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Effect of erythropoietin on staurosporine-induced apoptosis and differentiation of SH-SY5Y neuroblastoma cells

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Abstract

Since apoptosis appeared to be related to neurodegenerative processes, neuroprotection has been involved in investigation of therapeutic approaches focused upon pharmacological agents to prevent neuronal programmed cell death. In this regard, erythropoietin (Epo) seems to play a critical role. The present work was focused on the study of the Epo protective effect upon human neuroblastoma SH-SY5Y cells subjected to differentiation by staurosporine. Under this condition, profuse neurite outgrowth was accompanied by programmed cell death (35% of apoptotic cells by Hoechst assay, showing characteristic DNA ladder pattern). A previous treatment with recombinant human Epo (rHuEpo) increased the expression of the specific receptor for Epo while prevented apoptosis. Simultaneously, morphological changes in neurite elongation and interconnection induced by staurosporine were blocked by Epo. These Epo effects proved to be associated to the induction of Bcl-xL at the mRNA and protein levels (RT-PCR and Western blot after immunoprecipitation) and were mediated by activation of pathways inhibited by wortmannin. In conclusion, the fact that both events induced by staurosporine, cell apoptosis and differentiation, were prevented in SH-SY5Y cells previously exposed to rHuEpo suggests interrelated signaling pathways triggered by the Epo/EpoR interaction.

Keywords: Apoptosis; Erythropoietin; SH-SY5Y cell; Neuroprotection; Staurosporine; Bcl-X

1. Introduction

The cell death phenomenon, apart from being an important feature in the development of the nervous system, appears also to play a role in some neurodegenerative diseases and aging [1]. The fact that the basic mechanisms underlying these pathological processes are not clearly understood yet may explain the lack of targeted effective therapies for the treatment of these diseases. Therefore, the apoptosis phenomenon has attracted enormous attention during the past few years mainly to delineate the mechanistic details of the apoptotic pathways in the nervous system. In this regard, several studies in vivo and in vitro have shown that erythropoietin (Epo), a pleiotropic cytokine commonly identified by its role in bone marrow erythropoiesis, could have a direct neurotrophic and neuroprotective effect [1,2]. The possibility that Epo plays a significant biological role in tissues outside the hematopoietic system has triggered interest in this hormone as a cytoprotective agent [3–6]. It is now recognized that cells expressing specific receptors for Epo (EpoR) are not limited to the erythropoietic lineage, and include endothelial cells [7] and cells of neuronal origin [8]. Moreover, Epo and EpoR have been identified in specific areas of the embryonic, fetal and adult brain of rodents, nonhuman primates and humans [9–11].

It is known that signal transduction of Epo depends on EpoR dimerization to activate signaling pathways mediated by

Abbreviations: Epo, erythropoietin; EpoR, erythropoietin receptor; rHuEpo, recombinant human erythropoietin; D-MEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; PBS, phosphate buffered saline; EtBr, ethidium bromide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMSO, dimethylsulfoxide; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C

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phosphorylation of tyrosine residues which become docking sites for molecules responsible for subsequent activation of numerous other signaling pathways. The derived cascade of intracellular events is crucial for the biological activities emanating from the Epo/EpoR interaction and it appears certain that antiapoptotic members of the Bcl-2 family are involved in cell activation by Epo [12–14].

The detection of Epo synthesis and the expression of EpoR in the central nervous system was immediately followed by investigation of the Epo properties as a neuroprotective factor [2,15,16]. Given the role for Bcl-2 family members in regulating cell death during nervous system development, much interest has been given to their involvement in neuropathological conditions [17].

The human SH-SY5Y neuroblastoma cell line has the capability of undergoing neuronal maturation [18]. Morphologic and functional differentiation of these cells can be induced by staurosporine giving rise to a mature neuronal phenotype [19,20]. However, staurosporine has been extensively characterized as one of the best inducer of apoptosis in many neuronal cell types [20–22]. This can be explained by the fact that staurosporine is a potent and non-specific inhibitor of multiple protein kinases, including the protein kinase C, which plays an important role in cellular proliferation, differentiation and cell survival [23,24].

