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Research Article

TNF-alpha-induced apoptosis is prevented by erythropoietin treatment on SH-SY5Y cells

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ABSTRACT

The growth factor erythropoietin (Epo) has shown neuronal protective action in addition to its well known proerythroid activity. Furthermore, Epo has dealt with cellular inflammation by inhibiting the expression of several proinflammatory cytokines, such as IL-1 and TNF-α. The action of TNF can have both apoptotic and antiapoptotic consequences due to altered balance between different cell signalling pathways. This work has focused on the apoptotic effects of this cytokine and the potential protective action of Epo. The model we used was neuroblastoma SH-SY5Y cells cultured in the presence of 25 ng/ml TNF-α or pretreated with 25 U/ml Epo for 12 h before the addition of TNF-α. Apoptosis was evaluated by differential cell count after Hoechst staining, analysis of DNA ladder pattern, and measurement of caspase activity. Despite its ability to induce NF-κB nuclear translocation, TNF-α induced cell death, which was found to be associated to upregulation of TNF Receptor 1 expression. On the other hand, cells activated by Epo became resistant to cell death. Prevention of death receptor upregulation and caspase activation may explain this antiapoptotic effect of Epo, which may be also favoured by the induction of a higher expression of protective factors, such as Bcl-2 and NF-κB, through mechanisms involving Jak/STAT and PI3K signalling pathways.

Introduction

Erythropoietin (Epo) increases the number of circulating erythrocytes primarily by preventing apoptosis of erythroid progenitors. In addition to this proerythroid activity, evidence has shown neuronal protective action of Epo *in vivo* [1,2], as well as *in vitro* [3,4]. In view of these findings, a prominent role for Epo in the nervous system has been defined and there is growing interest in its potential therapeutic use for neuroprotection.

Epo initiates its cellular response by binding to the Epo receptor (EpoR), which induces phosphorylation of the transcription factor STAT5, which in turn induces activation of a signalling pathway cascade important for the regulation of cellular responses. Cell culture studies of erythroid progenitors have demonstrated that Epo functions as a survival factor by repressing apoptosis at least in part through the antiapoptotic proteins of the Bcl-2 family [5]. In this context, we had observed that Bcl-x_L upregulation was involved in the action of Epo to prevent staurosporine-induced programmed cell death of SH-SY5Y neuronal cells [6]. In accordance, Epo-facilitated neuritogenesis was coincident with upregulation of Bcl-x_L [7]. Furthermore, Epo was found to attenuate the inflammatory response in injured rat

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Abbreviations: TNF-α, tumour necrosis factor-alpha; Epo, erythropoietin; EpoR, erythropoietin receptor; D-MEM, Dulbecco's Modified Eagle Medium; FBS, foetal bovine serum; PBS, phosphate buffered saline; EtBr, ethidium bromide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PI3K, phosphatidylinositol 3-kinase; Jak2, Janus kinase 2; NF-κB, nuclear factor-κappaB; STAT5, signal transducer and activator of transcription

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brain by mechanisms leading to decreased levels of several cytokines, such as IL-1 and TNF-α [8].

TNF can initiate two distinct pathways, one leading to cell death while the other promoting antiapoptotic proteins. Therefore, the stimulation of cells with TNF can have both apoptotic and antiapoptotic consequences due to altered balance between different signalling pathways related to proliferative responses and destructive cellular outcomes [9,10]. Various members of the TNF super family mediate either proliferation and survival, or cell death. Despite of the existence of distinct receptors, all members share a common cell signalling pathway that mediates the activation of the nuclear factor-κappaB (NF-κB) and mitogen-activated protein kinases (e.g. c-jun N-terminal kinase). Both apoptotic and antiapoptotic signals can be activated simultaneously by the same cytokine in the same cell [11].

This disparity in the TNF-induced responses, involving different signals mediated by the two TNF receptors, TNFR1 and TNFR2, is not fully understood. Receptor activation by the TNF ligands causes recruitment of several intracellular adaptor proteins. These proteins activate multiple signal transduction pathways that modulate cellular actions, including immune responses, inflammatory reactions, cell death mechanisms, or cell survival, the latter via activation of the nuclear factor NF-κB [12]. Therefore, deregulation of intracellular signals might lead to the development of various diseases [13].

It is known that the survival of neurons depends on a complex interplay of several factors and any imbalance of this relationship may lead to cell death. The programmed cell death phenomenon, apart from being an important feature in the development of the nervous system, appears also to be responsible for many neurodegenerative diseases and is frequently associated to inflammatory processes. An experimental therapeutic strategy, using anti-TNF antibody, was employed in animal models of central nervous system inflammatory diseases to inhibit the cytokine effects [14]. In recent years, evidence has been reported regarding systemically administered Epo which proved to protect rats from cerebral ischemia, traumatic brain injury, and experimental autoimmune encephalomyelitis [15,16]. It has been suggested that an Epo-induced attenuation of the reactive inflammatory response might be a secondary effect of decreased apoptosis [17]. Therefore, we were interested in the investigation of possible apoptotic effects of TNF-α, resembling a proinflammatory condition and in the further elucidation of an Epo protective action as well as the related mechanisms. The human neuroblastoma SH-SY5Y cell line was used as the experimental model based on our previous experience of staurosporine-induced apoptosis, which was found to be prevented by the action of Epo in these cells [6].

Materials and methods

Materials

All chemicals used were of analytical grade. Dulbecco's Modified Eagle Medium (D-MEM) and Ham F12 Medium were obtained from GibcoBRL. Proteinase K, Trizol reagent, goat anti-mouse (IgG)-Alexa Fluor 488 (Molecular Probes), and specific primers for Bcl-2, Bcl-xL, TNFR1, TNFR2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Invitrogen Life Technologies. Monoclonal anti-Bcl-xL antibody was obtained from Chemicon International, monoclonal anti-PARP and monoclonal anti-Bcl-2 were obtained from BD Pharmingen, anti-IKBα, anti-NF-κB (P65) and anti-STAT5 antibodies were from Santa Cruz Biotechnology and monoclonal anti-human erythropoietin receptor antibody (Clone 38409.11) was from RD Systems. EDTA, Nonidet P40, AG490, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium o-vanadate, L-glutamine, paraformaldehyde, phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, pepstatin A, Hoechst 33258 dye, DNAse-free RNAse A, monoclonal anti-actin antibody, propydium iodide (PI), and the caspase substrates IETD-pNA (isoleucine, glutamic acid, threonine, aspartic acid-p-Nitroanilide) and Ac-DEVD-pNA (aspartic acid, glutamic acid, valine, aspartic acid-p-Nitroaniline) were obtained from Sigma-Aldrich. Ly294002 was obtained from Calbiochem. Ready To Go T-Primed First-Strand Kit, chemiluminiscent system kit (ECL), and nitrocellulose (NC) membranes (Hybond-ECL) were obtained from Amersham Biosciences and Sybr Green I nucleic acid stain from Roche. Human recombinant TNF-alpha and agarose was obtained from Promega, ethidium bromide (EtBr) from Mallinckrodt, sodium dodecylsulfate (SDS), acrylamide, bis-acrylamide, Triton X-100, Folin-Ciocalteu's reagent, Tween 20 and dimethylsulfoxide (DMSO) from Merck, and foetal bovine serum (FBS) and penicillin-streptomycin from PAA Laboratories GmbH. Protein A-agarose was from BD Transduction Laboratories. Recombinant human erythropoietin (rHuEpo, Hemax) was supplied by Biosidus (Argentina).

Cell line and cultures

Human SH-SY5Y neuroblastoma cells (CRL-2266, American Type Culture Collection ATCC) were used. Cells were grown in 25 cm^2 culture bottles (Falcon BD) containing 5 ml of 1:1 D-MEM:Ham F12 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) heat-inactivated foetal calf serum. The medium was routinely replaced every 2 days and cultures maintained at 37 °C in humidified atmosphere containing 95% air–5% CO_2.

Cellular extracts and subcellular fractions

Cellular extract

Cells (10^7) were washed with ice-cold phosphate buffer saline (PBS) and suspended in 200 μl of ice-cold hypotonic lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100), with protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin, 2 μg/ml leupeptin, 1.37 μg/ml pepstatin A). After 60 min-incubation on ice, the insoluble material was removed by centrifugation for 15 min at 15,000 *g* and 4 °C. Total proteins were quantified by the Lowry's method [18].

Nuclear and cytoplasm separation

After experimental treatments, cultured cells (2×10^6), placed on ice, were scraped, added to 1.5 ml cold PBS, pelleted for 5 min at 1000 *g*, at 4 °C, and suspended in 100 μl of cold buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) supplemented with 0.5% Nonidet P-40. The cytoplasm containing supernatant, added to 50 μl of buffer B (10 mM Tris pH 7.5, 7.0 M urea, 1% SDS, 0.3 M sodium acetate, 20 mM EDTA), was immediately mixed and stored at −20 °C. The nuclear pellet was resuspended in 50 μl of ice-cold buffer C (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT,

1 mM PMSF), and the tube was vigorously shacked at 4 °C for 15 min. The nuclear extract was centrifuged for 15 min at 15,000 *g*, at 4 °C and the supernatant was frozen in aliquots at −70 °C.

Determination of cell viability

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay modified from that of Mossman [19] was used to assess cell viability. Cells were cultured in 35 mm Petri dishes at a density of 1×10^5 cell/ml. After the cells had been subjected to appropriate treatments and the medium removed, they were incubated for 4 h at 37 °C with MTT at a final concentration of 0.5 mg/ml. The supernatant was removed and the pellet washed with PBS. Finally, 100 µl of 0.04 M HCl in isopropanol was added to dissolve the blue formazan product (reduced MTT), which was quantified by measuring the absorbance at 570 nm test wavelength and at 655 nm reference wavelength.

