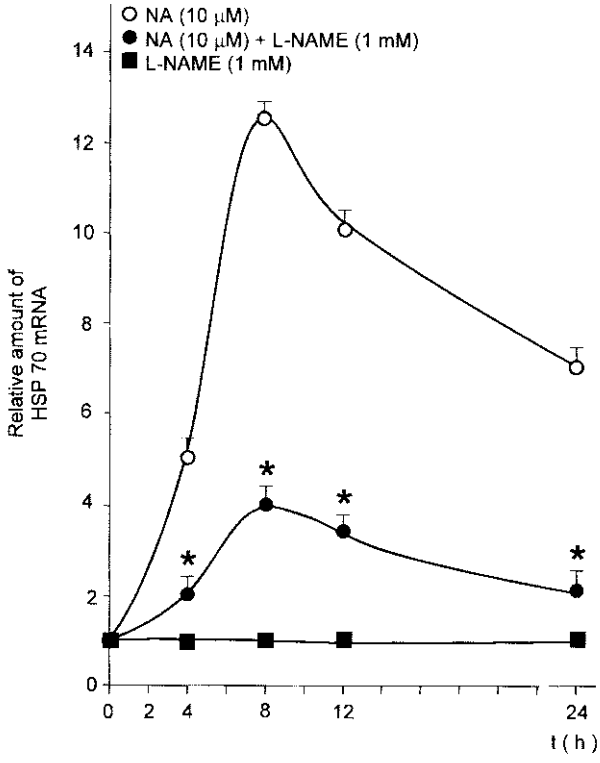

Figure 6 L-NAME antagonizes the time-dependent NA-induced increases in HSP70 mRNA levels. The brown adipocytes were exposed to NA 10 μM (open circles) or NA 10 μM plus L-NAME 1 mM (closed circles) for 24 h. L-NAME alone had no effect. The bars represent the mean values ± s.d. of three separate experiments plotted in relation to the area under the curve for the HSP70 mRNA levels in the untreated cells taken as one. *$P < 0.005$ *vs* NA-treated cells.

adipocytes (present and unpublished results), it is unlikely that this system is involved in the cytoprotective effects of NO. Thus, we investigated whether HSP70 may play a role in these effects.

Indeed, both time-course and dose–response studies have revealed that the induction of HSP70 mRNA and protein after cell exposure to both NA and chemically generated NO protects against apoptosis. Our results confirm previous findings published by others indicating that the exposure of rodents to cold ambient temperatures leads to a tissue-selective induction of HSP70 in BAT, which coincides with the induction of UCP-1 synthesis.[30,31] It has been found that adrenergic receptor antagonists block cold-induced HSP70 expression in BAT, whereas adrenergic agonists induce BAT HSP70 expression even in the absence of cold exposure.[30,31] These *in vivo* and our own *in vitro* findings suggest that NA induces HSP70 expression in BAT, and that this may affect cell survival by counteracting cytokine-induced apoptosis; this hypothesis is also supported by other recent evidence highlighting the substantial involvement of HSPs in protecting cells against apoptotic stimuli.[32–34]

In order to demonstrate the assumed role of HSP70 in protecting brown adipocytes against cytokine-induced toxicity, DNA fragmentation was studied in cells treated with the HSP70 antisense oligomer in which its expression was