This study was designed considering the strong relationship of Epo with proliferation, differentiation and survival of erythroid progenitors [25,26], and the evidences regarding protection on brain cells by Epo [2,27]. Then, staurosporine-treated SH-SY5Y cells were used to investigate whether a complex interplay between cell death and differentiation is related to the neuroprotective activity of Epo.

2. Materials and methods

2.1. Materials

All chemicals used were of analytical grade. Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (D-MEM) and Ham F12 Medium were obtained from GibcoBRL. Proteinase K, Trizol reagent, specific primers for EpoR, Bcl-2, Bcl-X and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Invitrogen Life Technologies. Monoclonal anti-Bcl-xL antibody was obtained from Chemicon International. Staurosporine, EDTA, Nonidet P40, wortmannin, L-glutamine, paraformaldehyde, phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, pepstatin A, Hoechst 33258 dye and DNAse-free RNAse were obtained from Sigma-Aldrich. Ready-To-Go T-Primed First-Strand Kit and chemiluminescent system kit and blots (ECL and Hyperfilm) were obtained from Amersham Biosciences. Agarose was obtained from Promega, ethidium bromide (EtBr) from Mallinckrodt, dimethylsulfoxide (DMSO) and absolute ethyl alcohol from Merck, penicillin-streptomycin from PAA Laboratories GmbH and Protein A-agarose from BD Transduction Laboratories. Recombinant human erythropoietin (rHuEpo, Hemax) was supplied by Biosidus (Argentina).

2.2. Cell culture

Human SH-SY5Y neuroblastoma cells (CRL-2266, American Type Culture Collection ATCC) have been used along this work. 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 fetal 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.

2.3. Experimental design

Time-course assays were developed to determine: a) the incubation period for staurosporine-induced apoptosis and b) the appropriate period of Epo treatment to prevent apoptosis. Sequential cell treatments were developed in the presence of wotmannin and addition of rHuEpo previous to the apoptosis induction with staurosporine.

Staurosporine, rHuEpo and wortmannin were added to complete medium and adjusted to final concentrations as referred below in text and figures. The staurosporine vehicle DMSO was added to every control assay.

2.4. 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 phosphate buffered saline (PBS). The pellet was homogenized in 500 μl of lysis buffer (0.1 mM NaCl, 25 mM EDTA, 10 mM Tris–HCl pH 8.0, 1% (v/v) Nonidet P40). 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 and each sample incubated for 16 h at 37 °C. Precipitation with half a volume of 10 M ammonium acetate and 2 volumes of absolute ethyl alcohol was performed. Electrophoresis of DNA samples were carried out on 2% agarose gel (w/v), containing 0.5 mg/l EtBr during 90 min at 100 V. Bands were observed under an UV transilluminator and digitalized with a Kodak DC240 equipment.

2.5. Fluorescent nuclear staining of apoptotic cells

SH-SY5Y cells were cultured on slide covers plated in 35 mm Petri dishes during 48 h and then, incubated in the presence of staurosporine at final concentrations ranging from 10 nM to 200 nM during 12 h, or left untreated. Four plates per experimental condition were simultaneously run.