Assessment of nuclear morphology

Cells were cultured on slide covers plated in 35 mm Petri dishes. Different treatments were assayed after obtaining 50% confluence (48 h). Cells were then fixed with 4% paraformaldehyde in PBS (v/v) for 20 min at 4 °C, exposed to 0.05 g/l Hoechst 33258 dye in PBS for 30 min at room temperature, washed thrice with PBS and, finally, mounted by using mounting buffer (50% glycerol in PBS, v/v). Four plates per experimental condition were simultaneously run. Fluorescent nuclei with apoptotic characteristics were detected by microscopy under UV illumination at 365 nm (Eclypse E600 Fluorescent Microscope, Nikon, Japan). Differential cell counting was performed by analysing at least 500 cells [20]. The images were photographed by Nikon Coolpix 5000 equipment and then digitalised.

Detection of DNA fragmentation by electrophoresis

Approximately 5×10^6 cells were collected in culture medium, pelleted at 1000 *g* for 5 min at 4 °C and washed twice with PBS. The pellet was suspended in 500 µl of lysis buffer (0.1 mM NaCl, 25 mM EDTA, 10 mM Tris–HCl pH 8.0, 1% Nonidet P40, v/v). Digestion in the presence of 100 µM DNAse-free RNAse A was allowed to proceed during 2 h at 56 °C and then, proteinase K was added at 10 µM final concentration. Samples were incubated for 16 h at 37 °C and afterwards, subjected to precipitation with 10 M ammonium acetate-absolute ethyl alcohol (1:6 v/v). Electrophoresis of DNA samples were carried out during 90 min at 100 V on 2% agarose gels (w/v), containing 0.5 mg/l EtBr. Bands were visualised under an UV transilluminator and digitalised with Kodak DC240 equipment.

Assay for caspase 3 activity

After appropriate treatments, 1×10^7 cells were harvested by centrifugation at 1000 *g* for 5 min at 4 °C. Cell pellets were washed with 1 ml of PBS, then suspended in 100 µl of lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT) containing 1 mM PMSF protease inhibitor, and maintained for 30 min on ice with occasional agitation. Cell debris was removed by centrifugation at 15,000 *g* for 20 min at 4 °C. Cell lysates were transferred to a microplate, placing 50 µl per well. Then, 10 µl of 7.8 mM Ac-DEVD-pNA were added and the volume completed to 100 µl with the reaction buffer (100 mM HEPES pH 7.5, 0.5 mM EDTA, 5 mM DTT, 20% glycerol). Plates were covered and incubated at 37 °C for 2–4 h until a yellowish colour was observed. The amount of released p-nitroaniline was measured spectrophotometrically at 405 nm in a microplate reader (BioRad).

Assay for caspase 8 activity

Cells were collected by centrifugation at 1000 *g* for 5 min at 4 °C. Supernatants were gently removed and discarded while cell pellets were lysed by addition of 50 µl of the same lysis buffer as that used to measure caspase 3 activity. Cell lysate was incubated on ice for 10 min and then centrifuged at 15,000 *g* for 10 min. For the reaction, 50 µl of cell lysate, 50 µl of the reaction buffer (20 mM HEPES pH 7.4, 2.0 mM EDTA, 5 mM DTT, 1% CHAPS, 50% sucrose), and 5 µl of 20 mM IETD-pNA, the chromogenic substrate for caspase 8, were added to each well. The absorbance at 405 nm was read in a microplate reader every 10 min during 3 h.

Western blotting

Aliquots of whole cell extracts or subcellular fractions corresponding to 100 µg of total proteins were boiled for 1 min in the Laemmli sample buffer [21] and analysed by polyacrylamide gel electrophoresis (T=10%) with sodium dodecylsulfate (SDS-PAGE) using Tris–Glycine pH 8.3 (25 mM Tris; 192 mM Glycine; 0.1% SDS) as running buffer. The gels were then electroblotted onto nitrocellulose membranes during 1.5 h using transfer buffer pH 8.3 (25 mM Tris, 195 mM glycine, 0.05% SDS, and 20% (v/v) methanol). The blots were blocked by 1 h-incubation in TBS (25 mM Tris, 137 mM NaCl, 3 mM KCl, pH 7.4) containing 0.1% Tween 20 and 0.5% skim-milk powder, and then incubated in the presence of appropriate concentrations of the corresponding specific antibody. After washing three times with TBS–0.1% Tween 20, the immunoblots were probed with horseradish peroxidase-conjugated second antibody (1:1000) for 1 h at 25 °C and washed. Immunoreactive blotted proteins were visualised by chemiluminiscence (enhanced chemiluminiscence system, ECL kit, Amersham Biosciences) and the bands detected by using Fujifilm Intelligent Dark Box II equipment (Fuji, Japan) coupled to a LAS-1000 digital camera. Appropriate controls of protein amount loaded in electrophoretic runs and used for band density comparison are indicated in the figures.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated by means of Trizol Reagent, according to Chomczynski and Sacchi [22]. RNA was reverse transcribed by using Ready-To-Go T-Primed First-Strand Kit and the cDNA was amplified by PCR. Specific primers were used for Bcl-2 and Bcl-xL [23] and GAPDH [24]. PCR reactions were performed in a Mastercycler Gradient (Eppendorf) at the following PCR cycling conditions: an initial denaturing step at 94 °C for 5 min, 30 cycles repeating 94 °C for 45 s, 60 °C for 45 s and 72 °C for 45 s, and a final elongation step at 72 °C for 10 min. The PCR products were analysed by electrophoresis on 1.5% agarose gel containing EtBr. Gels were photographed and analysed with the Gel-Pro Analyzer software using GAPDH as gene control. Primers sequence: EpoR sense: 5′ - tctgaagcagaagatctggcc - 3′, antisense: 5′ - gatcatctgcagcctggtgt - 3′; GAPDH sense: 5′ - tgatgacatcaagaaggtggtgaag - 3′

and antisense: 5′ - tccttggaggccatgtaggccat - 3′; Bcl-2 sense: 5′ - agatgtccagccagctgcacctgac - 3′ and antisense: 5′ - agcttgcat-caccctgggtgcctatct - 3′; Bcl-xL sense: 5′ - cgggcattcagtgacctgac - 3′ and antisense: 5′ - tcaggaaccagcggttgaag - 3′.

Quantitative real-time PCR

Real-time PCR assays for EpoR, TNFR1, TNFR2 and GAPDH were performed in a 25 µl-final volume containing 1 µl of cDNA (1:10 dilution), 0.20 mM dNTPs, 0.25 µM specific primers, 3 mM $MgCl_2$, 2 U Taq DNA polymerase, and 1:30,000 dilution of Sybr Green. Specific primers were used for TNFR1 and TNFR2 [25]. Real-time PCR reactions were performed in a DNA Engine Opticon (MJ Research Inc.) and consisted of an initial denaturing step (94 °C for 5 min), followed by 40 cycles (each of 94 °C for 45 s, 60 °C for 45 s and 72 °C for 45 s). Sample quantification was normalised to endogenous GAPDH. Each assay included a DNA minus control and a standard curve performed with serial dilutions of control cDNA coming from SH-SY5Y cells maintained in D-MEM:HamF12, 10% FBS. All samples were run in duplicate and the experiment was repeated three times with independently isolated RNA. Primers sequence: EpoR sense: 5′ - tggtatctgactctggcatctc - 3′ and antisense: 5′ - tccctgatcatctgcagcc - 3′; TNFR1 sense: 5′ - tcgatttgctgtaccaagtg - 3′ and antisense: 5′ - gaaaatgaccaggggcaacag - 3′; TNFR2 sense: 5′ - ccagtgcgttggacagaa - 3′ and antisense: 5′ - ggcttcatcccagcatca - 3′.

Confocal laser scanning microscopy

Cells were cultured on slide covers plated in 35 mm Petri dishes. After obtaining 50% confluence (48 h), cell cultures were subjected to different treatments. During the entire following procedure, dishes were maintained on ice and washings with cold PBS for 10 min were performed between the different procedure steps. After culture medium removal, cells were fixed with methyl alcohol during 30 min. After blocking with 3% albumin in PBS for 1 h, an overnight incubation with the specific primary antibody anti-NFκB (1:100 dilution) was performed. An Alexa Fluor 488 dye-labelled secondary antibody was used and nuclei were stained with propydium iodide. After mounting, confocal laser microscopy was carried out on Laser Confocal Olympus Fluoview equipment, under appropriate lasers. Co-localisation sequential scanning was performed and digital images were obtained.

Statistics

Results are expressed as Mean±SEM. When corresponded, the non-parametric Mann–Whitney *U*-test or the Kruskal–Wallis One Way Analysis of Variance Test was employed. At least differences with $P<0.05$ were considered the criterion of statistical significance.

Results

Apoptosis induced by TNF-α in SH-SY5Y cells

Time-course and dose-response analysis was developed to investigate a possible proapoptotic effect of TNF-α upon cells of neuronal origin. SH-SY5Y cell cultures were exposed to different TNF-α concentrations (10, 25, 50 or 100 ng/ml), varying the length of the incubation periods (6, 12 or 24 h).