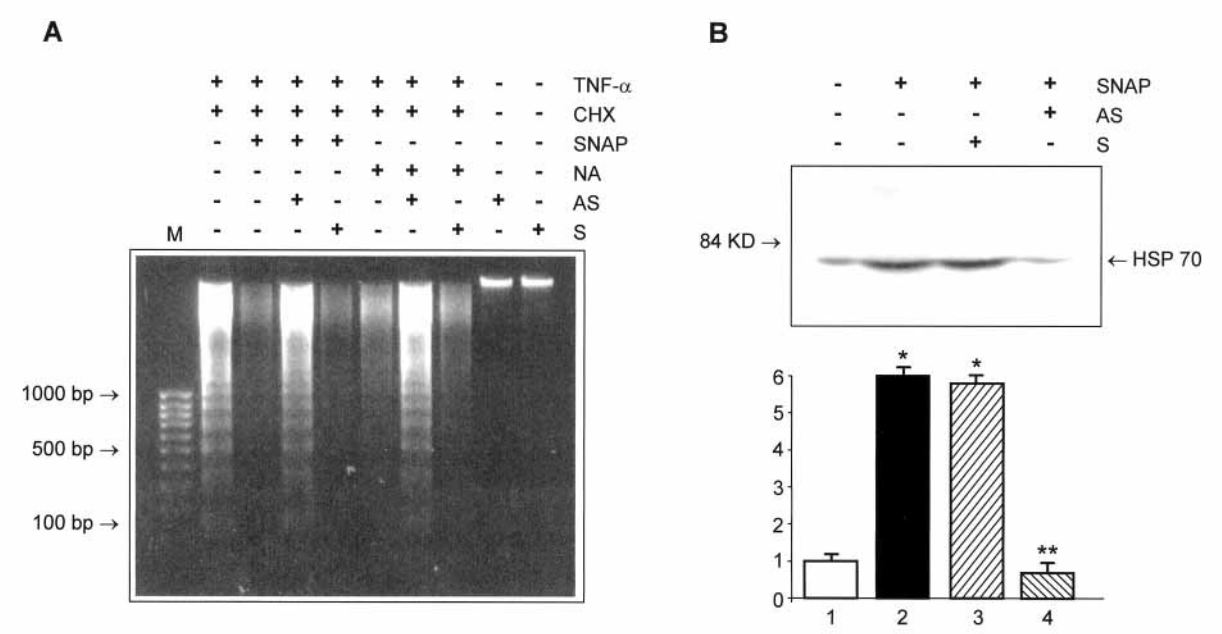

Figure 7 Effects of the HSP70 antisense oligomer on SNAP- and NA-induced protection against TNF-α plus CHX-mediated apoptosis, and on SNAP-induced HSP70 expression. (A) The brown adipocytes were pretreated with SNAP 500 μM or NA 10 μM, in the presence or absence of the HSP70 sense (S) or antisense (AS) oligomers, or with S or AS oligomers alone, for 24 h. The cells were washed twice with fresh medium, and then exposed to TNF-α (1 nM) plus CHX (10 μg/ml) for 4 h in the continued presence of the oligonucleotides. DNA fragmentation was measured as described in Figure 1. M, molecular weight marker (Gene Ruler 100 bp DNA ladder, Genenco, Milan, Italy). The gel shown is representative of the data obtained from three independent experiments. (B) Western blot analysis of SNAP-induced HSP70 expression in brown adipocytes treated as decribed above. The bars represent the mean values ± s.d. of three separate experiments plotted in relation to the area under the curve for the HSP70 protein levels in the untreated cells (lane 1) taken as one. *$P < 0.005$ *vs* untreated-cells; **$P < 0.005$ *vs* SNAP-treated cells.

markedly reduced. This suppression was highly specific for inducible HSP70. HSP70 antisense mRNA (but not the HSP70 sense oligomer) inhibited NO-induced HSP70 expression and restored the susceptbility of brown adipocytes to TNF-α plus CHX-induced apoptosis. It was particularly interesting to find that NA, which has been shown to induce NO production in brown adipocytes and protect them against TNF-α plus CHX-induced apoptosis,[8,12] lost its anti-apoptotic effects when the cells were pretreated with the NOS inhibitor L-NAME or the antisense oligomer to HSP70. These results suggest that the antiapoptotic effect of NO in brown fat cells is mediated by HSP70 induction, and that NA can at least partially antagonize TNF-α plus CHX-induced apoptosis by means of the NO-mediated induction of HSP70 expression.

The decomposition products of SNAP 1 mM (unpublished data), and 8 Br-cGMP 250 and 500 μM had no effect on HSP70 induction or cytoprotection, thus suggesting that the NO released by SNAP acted as a mediator by means of a cGMP-independent mechanism. It has been previously shown that NO inhibits TNF-α-induced apoptosis by means of mechanisms that are both dependent on, and independent of, guanylyl cyclase activation and cGMP formation.[21–23] The cGMP-independent mechanism may be due to *S*-nitrosylation and the consequent inhibition of various active caspases.[21,22] It has been found that NO inhibits both early and late apoptosis: its late-stage protection is cGMP-independent and seems to depend on the oxidative state of the cells, as is suggested by the fact that it is inhibited by the reducing agent DTT.[35] It is known that NO can protect cells against apoptosis by directly scavenging O_2^-,[36] but this seems to be unlikely in our cell system because the NO donor was added 16–24 h before TNF-α plus CHX, and no protective effect against DNA fragmentation was observed when brown adipocytes were simultaneously treated with 500 μM of the NO donor SNAP and TNF-α.