Control non-induced and staurosporine-treated cells were fixed with 4% (v/v) paraformaldehyde in PBS solution for 20 min at 4 °C, exposed to 0.05 g/l Hoechst 33258 dye in PBS for 30 min at room temperature,

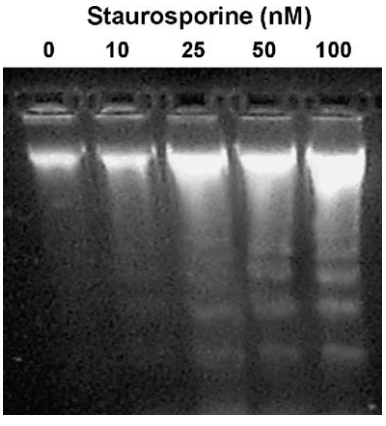

Fig. 1. Programmed cell death induction by staurosporine evaluated by DNA fragmentation. SH-SY5Y cells were cultured without or in the presence of 10–100 nM staurosporine and then the corresponding DNA was extracted. Internucleosomal DNA degradation was detected by electrophoretic analysis in 2% agarose gels containing EtBr. Bands were observed under UV transillumination and digitalized.

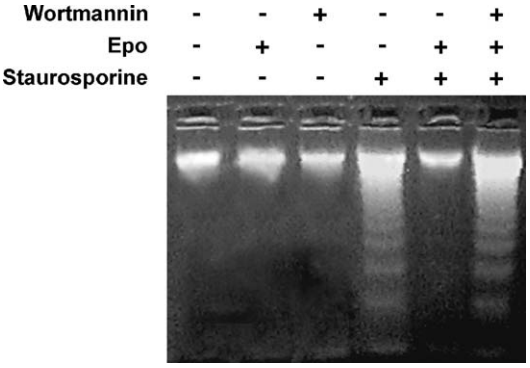

Fig. 2. Antiapoptotic effect of Epo demonstrated by DNA fragmentation. Untreated SH-SY5Y cells were incubated in conventional medium (Lane 1), with Epo (Lane 2) or wortmannin (Lane 3) alone, induced to apoptosis by incubation with 25 nM staurosporine for 12 h (Lane 4), treated with 25 U/ml rHuEpo for 12 h before addition of staurosporine (Lane 5), or subjected to treatment with 200 nM wortmannin for 2 h, rHuEpo for 12 h and then, staurosporine during additional 12 h (Lane 6). The electrophoretic result shown here is representative of three independent experiments.

washed thrice with PBS and, finally, mounted by using mounting buffer (50% glycerol in PBS, v/v).

Fluorescent nuclei with apoptotic characteristics were detected by microscopy under UV illumination at 365 nm (Eclypse E600 Fluorescent Microscope, Nikon, Japan). The images were photographed by a Nikon Coolpix 5000 equipment and digitalized. Differential cell counting was performed by analyzing at least 500 cells [26].

2.6. Analysis of mRNA levels by reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated by means of Trizol reagent. For qualitative analysis, electrophoresis of RNA was performed in 2% agarose gels (w/v), stained with EtBr and photographed. The RNA amount and the purity of extraction were evaluated by measuring absorbance at 260 and 280 nm [28]. Complementary DNA was prepared by reverse transcription using Ready-to-Go T-Primed First-Strand Kit starting from a sample of 2 μg of total RNA.

An aliquot of cDNA was subjected to 30 PCR amplification cycles (94 °C for 20 s, primer annealing at 62 °C for 30 s, extension at 72 °C for 40 s) with an initial incubation at 94 °C for 5 min and a final incubation at 60 °C for 7 min for EpoR and 30 cycles (94 °C for 45 s, 60 °C for 30 s, and 72 °C for 45 s) with initial incubation at 94 °C for 5 min and final incubation at 72 °C for 10 min for Bcl-X and Bcl-2. Specific primers were employed for EpoR [29], for Bcl-X and Bcl-2 [13] and for the internal standard GAPDH [30].

Primers for human EpoR: 5′-TCTGAAGCAGAAGATCTGGCC-3′ and 5′-GATCATCTGCAGCCTGGTGT-3′.

Primers for human Bcl-2: 5′-AGATGTCCAGCCAGCTGCACCTGAC-3′ and 5′-AGATAGGCACCAGGGTGAGCAAGCT-3′.

Primers for human Bcl-X: 5′-CGGGCATTCAGTGACCTGAC-3′ and 5′-TCAGGAACCAGCGGTTGAAG-3′.