Decreased cell viability and characteristic features of apoptosis were already observed after a 12 h-incubation in the presence of 25 ng/ml TNF-α. As can be seen in Fig. 1, at this concentration, TNF-α induced a significant decrease ($P<0.001$) in cell viability

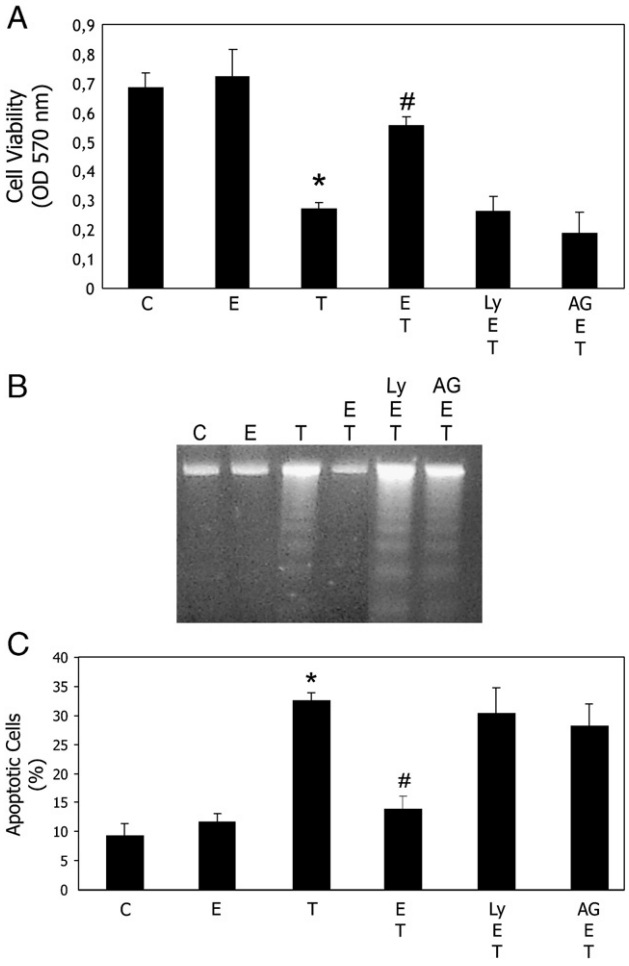

Fig. 1 – Proapoptotic effect of TNF-α and neuroprotective action of erythropoietin. Cells, preincubated with 25 U/ml rhuEpo during 12 h, were then cultured with or without 25 ng/ml TNF-α for 12 h (ET and E, respectively). Non Epo-pretreated cells were also incubated in the presence of 25 ng/ml TNF-α for a 12 h (T) and control cells received no treatment (C). Some cultures were developed in the presence of 25 µM Ly294002 (PI3K inhibitor) or 25 µM AG490 (Jak2 inhibitor) before the addition of Epo (LyET and AGET). At the end of the whole treatment period, cell viability and apoptosis signals were determined by different assays with congruent results. (A) Cell viability, determined by the MTT assay, showed a significant decrease induced by TNF-α (*T *vs.* C, $P<0.001$, $n=5$) which was almost prevented by the Epo pre-treatment (#ET *vs.* T, $P<0.01$, $n=5$). (B) The typical ladder pattern induced by TNF-α was not observed under the protective effect of Epo. (C) Significant changes in nuclear morphology was observed under fluorescence microscopy by staining with Hoechst dye after treatment with TNF-α (*T *vs.* C, $P<0.01$, $n=5$). Cell protection was observed by the Epo pre-treatment (#ET *vs.* T, $P<0.01$, $n=5$). This effect was abrogated when cultures were performed in the presence of either Ly294002 or AG490 ($n=3$, A–C). Each bar represents Mean±SEM.

measured by the MTT assay (Fig. 1A), the appearance of a DNA degradation ladder pattern analysed by electrophoresis in agarose gels (Fig. 1B), and a significant increase ($P<0.001$) in the number of apoptotic cells showing nuclear chromatin condensation evaluated by fluorescence microscopy after Hoechst 33258 staining (Fig. 1C).

Activation of caspases associated to TNF-α-induced apoptosis

The extrinsic apoptotic pathway activated by ligand-bound death receptors such as TNF-α is closely related to the activation of caspase 8 [26]. We then investigated the activation of caspases and PARP cleavage as signals of SH-SY5Y cell response to TNF-α treatment. Caspase activities were measured by spectrophotometric methodology, using two different chromogenic substrates, IETD-pNA for caspase 8, and Ac-DEVD-pNA for caspase 3, whereas PARP cleavage was evaluated by Western blotting.

Both, caspase 8 and caspase 3 activities increased due to activation of death receptors by TNF-α (Figs. 2A and 2B) and cultures performed in the presence of the proinflammatory cytokine resulted in endogenous PARP cleavage, yielding an 85-kDa fragment (Fig. 2C). Therefore, results obtained on caspase 3 activity, in accordance with those of the PARP cleavage, confirmed the activation of caspase 3.

The results support that TNF-α-induced SH-SY5Y cell killing activated apoptotic pathways that involve caspase activation mostly through extrinsic apoptotic mechanisms.

Prevention of TNF-α-induced apoptosis by erythropoietin

Previously, we have demonstrated the expression of Epo receptors in the SH-SY5Y cell line and an Epo antiapoptotic effect by treating these cells with 25 U/ml Epo for 12 h before the induction of apoptosis by staurosporine [6]. In a previous work, we investigated the protective effect of Epo at different concentrations and found that even though at 10 U/ml it showed antiapoptotic action, this dose proved not to be suitable to completely prevent the effect of 25 nM staurosporine [6]. Therefore, in the present work, similar pretreatment was used as a model to study whether Epo might be able to prevent the apoptosis induced by TNF-α.

Epo did not promote cell growth but it prevented apoptosis, as reflected by the fact that DNA degradation and characteristic apoptotic nuclear images as well as caspase activation induced by TNF-α were found to be almost completely abolished by the Epo pretreatment (Figs. 1 and 2). Taking together, these findings confirmed that Epo prevents the apoptosis of SH-SY5Y cells induced by the proinflammatory cytokine. However, this protective effect was not detected when Epo and TNF-α were simultaneously added to cell cultures (data not shown).

Erythropoietin-mediated neuroprotection involves Jak2 and PI3K pathways

It is known that Jak2 and STAT molecules are involved in Epo-mediated signal transduction pathways activated by Epo binding

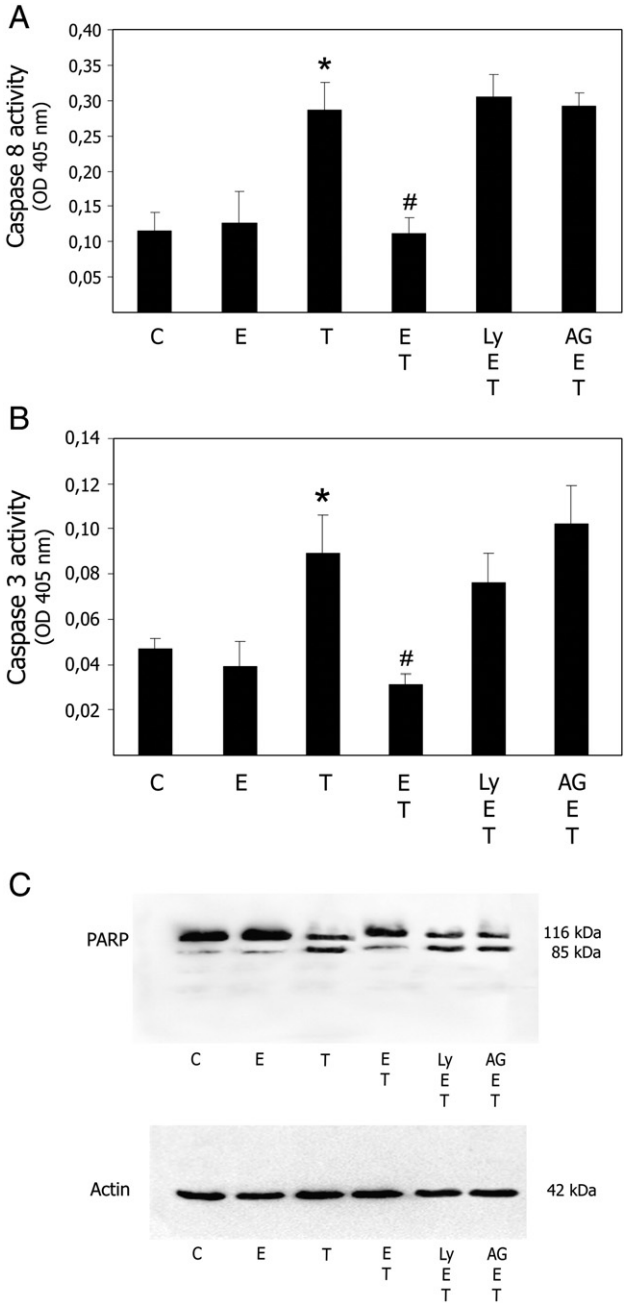

Fig. 2 – Activation of caspases by TNF-α. Cells, preincubated with 25 U/ml rhuEpo during 12 h, were then cultured with or without 25 ng/ml TNF-α for additional 12 h (ET and E, respectively). Non Epo-pretreated cells were also incubated in the presence of 25 ng/ml TNF-α for 12 h (T), and control cells received no treatment (C). Assays in the presence of the inhibitors Ly294002 or AG490 were carried out for 2 h previously to the Epo-pretreatment. Caspase activity was determined by cleavage of two different chromogenic substrates, IETD-pNA for caspase 8 (A) and Ac-DEVD-pNA for caspase 3 (B), and the product absorbance after a 4 h-incubation was measured at 405 nm. PARP cleavage was evaluated by the presence of the 85 kDa band observed after Western blotting (C). Both caspase 8 and caspase 3 activities were enhanced due to the action of TNF-α (*T *vs.* C, $P<0.01$). Epo prevented caspase activation (#ET *vs.* T, $P<0.005$) whereas cell pretreatments in the presence of either Ly294002 or AG490 cancelled the Epo protective effect (A–C). Each bar represents Mean±SEM corresponding to 3 independent assays.

to the EpoR [3,27]. On the other hand, pathways mediated by phosphatidylinositol 3-kinase (PI3K) were found to be involved in Epo-mediated erythroid and neuronal cell protection [6,7,28]. To test the contribution of Jak2 and PI3K signalling pathways to Epo-mediated SH-SY5Y neuronal cell survival when treated with TNF-α we incubated cells with the selective inhibitors AG490 and LY294002, respectively. Sequential cell treatments were carried out for 2 h in medium containing 25 μM AG490 or 25 μM Ly294002, additional 12 h-incubation with 25 U/ml Epo, and then, cultured in the presence of 25 ng/ml TNF-α.

Results in Figs. 1 and 2A–C showing that Jak2 inhibition completely abolished the antiapoptotic effect of Epo point to the direct substrate of Jak2 as an early regulator in the antiapoptotic cascade activated by Epo in our experimental model.

On the other hand, a significant decrease in cell viability and an increase in apoptotic signs observed in cells cultured in the presence of Ly294002 suggest an important role for the PI3K signalling pathway in the cell protective role of Epo (Figs. 1 and 2).