Taken together, our present and previous results suggest that NA can counteract the TNF-α-induced apoptosis of brown adipocytes through different signaling pathways, two of which seem to be independent of each other and to involve Bcl-2 protein family and NO systems. In turn, NO may act through HSP70 induction (see Figure 8). Further experiments will be required to establish whether these signaling processes actually play a role in the pathophysiology of obesity and related disorders, but it is interesting to note that TNF-α induces NO production in BAT.[37–39] We can therefore speculate that this induction is insufficient to replace the anti-apoptotic NO signaling system triggered by NA in the case of defective sympathetic nervous system

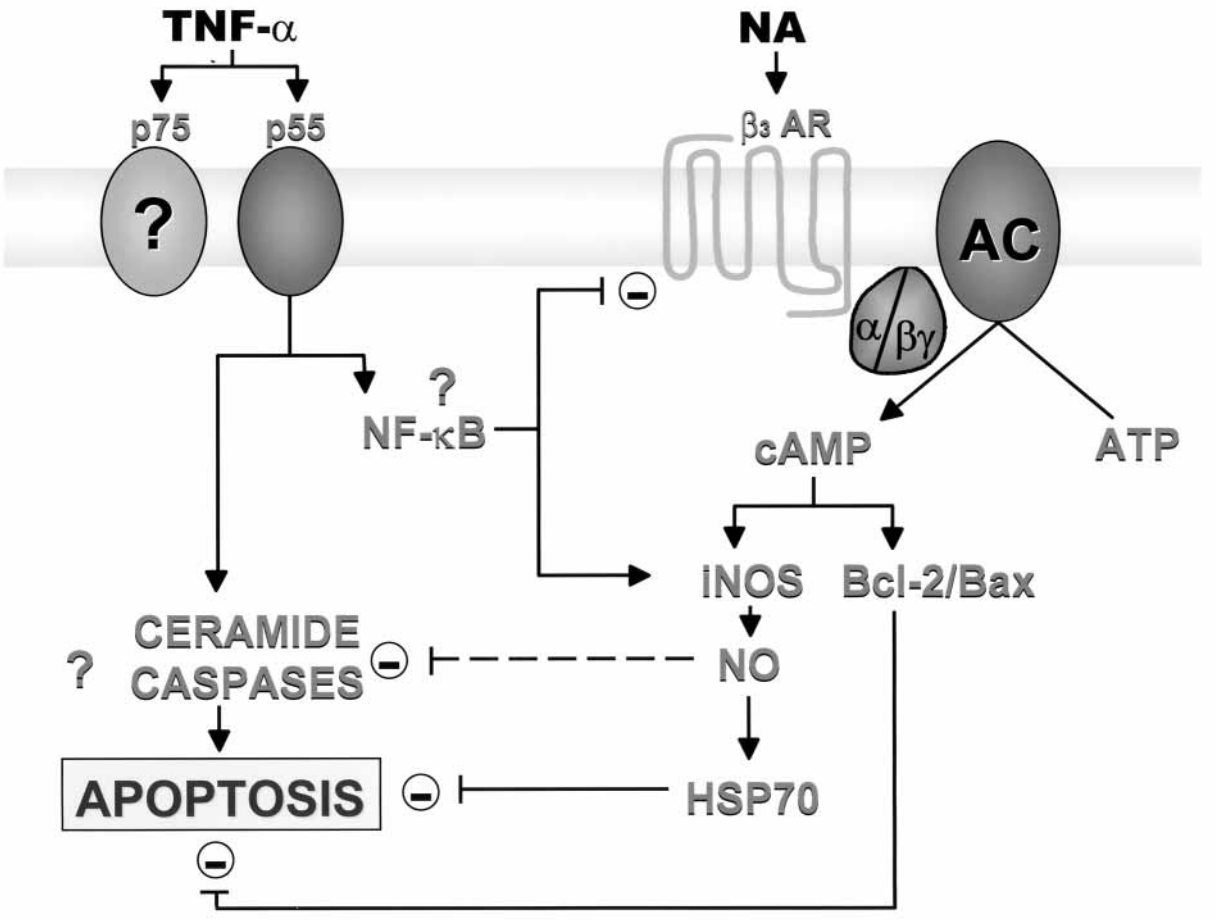

Figure 8 See Discussion for major details. p55 and p75, TNF-α receptor type I and II; β_3 AR, β_3-adrenoceptor; AC, adenylyl cyclase; $\alpha/\beta\gamma$, G-protein heterotrimer coupling the β_3 AR to AC.

activity and markedly increased TNF-α synthesis, such as that observed in obese subjects: indeed, in this situation BAT is apoptotic and functionally atrophied.[10]

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