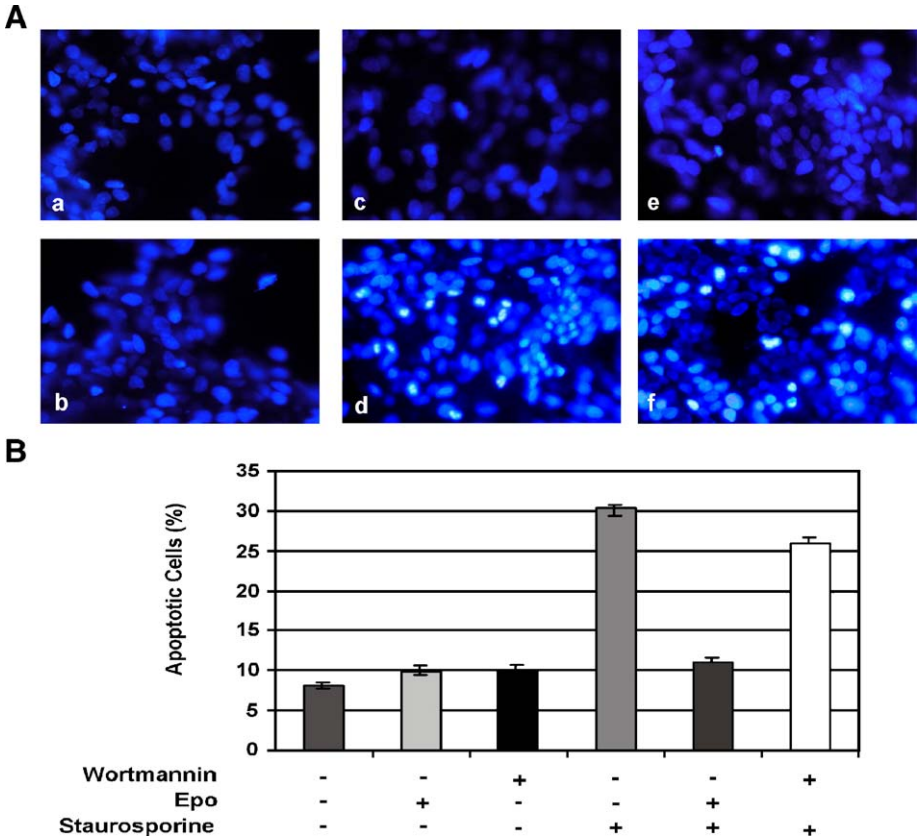

Fig. 3. Antiapoptotic effect of Epo detected by fluorescence microscopy. (A) Fluorescence photomicrographs after Hoechst 33258 staining of untreated control cells (a), cells induced to apoptosis by 25 nM staurosporine (d), protected cells treated with rHuEpo for 12 h before being induced with staurosporine (e) and cells which received a sequential treatment with wortmannin for 2 h, rHuEpo for 12 h and staurosporine during 12 h (f). Apoptosis-induced cells show fluorescent nuclei with typical characteristics of condensed chromatin detected by microscopy under UV illumination at 365 nm. No effect of Epo or wortmannin alone was detected (b and c, respectively). (B) Positive apoptotic cells counting performed by analyzing 500 cells/sample. Each bar represents the percentage value (mean±S.E.M.) of apoptotic cells with respect to the total cell number corresponding to four independent assays. Significant differences ($P<0.01$) of assays induced to apoptosis (d) with respect to controls (a, b and c) and cultures protected by Epo (e).

The resulting PCR fragments were visualized on 1.5% agarose gels (w/v) containing EtBr and then digitalized with a Kodak DC240 equipment.