Antiapoptotic mechanisms induced by erythropoietin

Modulation of TNF receptors

It is known that TNF signalling is mediated via two distinct receptors, TNFR1 and TNFR2, which showed partially overlapping signalling mechanisms and biological roles depending on cell type [29]. By Real time PCR, TNFR1 was found constitutively expressed in SH-SY5Y cells at basal levels, while significant upregulation ($P<0.05$) was observed by incubation in the presence of TNF-α (Fig. 3).

To further analyse whether the observed antiapoptotic contribution of Epo could be related to modulation of death receptors, we examined the expression of TNFR1 in cells subjected to Epo

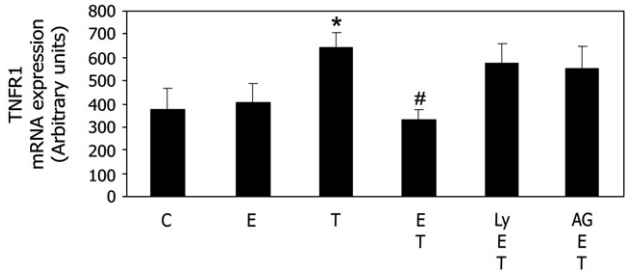

Fig. 3 – Modulation of TNFR1 mRNA expression by erythropoietin. Real time-PCR was performed to analyse TNFR1 mRNA levels, using GAPDH as internal control. Cells, preincubated with 25 U/ml rhuEpo for 12 h, were then cultured with or without 25 ng/ml TNF-α for another 12 h-period (ET and E). Non Epo-pretreated cells were also incubated in the presence of 25 ng/ml TNF-α for 12 h (T) while control cells received no additional treatment (C). Assays in the presence of the inhibitors Ly294002 or AG490 were carried out for 2 h before the addition of Epo to cultures. TNFR1 was found constitutively expressed and significantly upregulated (*T *vs.* C, $P<0.05$) by the presence of TNF-α. This cytokine-induced expression of TNFR1 was counteracted by the Epo-pretreatment (#ET *vs.* T, $P<0.05$) through mechanisms involving Jak2 and PI3K activation. Bars indicate Mean ± SEM of TNFR1 mRNA levels expressed as arbitrary units with respect to GAPDH ($n=3$ independent assays).

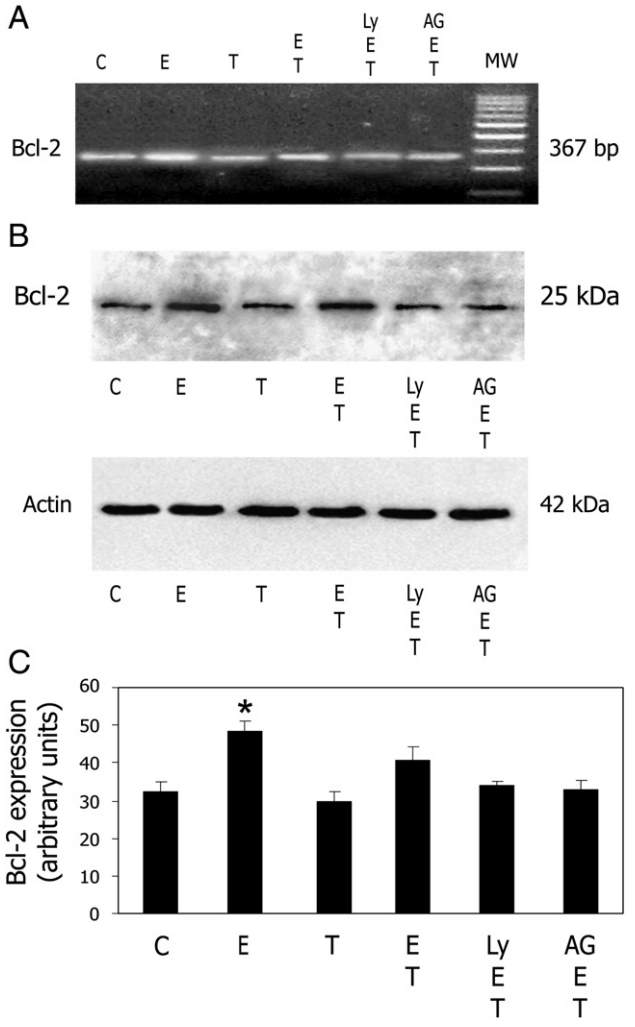

Fig. 4 – Upregulation of Bcl-2 by erythropoietin. Cells preincubated with 25 U/ml rhuEpo for 12 h were then cultured with or without 25 ng/ml TNF-α for another 12 h-period (ET and E). Non Epo-pretreated cells were also incubated in the presence of 25 ng/ml TNF-α during 12 h (T) while control cells received no additional treatment (C). Assays in the presence of the inhibitors Ly294002 or AG490 were carried out for 2 h before the period of Epo-pretreatment. Bcl-2 expression was detected at mRNA and protein levels by using RT-PCR (A) and Western blotting (B) assays, respectively. The photographs are representative of 3 different assays. Density of chemiluminiscent bands was estimated by an appropriate software and expressed as arbitrary units with respect to actin (C). Each bar represents Mean ± SEM of 3 different independent assays. Bcl-2 expression was significantly induced by Epo (*E *vs.* C, $P<0.05$) but this effect was only partially demonstrated in the presence of TNF-α.

treatment previously to the TNF-α-induced apoptosis. The increased expression of TNFR1 induced by TNF-α was completely abolished by Epo (Fig. 3, ET *vs.* T, $P<0.05$), through mechanisms mediated by Jak2 and PI3K activation (Fig. 3). These results contributed to explain not only the apoptotic effect of TNF-α but at least one mechanism related to the Epo protective action in the presence of this proinflammatory cytokine.

Conversely, the very low levels of TNFR2 detected in SH-SY5Y cells were not found modified under the effect of TNF-α (data not shown).

Modulation of members of the Bcl-2 family

It has been reported that modulation of members of the Bcl-2 family are associated to cell protection by Epo. On the basis that overexpression of *bcl-2* gene delays caspase 3 activation [30], we decided to further investigate whether Bcl-2 factors are involved in the antiapoptotic effect of Epo. Consistent with its action upon the SH-SY5Y cells, Epo was found to induce an increased expression of Bcl-2 at both mRNA and protein levels (Fig. 4). As mentioned before, cell stimulation by Epo is mediated by the activation of Jak2 and PI3K pathways. Therefore, inhibitors of these signalling pathways modulated negatively the Epo-induced expression of Bcl-2 (Fig. 4). This effect was only partially observed in the presence of TNF-α. On the other hand, neither TNF-α nor Epo were able to modulate the expression of other members of this family, such as the antiapoptotic factor Bcl-x_L and the proapoptotic factor Bax (Data not shown).

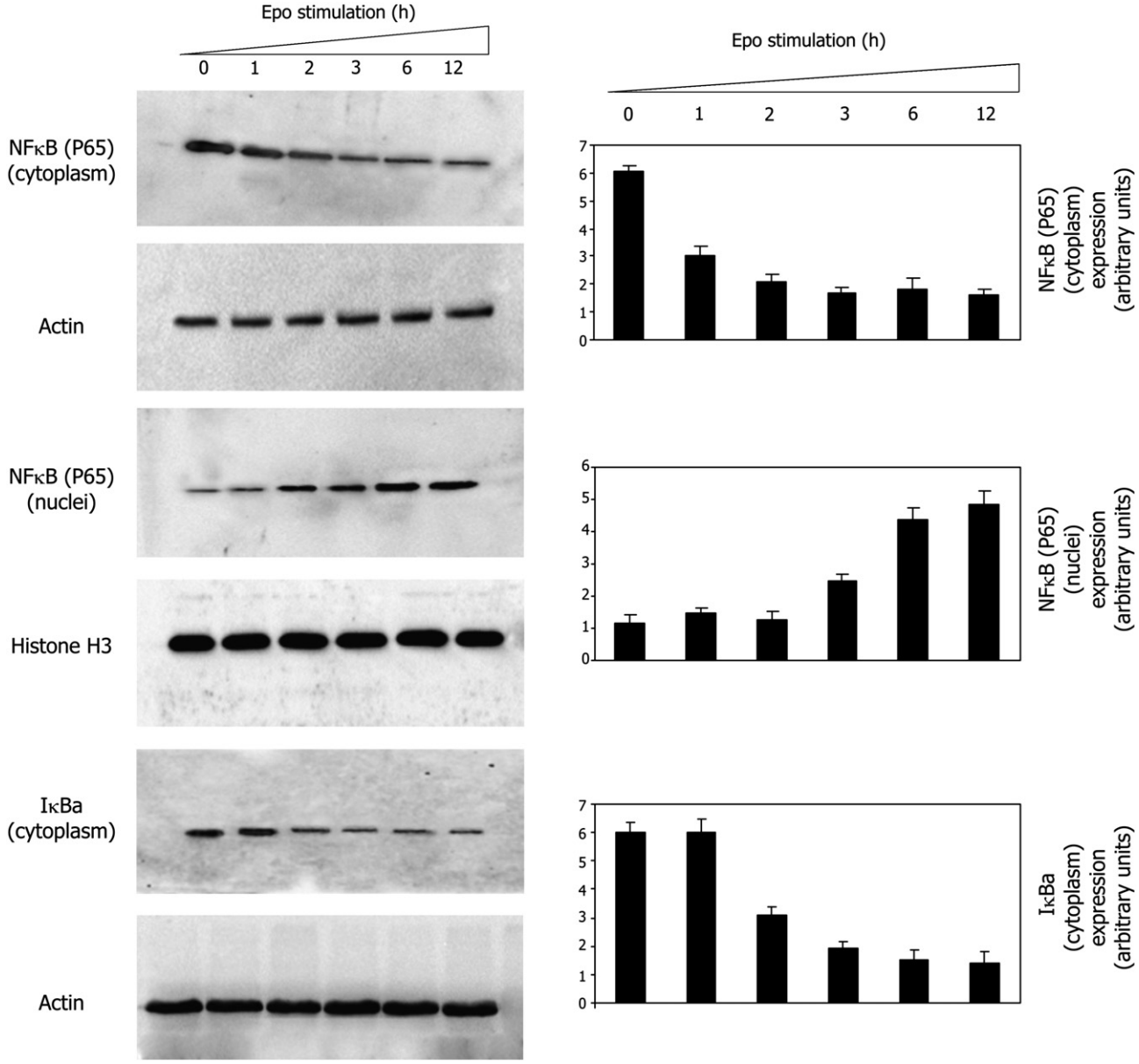

Fig. 5 – NF-κB involvement in cell activation by erythropoietin. Kinetic assays were performed in the presence of Epo to detect nuclear translocation of NF-κB. Cell subfractions were prepared from cell cultures treated with 25 U/ml Epo for 0, 1, 2, 3, 6 and 12 h. Samples from nucleus and cytosol were analysed by Western blotting with anti-p65 antibody or anti-IκB antibody. Either actin or histone was used as internal control in assays using cytosol or nuclear fractions, respectively. The time-course of disappearance of NF-κB from the cytosol is consistent with the appearance of this factor in the nuclear fraction. IκB degradation, detected along the incubation period with Epo, also support the process of NF-κB activation. The photographs are representative of 3 different assays. Density of chemiluminiscent bands was estimated by appropriate software and expressed as arbitrary units. Each bar represents Mean±SEM of different 3 independent assays.