2.7. Immunoprecipitation of cell extracts and Western blotting

Cells were washed with ice-cold PBS solution and lysed with hypotonic buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecylsulfate and 0.5% sodium deoxycholate) containing protease inhibitors (1 mM PMSF, 4 μM leupeptin, 2 μM pepstatin A, 1 μg/ml aprotinin), in a ratio of 200 μl/10^7 cells. After 30 min incubation on ice, insoluble material was removed by centrifugation at 15,000×*g* for 15 min and protein concentration was measured in supernatants [31].

Two hundred μl of cell extracts were incubated with 3 μg/ml monoclonal specific antibody (anti-Bcl-X or anti-EpoR antibody) during 1 h at 4 °C. Then, Protein A-agarose was added and, after overnight incubation at 4 °C in a rotating shaker, immunoprecipitates were collected by centrifugation at 15,000×*g* during 15 min and washed twice with the lysis buffer, containing protease inhibitors (1 mM PMSF, 4 μM leupeptin, 2 μM pepstatin A, 1 μg/ml aprotinin) as previously reported [26].

Immunoprecipitates were boiled for 3 min in the Laemmli buffer [32], resolved by SDS-polyacrylamide gel electrophoresis (*T*=8%) and then, electroblotted onto a Hybond nitrocellulose membrane (transfer buffer: 25 mM Tris, 195 mM glycine, 0.05% SDS, pH 8.3, and 20% (v/v) methanol). Non-specific binding sites on the membrane were blocked with 5% ECL membrane blocking agent in Tris-buffered saline (25 mM Tris, 137 mM NaCl, 3 mM KCl, pH 7.4) containing 0.1% Tween 20 (TBS-Tween) for 1 h at room temperature. The blots were briefly rinsed using two changes of TBS-Tween buffer, and washed three times for 10 min each with fresh changes of the washing buffer. Then, the blots were incubated with the appropriate concentration of the adequate antibody (anti-Bcl-X or anti-EpoR antibody) during 1 h at 4 °C, washed again three times for 10 min each with TBS-Tween and probed with a 1:1000 dilution of anti-mouse horseradish peroxidase-conjugated antibody for 1 h at 20 °C. After washing, the blots were incubated with the enhanced chemiluminescence substrate (ECL kit) and the bands detected by using a Fujifilm Intelligent Dark Box II (Fuji) equipment coupled to a LAS-1000 digital camera. To visualize the bands the Image Reader LAS-1000 and LProcess V1.Z2 programs were employed.

2.8. Cell morphology determined by light microscopy

Cells grown at a density of 1×10^6 cells/ml were treated by the apoptosis inducer and/or signaling inhibitors as indicated in the legend of the corresponding figures. After the incubation period, cultures were observed under phase-contrast, using a 40× objective in an inverted microscope (Axiovert 135, Carl Zeiss, Germany) and then photographed by using a Nikon Coolpix 5000 digital camera. Multiple independent images were taken and at least 10 neurites per field were selected for neurite outgrowth measurement. Finally, means of neurite length were calculated for each assay. This neurite tracing technique was implemented in the form of a plugin (NeuronJ) for ImageJ (National Institutes of Health, Bethesda, MD), the computer-platform independent public domain image analysis program inspired by NIH-Image [33].

2.9. Statistics

Results are expressed as mean±S.E.M. When corresponding, the non-parametric Mann–Whitney *U*-test or the Kruskal–Wallis one-way analysis of variance test were employed. At least differences with $P<0.05$ were considered the criterion of statistical significance. Correlation between variables was described by the Pearson *r* coefficient.

3. Results

3.1. Programmed cell death induction by staurosporine

The ability of staurosporine to act as an effective inducer of apoptosis upon the SH-SY5Y cells was initially characterized by analyzing its effect in a range from 10 to 100 nM. Incubation with staurosporine during a period of 12 h induced clear signs of cell apoptosis.

One of the apoptosis signs derives from activation of endogenous endonucleases that attack and degrade preserved chromatin. This phenomenon was analyzed by means of electrophoresis in agarose gels. Through this methodology, the staurosporine-induced cell death was detected by the appearance of a ladder pattern of DNA degradation (Fig. 1). This effect was concentration-dependent and no further changes were observed at staurosporine concentration as high as 100 nM.