Induction of NF-κB

Evidence has suggested an important role of NF-κB proteins as intracellular messengers transmitting Epo-derived signals to the nucleus [31,32]. Besides, cross-talk between Jak2 and NF-κB has been related to erythropoietin-mediated neuroprotection [33]. Since activation of the Jak2 pathway was found to be involved in Epo-mediated protection of SH-SYY cells against the apoptosis induced by TNF-α (Figs. 1–4), we investigated the role of NF-κB in cell activation by Epo. After SH-SY5Y cell exposure to Epo for different periods, cytosol and nuclear fractions were separated to analyse the NF-κB and IκB localisation by immunoblotting. Fig. 5 shows that the time-course of disappearance of NF-κB from the cytosol was found to be closely associated to increasing levels of this factor in the nuclear fraction. At the same time, the disappearance of IκB was consistent with its ubiquitination and proteasomal degradation, allowing NF-κB translocation to the nucleus where it activates the transcription of its target genes.

Based on these results, we decided to further investigate possible modulation of c-FLIP$_L$ (cellular FLICE-inhibitor protein), the expression of which is regulated by the transcriptional activity of NF-κB [34]. This protein, a catalytically inactive caspase 8 homologue, functions as a caspase 8 dominant negative, blocking the apoptosis triggered by death receptors [35]. Notwithstanding, the levels of c-FLIP$_L$ RNAm were found unchanged at different cell treatments (data not shown), therefore suggesting that this antiapoptotic factor is not affected by the TNF-α-induced apoptosis or by the neuroprotection due to Epo.

Activation of STAT5

Once the activation kinetics of NF-κB was established, it was interesting to study other classic signalling pathways activated by EpoR. Because of the role of Jak2 signalling pathways in the Epo antiapoptotic effect, time-course of STAT5b activation was determined by detecting this transcription factor in cytosolic and nuclear subfractions at different times of Epo exposure.

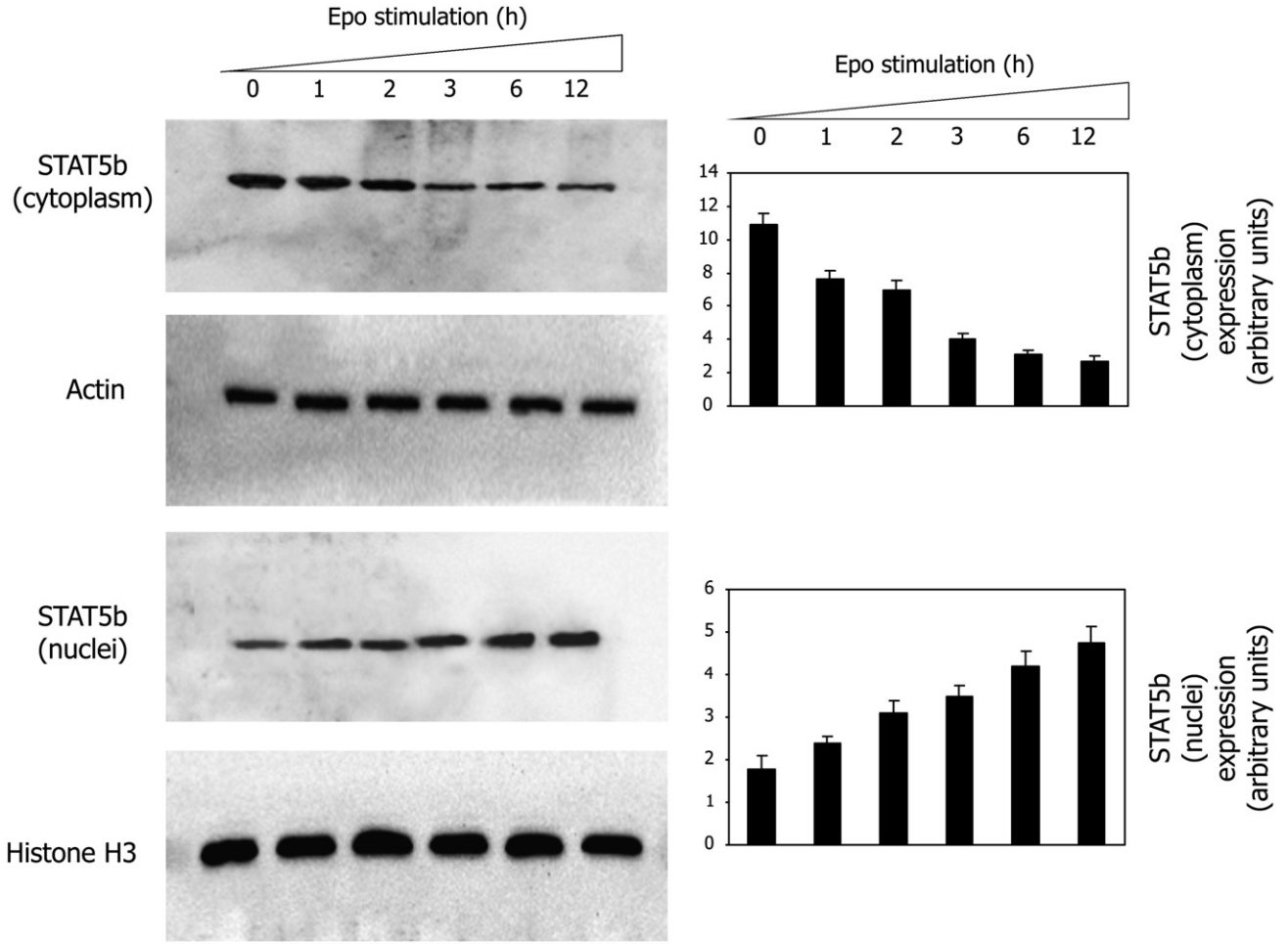

Fig. 6 – STAT5 involvement in Epo activated effect. Cells incubated in the presence of 25 U/ml Epo for different periods (from 0 to 12 h) were used to analyse nuclear translocation of STAT5b. Cell subfractions were obtained as indicated in Materials and methods and analysed by Western blotting using anti-STAT5b antibody. Either actin or histone was used as internal control of protein loading in assays corresponding to the cytosolic or nuclear fraction, respectively. The photographs are representative of 3 different assays. Density of chemiluminiscent bands was estimated by appropriate software and expressed as arbitrary units. Each bar represents Mean±SEM of different 3 independent assays. The time-course of disappearance of STAT5b from the cytosol was found to be consistent with that of the appearance of this factor in the nuclear fraction. The blots are representative of 3 independent assays with similar findings.

It can be seen in Fig. 6 that the disappearance of STAT5 from the cytosol is closely associated to increasing levels of this factor in the nuclear fraction, a showing STAT5 activation kinetic similar to that observed for NF-κB.

Interplay between signalling pathways induced by erythropoietin and TNF-α

NF-κB: a common transcription factor between Epo and TNF-α

Although cells treated with TNF-α may undergo apoptosis, the activation of additional antiapoptotic pathways by the proinflammatory cytokine may also modulate programmed cell death. Taking into account that cell death receptors are known to initiate signals that regulate NF-κB activation, we investigated this nuclear factor activation by Western blotting in a set of experimental assays. Fig. 7 shows increasing nuclear localisation of NF-κB due to the effect of either Epo or TNF-α. Moreover, under our experimental conditions, TNF-α and Epo appeared to have additive effects (Fig. 7A, ET *vs*. E or T). Despite of the decreased activation of NF-κB detected in cell cultures developed in the presence of the inhibitors Ly294002 and AG490, this additive action of Epo plus TNF-α could not be completely blocked (Fig. 7A).

To corroborate these data, NF-κB nuclear localisation was observed by confocal laser scanning microscopy (Fig. 8B). Consistent with results of NF-κB protein levels, the analysis of confocal images demonstrated the presence of NF-κB in nuclei after cell incubation in the presence of TNF-α, Epo, or both factors simultaneously.

Modulation of erythropoietin receptor by TNF-α

The NF-κB activation induced by the presence of either TNF-α or Epo suggested the involvement of common mediators in the regulatory mechanism of our experimental model. The way in which pathways activated by Epo and TNF-α interact and regulate each other may have an effect on cell fate to apoptosis or survival. This suggestion and the lack of an antiapoptotic effect of Epo when added to cell cultures simultaneously with TNF-α lead us to further investigate potential cross-talk between signalling pathways activated by both factors. In this context, the following question was analysed: is TNF-α capable of regulating the expression of the erythropoietin receptor?. By using Real time-PCR and Western blotting, EpoR was found to be negatively modulated by the inflammatory cytokine and this effect was at least in part counteracted by the cell pretreatment with Epo (Fig. 9).

Discussion

In this work, we have characterised some aspects of the process of cell death triggered by the inflammatory cytokine TNF-α in the human SH-SY5Y cell line derived from neuroblastoma. We have identified apoptosis as the type of cell death induced by TNF-α. Results indicate that exposure to 25 ng/ml TNF-α for at least 12 h induced apoptosis of SH-SY5Y cells (Figs. 1 and 2). Since it is known that an apoptotic cell is characterised by morphological, biochemical and genetic changes and that these signs are not always simultaneously present, special care was taken to detect independent or dependent death phenomena by using different methods. Congruent results were found among qualitative and quantitative data, such as those determining chromatin condensation (Hoechst staining and fluorescent measurement), DNA fragmentation (ladder electrophoresis), and caspase activation (enzymatic ability to cleave chromogenic substrates and to produce PARP degradation) (Figs. 1 and 2).

Tumour necrosis factor (TNF), a potent cytokine that exerts pleiotropic functions related to immunity, inflammation, and apoptosis, is the prototypical member of a family of cytokines with still growing functions. Although most TNF-α is recognised as a potent inducer of the signalling pathways that lead to activation of the transcription factor NF-κB, it can also induce apoptosis by activation of death receptors. TNF-α binds and induces signals through two distinct receptors, TNFR1 and TNFR2, which are present in most cells. In the SH-SY5Y cell line, we detected mainly TNFR1 and only a slight expression of TNFR2. However, it has been

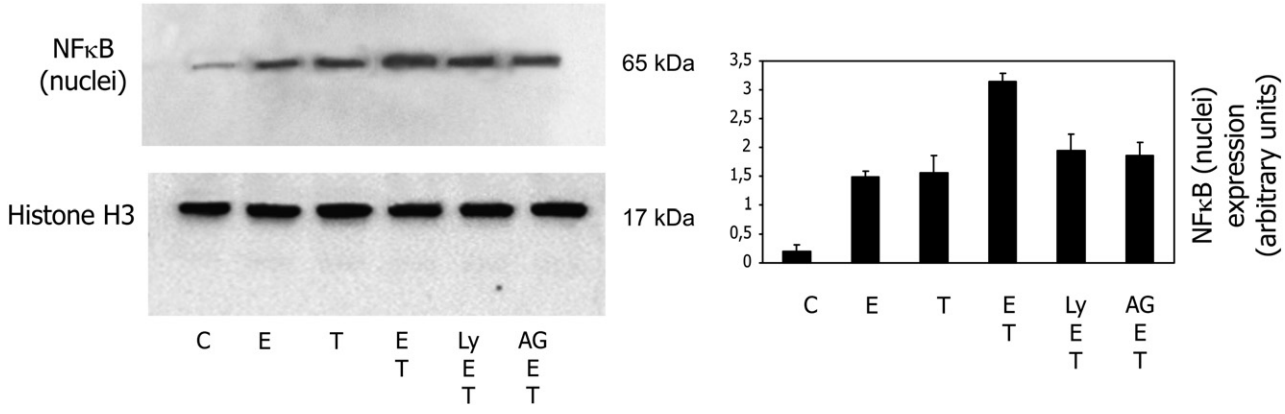

Fig. 7 – NF-κB activation by TNF-α and Epo. Cell, preincubated with 25 U/ml Epo for 12 h, were then cultured with or without 25 ng/ml TNF-α for another 12 h-period (ET and E). Non Epo-pretreated cells were also incubated in the presence of 25 ng/ml TNF-α during 12 h (T) while control cells received no treatment (C). Assays in the presence of the inhibitors Ly294002 or AG490 were carried out during 2 h before the period of Epo-pretreatment. Western blots show increased NF-κB nuclear levels in cell cultures incubated in the presence of Epo, TNF-α or both factors at the same time. Equal loading of nuclear protein was confirmed by histone. The blot is representative of three independent assays. Density of chemiluminiscent bands was estimated by appropriate software and expressed as arbitrary units with respect to histone. Each bar represents Mean±SEM of different 3 independent assays.

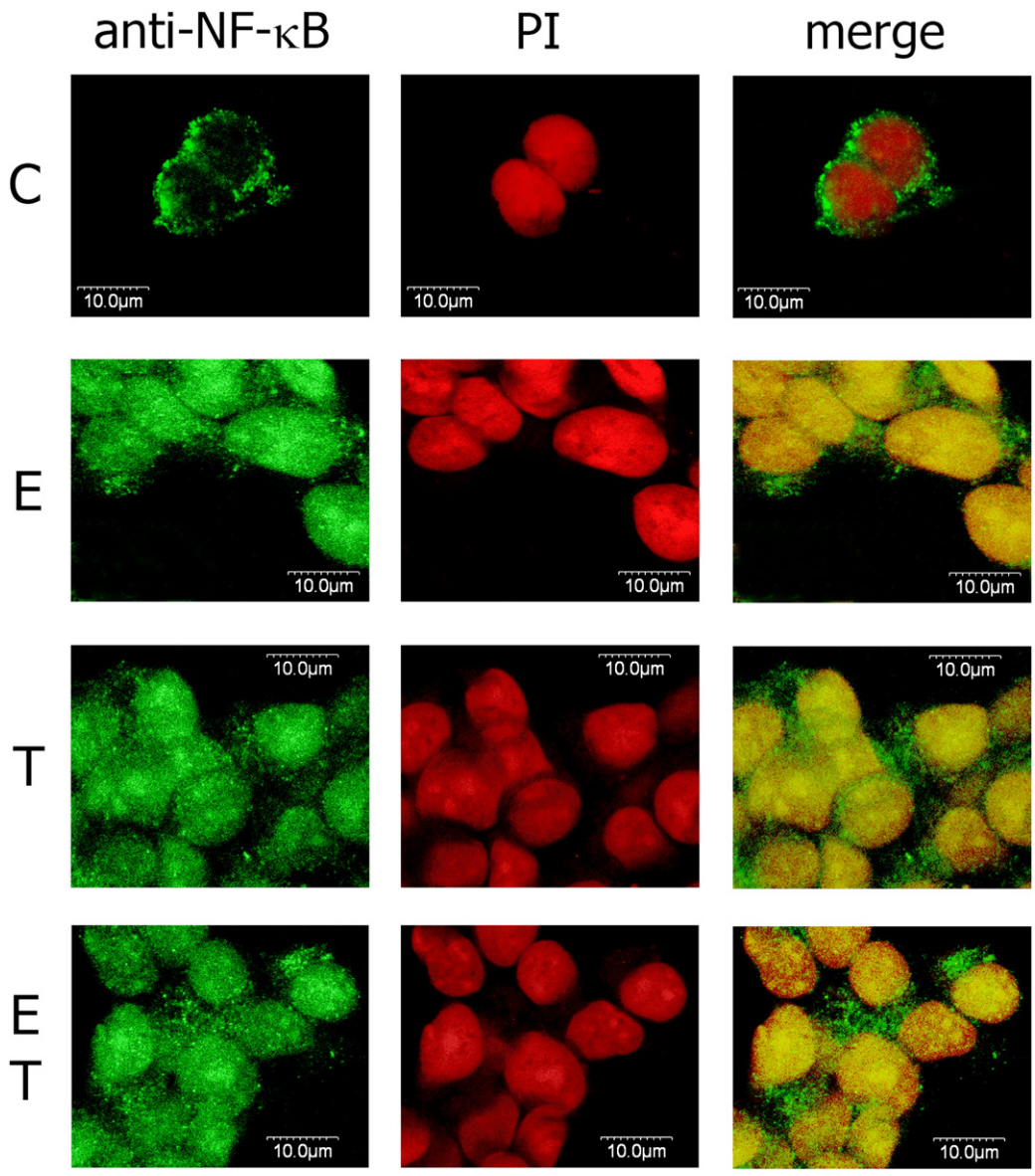

Fig. 8 – Confocal analysis of NF-κB activation by TNF-α and Epo. Similar results to Western blotting analysis was observed when nuclear translocation of NF-κB, induced by Epo and TNF-α, was detected by confocal laser scanning microscopy. Cell, preincubated with 25 U/ml Epo for 12 h, were then cultured with or without 25 ng/ml TNF-α for another 12 h-period (ET and E). Non Epo-pretreated cells were also incubated in the presence of 25 ng/ml TNF-α during 12 h (T) while control cells received no treatment (C). Cells were then processed for immunofluorescence staining as described in Materials and methods. Green stain indicates the localisation of protein p65, and red stain (PI) indicates the nucleus. The images shown are representative experiments from two other independent determinations with essentially the same findings.

reported that TNFR1 appeared to be sufficient to induce most common TNF responses such as activation of NF-κB, cytotoxicity and proliferation. Nevertheless, it has been demonstrated that in some cell types, TNFR2 can independently mediate cellular responses like activation of NF-κB, proliferation and cell death [29]. Even though more research is needed to fully clarify the role of these TNFRs, some reports indicate diverse involvement of the two receptors in the large repertoire of cellular TNF responses. These differential effects might explain the result found in our work, which shows close association between TNF-induced upregulation of the TNFR1 expression and a proapoptotic effect of the cytokine (Figs. 1C and 3).

Apoptosis mediated by TNFR1 involves two sequential signalling complexes. The initial plasma membrane bound complex (complex I), consisting of TNFR1, the adaptor TRADD (TNFR1-associated death domain protein), the kinase RIP (receptor-interacting protein), and TRAF2, rapidly signals activation of NF-κB. In a second step, TRADD and RIP1 associate with FADD (Fas-associated death domain protein) and procaspase 8, forming a cytoplasmic complex (complex II). Thus, TNFR1-

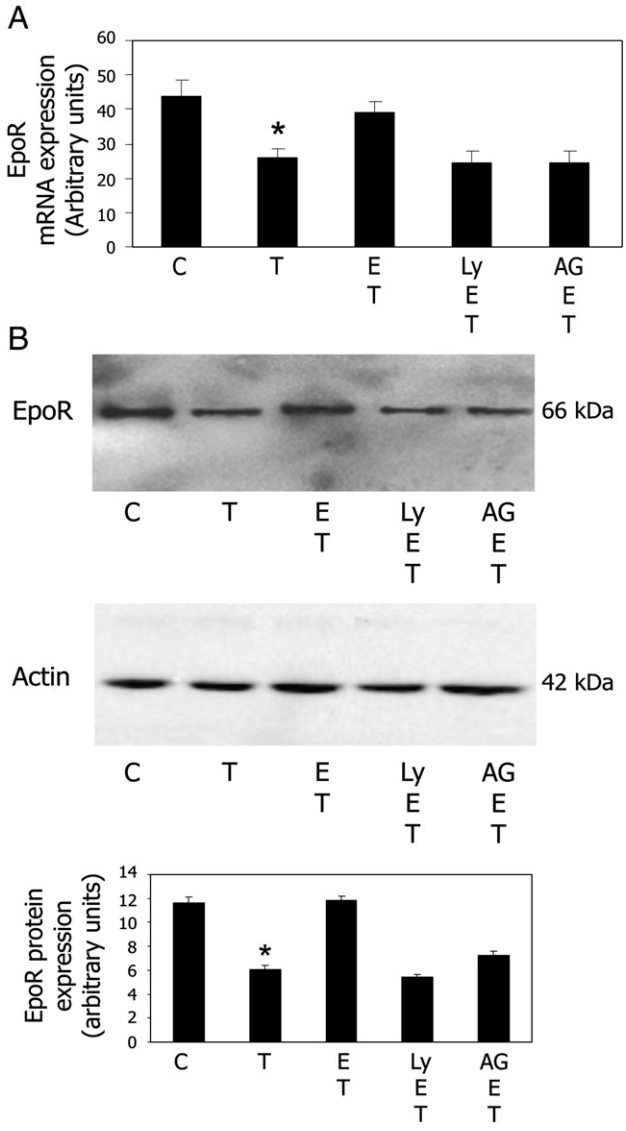

Fig. 9 – Modulation of erythropoietin receptor by TNF-α. Cells preincubated with 25 U/ml Epo for 12 h were cultured with 25 ng/ml TNF-α for another 12 h-period (ET). Non Epo-pretreated cells were also incubated in the presence of 25 ng/ml TNF-α during 12 h (*T*) while control cells received no treatment (*C*). Additional assays were performed in the presence of the inhibitors Ly294002 or AG490 for 2 h previous to the period of incubation with Epo. A negative modulation of EpoR by TNF-α was detected by Real time-PCR (A, **P*<0.05) and Western blotting (B). Results are Mean±SEM of at least 3 experiments in A (expressed as arbitrary units with respect to GAPDH) and representative of 3 experiments in B (expressed as arbitrary units with respect to actin). This effect of TNF-α was counteracted by cell pretreatment with Epo through mechanisms involving Jak2 and PI3K signalling pathways.

mediated signal transduction includes a checkpoint, resulting in cell death in instances where the initial signal fails to be activated [34]. As a consequence of this, activation of procaspase 8 starts a cascade of caspase activation. In accordance with this mechanism, we found increased activity of caspase 8 after the SH-SY5Y cells had been treated with TNF-α (Fig. 2). Caspase 8 then activates downstream caspases, such as caspase 3, which participates in the execution of the apoptotic process. Therefore, consistent results were observed among measurements of caspase 8 and caspase 3 activities, as well as in assays of PARP cleavage, the substrate of activated caspase 3 (Fig. 2).

We have previously demonstrated that the process of apoptosis induced in SH-SY5Y cells by staurosporine could be prevented by cell pretreatment with erythropoietin [6]. In a similar model, complete protection against the apoptotic effect of 25 ng/ml TNF-α was also demonstrated in this work when cells of the SH-SY5Y line were previously treated for 12 h with 25 U/ml Epo TNF-α (Fig. 1). However, Epo failed to prevent the cytokine-induced apoptosis when both compounds were simultaneously added to the culture medium (data not shown). These results suggest activation of cellular pathways during the period of preincubation with Epo, causing increased cell resistance to the subsequent TNF-α-induced apoptosis. Indeed, the negative modulation of EpoR by TNF-α (Fig. 9) may support this hypothesis.

In order to gain further insight into the mechanisms involved in this antiapoptotic effect of Epo, modulation of specific receptors and expression of transcription factors have been evaluated. A significant prevention by Epo of upregulation of the TNFR1 expression induced by TNF-α coincided with an attenuated deleterious effect of the proinflammatory cytokine. This means that the sensitivity shown by SH-SY5Y cells to TNF-α may be partly due to the upregulation of the death receptor expression observed after cell incubation with the cytokine. Therefore, at least one mechanism leading to the Epo neuroprotective effect may be related to the prevention of the increase in binding sites for TNF-α. On the other hand, by using AG490, an inhibitor of Jak2, we have demonstrated the protective action of Epo to be mediated by this signalling pathway. In the hematopoietic system, the mechanisms by which Epo signals are linked to gene transcription have been extensively investigated. Once Epo binds to its receptor EpoR, Jak2 associated to the EpoR cytoplasmic domain rapidly becomes activated by transphosphorylation. Subsequently, tyrosine residues in the EpoR become phosphorylated and recruit multiple signalling molecules that contain Src homology 2 domains, including STAT5 and PI3K. In our model, Epo exerted its protective action on SH-SY5Y cells induced to apoptosis in a PI3K dependent manner; thus, the ability of Epo to exert neuroprotection was found to be decreased in the presence of the specific PI3K inhibitor LY294002 (Figs. 1–4 and 7). In cells stimulated by Epo, the time course analysis of the disappearance of STAT5 from the cytosol was closely associated to increasing levels of this factor in the nuclear fraction (Fig. 6). Therefore, the antiapoptotic effect of Epo in neuronal cells, like its effect upon erythroid progenitors, appeared to be mediated by activation of Jak2/STAT5 and PI3K signals. The results presented in this paper are consistent with studies showing the ability of Epo to exert neuroprotective and cardioprotective action originated in upstream pathways that involve Jak2 activation [33,36]. In this regard, Um and Lodish found an Epo protective action upon differentiated SH-SY5Y cells carrying a prolactin/erythropoietin receptor chimera system, and this Epo effect was associated to activation of STAT5, AKT, and MAPK signalling pathways [3].

As regards the antiapoptotic effect of Epo, we detected the induction of higher Bcl-2 at mRNA and protein levels (Fig. 4). This result agrees with other reports since cell activation by Epo is

known to be controlled by anti-death members of the Bcl-2 family, such as Bcl-2 and/or Bcl-x_L [37,38]. We have previously observed that preincubation with Epo prevented the subsequent staurosporine-induced apoptosis of SH-SY5Y cells through mechanisms mediated by Bcl-x_L, instead of Bcl-2 [6]. However, in the present work, in similar assays run in the presence of TNF-α, Bcl-x_L was not found to be induced. These results suggest that modulation of this antiapoptotic factors depends on the type of apoptotic agent and/or the degree of cell injury. Increased Bcl-2 immunoreactivity was demonstrated in rodent models of hypoxic-ischemic injury but it was not observed in all animals, suggesting that Bcl-2 expression may be influenced by the degree of the insult, species-specific factors, and gender-specific factors [37].

In a physiological context, variable regulatory effects of NF-κB on different promoters offer the potential for target genes to be under the transcriptional control of distinct NF-κB subunits. How it operates within the nervous system and whether it might be a distinguishing feature of NF-κB activation by discrete stimuli and in distinct cell types remains unknown. It has been shown that NF-κB resides in the cytoplasm in an inactive form consisting of three protein subunits, a transcription factor dimer and an inhibitory subunit called IκB. Several different NF-κB DNA-binding subunits have been identified, including p65, p50 and p52 [39]. In this work, we found that Epo treatment of SH-SY5Y cells induced the expression and translocation of NF-κB p65. Kinetic assays showed the disappearance of this factor from the cytoplasm consistent with the increasing levels in the nuclear subfraction (Figs. 5 and 8). The simultaneous decrease in IκB expression suggested rapid degradation of this inhibitor due to activated signalling pathways. These results are consistent with Epo-activation of IKKs, the kinases that phosphorylate IκB and lead to its subsequent ubiquitination and proteasomal degradation, thereby allowing NF-κB to translocate to the nucleus where it activates the transcription of target genes [31].

Interestingly, in our experimental model, we also found activation of NF-κB by TNF-α. It is known that the proinflammatory cytokine TNF plays a key role in a wide variety of physiological processes, including inflammation, proliferation and programmed cell death. These pleiotropic biological effects of TNF result from its ability to initiate different intracellular signalling pathways. It has been reported that activation of TNFR1 is capable of activating two pathways simultaneously, leading to the induction of apoptosis and the activation of NF-κB at the same time [40]. This can explain our finding of increased nuclear localisation of NF-κB not only due to cell activation by Epo but also caused by the presence of TNF-α (Figs. 5, 7 and 8). Moreover, additive NF-κB levels were observed when cell cultures were developed in the presence of both factors, Epo and TNF-α. Nevertheless, the protective effect of NF-κB was not enough to impede TNF-α-induced apoptosis of SH-SY5Y cells (Fig. 1).

In conclusion, we have described mechanisms related to the antiapoptotic effect of Epo against TNF-α-induced cell death and obtained data suggesting a cross-talk between death receptor and Epo/EpoR signalling pathways. A similar cross-talk has been recently identified by the finding that TNFR1 was necessary for the expression of EpoR and then, for Epo mediated neuroprotection in cerebral tissue [41].The results provide evidence for the complexity of the role of individual cytokines and let us suggest that cell sensitivity to death receptor-induced apoptosis may be regulated by several factors, such as cytokine concentration, time of activation, and cross-talk between different signals. Therefore, the balance of these factors, which might decide between cell survival and cell death, might be essential for the cellular pathological outcome. This can explain why, in our experimental design, the incubation of SH-SY5Y cells with TNF-α leads to apoptosis in spite of the cytokine ability to induce NF-κB nuclear translocation. In accordance, Taoufik et al. have suggested that NF-κB may represent the link between pathways activated by TNFR1 and EpoR [41]. The high expression of TNFR1 under TNF-α exposure and the lack of contribution of the Bcl-2 family members may increase neuronal cell sensitivity to the proinflammatory cytokine but it may also sensitise cells to the antiapoptic action of Epo. Our present study also shows further evidence that cells activated by Epo become more resistant to cell death. Prevention of death receptor upregulation and caspase activation may explain this antiapoptotic effect of Epo, which may be favoured by induction of higher expression of protective factors, such as Bcl-2 and NF-κB, through mechanisms involving Jak/STAT and PI3K signalling pathways.

This study underlined the Epo capacity as an anti-inflammatory factor which may play a significant role in stimulating neuroprotection, presumably through its antiapoptotic effects.

Acknowledgments

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REFERENCES

[1] P. Ghezzi, M. Brines, Erythropoietin as an antiapoptotic, tissue-protective cytokine, Cell Death Differ. 11 (Suppl. 1) (2004) S37–S44.
[2] H. Ehrenreich, C. Aust, H. Krampe, H. Jahn, S. Jacob, M. Herrmann, A.-L. Sirén, Erythropoietin: novel approaches to neuroprotection in human brain disease, Metab. Brain Dis. 19 (2004) 195–206.
[3] M. Um, H.F. Lodish, Antiapoptotic effects of erythropoietin in differentiated neuroblastoma SH-SY5Y cells require activation of both the STAT5 and AKT signalling pathways, J. Biol. Chem. 281 (2006) 5648–5656.
[4] Z-Y. Chen, P. Asavaritikrai, J.T. Prchal, C.T. Noguchi, Endogenous erythropoietin signaling is required for normal neural progenitor cell proliferation, J. Biol. Chem. 282 (2007) 25875–25883.
[5] M. Silva, D. Grillot, A. Benito, C. Richard, G. Nunez, J.L. Fernandez-Luna, Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-XL and Bcl-2, Blood 88 (1996) 1576–1582.
[6] N. Pregi, D. Vittori, G. Perez, C.P. Leirós, A. Nesse, Effect of erythropoietin on staurosporine-induced apoptosis and differentiation of SH-SY5Y neuroblastoma cells, Biochim. Biophys. Acta 1763 (2006) 238–246.
[7] A. Kretz, C.J. Happold, J.K. Marticke, S. Isenmann, Erythropoietin promotes regeneration of adult CNS neurons via Jak2/AKT pathway activation, Mol. Cell. Neurosci. 29 (2005) 569–579.
[8] G. Chen, J.X. Shi, C.H. Hang, W. Xie, J. Liu, X. Liu, Inhibitory effects on cerebral inflammatory agents that accompany traumatic brain

injury in a rat model: a potential neuroprotective mechanism of recombinant human erythropoietin (RHUEPO), Neurosci. Lett. 425 (2007) 177–182.
[9] B.B. Aggarwal, Signalling pathways of the TNF superfamily: a double-edged sword, Nat. Rev. Immunol. 3 (2003) 745–756.
[10] T. Hehlgans, K. Pfeffer, The intriguing biology of the tumor necrosis factor/tumor necrosis factor receptor superfamily: players, rules and the games, Immunology 115 (2005) 1–20.
[11] S.J. Tucker, C. Rae, A.F. Littlejon, A. Paul, D.J. MacEwan, Switching leukemia cell phenotype between life and death, Proc. Natl. Acad. Sci. 101 (2004) 12940–12945.
[12] E.E. Varfolomeev, A. Ashkenazi, Tumor necrosis factor: an apoptosis JunKie? Cell 116 (2004) 491–497.
[13] O.J. Jupp, S.M. McFarlane, H.M. Anderson, A.F. Littlejohn, A.A. Mohamed, R.H. MacKay, P. Vandenabeele, D.J. MacEwan, Type II tumor necrosis factor-α (TNFR2) activates c-Jun N-terminal kinase (JNK) but not mitogen-activated protein kinase (MAPK) or p38 MAPK pathways, Biochem. J. 359 (2001) 525–535.
[14] D. Baker, D. Butler, B.J. Scallon, J.K. O, Neill, J.L. Turk, M. Feldmann, Control of established experimental allergic encephalomyelitis by inhibition of tumor necrosis factor (TNF) activity within the central nervous system using monoclonal antibodies and TNF receptor-immunoglobulin fusion proteins, Eur. J. Immunol. 24 (1994) 2040–2048.
[15] A.L. Sirén, M. Fratelli, M. Brines, C. Goemans, S. Casagrande, P. Lewczuk, S. Keenan, C. Gleiter, C. Pasquali, A. Capobianco, T. Mennini, R. Heumann, A. Cerami, H. Ehrenreich, P. Ghezzi, Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 4044–4049.
[16] D. Agnello, P. Bigini, P. Villa, T. Mennini, A. Cerami, M.L. Brines, P. Ghezzi, Erythropoietin exerts an anti-inflammatory effect on the CNS in a model of experimental autoimmune encephalomyelitis, Brain Res. 952 (2002) 128–134.
[17] M. Brines y, A. Cerami, Emerging biological roles for erythropoietin in the nervous system, Nat. Rev. Neurosci. 6 (2005) 484–494.
[18] O.H. Lowry, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[19] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[20] D. Vittori, N. Pregi, G. Pérez, G. Garbossa, A. Nesse, The distinct erythropoietin functions that promote cell survival and proliferation are affected by aluminium exposure through mechanisms involving erythropoietin receptor, Biochim. Biophys. Acta 1743 (2005) 29–36.
[21] U.K. Laemmli, L.A. Amos, A. Klug, Correlation between structural transformation and cleavage of the major head protein of T4 bacteriophage, Cell 7 (1976) 191–203.
[22] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[23] A. Benito, M. Silva, D. Grillot, G. Núñez, J.L. Fernández-Luna, Apoptosis induced by erythroid differentiation of human leukaemia cell lines is inhibited by Bcl-xL, Blood 87 (1996) 3837–3843.
[24] M. McKinney, M. Robbins, Chronic atropine administration up-regulates rat cortical muscarinic m 1 receptor mRNA molecules: assessment with the RT/PCR, Brain Res. Mol. Brain Res. 12 (1992) 39–45.
[25] L. Yang, K. Lindholm, Y. Konishi, R. Li, Y. Shen, Target depletion of distinct tumor necrosis factor receptor subtypes reveals hippocampal neuron death and survival through different signal transduction pathways, J. Neurosci. 22 (2002) 3025–3032.
[26] Thorburn, Death receptor-induced cell killing, Cell Signal. 16 (2004) 139–144.
[27] M. Socolovsky, A.E. Fallon, S. Wang, C. Brugnara, H.F. Lodish, Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction, Cell 98 (1999) 181–191.
[28] Z.Z. Chong, J.-Q. Kang, K. Maiese, Erythropoietin fosters both intrinsic and extrinsic neuronal protection through modulation of microglia, Akt, Bad, and caspase-mediated pathways, Br. J. Pharmacol. 138 (2003) 1107–1118.
[29] L. Thommesen, A. Lægreid, Distinct differences between TNF receptor 1- and TNF Receptor 2-mediated activation of NFκB, J. Biochem. Mol. Biol. 38 (2005) 281–289.
[30] O. Nicolas, R. Gavin, N. Braun, J.M. Ureña, X. Fontana, E. Soriano, A. Aguzzi, J.A. del Río, Bcl-2 overexpression delays caspase-3 activation and rescues cerebellar degeneration in prion-deficient mice that overexpress amino-terminally truncated prion, FASEB J. 21 (2007) 3107–3117.
[31] T. Bittorf, T. Büchse, T. Sasse, R. Jaster, J. Brock, Activation of the transcription factor NF-κB by the erythropoietin receptor. Structural requirements and biological significance, Cell Signal. 13 (2001) 673–681.
[32] Z.Z. Chong, F. Li, K. Maiese, Erythropoietin requires NF-κB and its nuclear translocation to prevent early and late apoptotic neuronal injury during β-amyloid toxicity, Curr. Neurovasc. Res. 2 (2005) 387–399.
[33] M. Digicaylioglu, S.A. Lipton, Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades, Nature 412 (2001) 641–647.
[34] O. Micheau, J. Tschopp, Induction of TNF Receptor I-mediated apoptosis via two sequential signalling complexes, Cell 114 (2003) 181–190.
[35] A. Krueger, S. Baumann, P. Krammer, S. Kirchhoff, FLICE-Inhibitory proteins: regulators of death receptor-mediated apoptosis, Mol. Cell. Biol. 21 (2001) 8247–8254.
[36] P. Rafiee, Y. Shi, J. Su, K.A. Pritchard, J.S. Tweddell, J.E. Baker, Erythropoietin protects the infant heart against ischemia-reperfusion injury by triggering multiple signaling pathways, Basic Res. Cardiol. 100 (2005) 187–197.
[37] R.S. Akhtar, J.M. Ness, K.A. Roth, Bcl-2 family regulation of neuronal development and neurodegeneration, Biochim. Biophys. Acta 1644 (2004) 189–203.
[38] K. Maiese, F. Li, Z.Z. Chong, Erythropoietin in the brain: can the promise to protect be fulfilled? Trends Pharmacol. Sci. 25 (2004) 577–583.
[39] P.A. Baeuerle, D. Baltimore, NF-kappa B: ten years after, Cell 87 (1996) 13–20.
[40] L. Marchetti, M. Klein, K. Schlett, K. Pfizenmaier, U.M.L. Eisel, Tumor necrosis factor (TNF)-mediated neuroprotection against glutamate-induced excitotoxicity is enhanced by *N*-methyl-D-aspartate receptor activation, J. Biol. Chem. 279 (2004) 32861–32881.
[41] E. Taoufik, E. Petit, D. Divoux, V. Tseveleki, M. Mengozzi, M. Roberts, S. Valable, P. Ghezzi, J. Quackenbush, M. Brines, A. Cerami, L. Probert, TNF receptor I sensitizes neurons to erythropoietin- and VEGF-mediated neuroprotection after ischemic and excitotoxic injury, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 6185–6190.