3.2. Antiapoptotic effect of Epo

The effect of Epo on DNA fragmentation induced by staurosporine was evaluated by incubating cells with 25 U/ml rHuEpo for 12 h previous to the induction of apoptosis. This

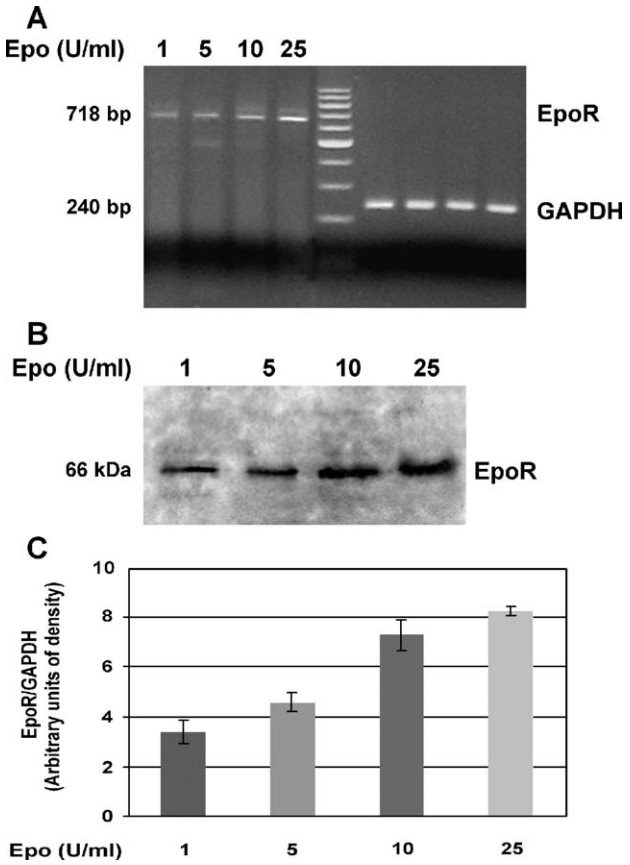

Fig. 4. Regulation of EpoR expression by Epo in SH-SY5Y cells. Cells were incubated during 12 h in the presence of different rHuEpo concentrations, ranging from 1 to 25 U/ml. After total RNA extraction, EpoR mRNA was analyzed by RT-PCR (A) and the protein expression was evaluated by immunoprecipitation and Western blot (B). (A) PCR fragments were visualized on 1.5% agarose gels containing EtBr and digitalized. (B) Western blots were revealed by chemiluminescence using anti-EpoR antibody. (C) The analysis of EpoR signals, normalized with GAPDH internal standard, are expressed as arbitrary units of mRNA band density. Results shown in (A) are representative of four assays similarly run, and densitometric analysis shown in (C) represents the mean±S.E.M.

pretreatment completely prevented the typical electrophoretic pattern of DNA fragmentation induced by 25 nM staurosporine (Fig. 2). This complete protective effect was less pronounced due to a shorter Epo exposure. Only a slight reduction of apoptosis was observed after a pretreatment of 6 h and programmed cell death was not prevented when the rHuEpo was co-administered with staurosporine. rHuEpo doses lower than 25 U/ml proved not to be suitable to completely prevent the effect of 25 nM staurosporine (data not shown).

Assays in the presence of wortmannin were carried out to study signaling pathways involved in the antiapoptotic effect of Epo. Phosphatidylinositol 3-kinase (PI3K), as well as phospholipases A_2, C and D, are known as enzymes potentially inhibited by wortmannin [34]. Cell incubation with 200 nM wortmannin previously to the addition of rHuEpo inhibited the protective effect of the hormone (Fig. 2).

Another relevant feature of apoptosis is a specific pattern of chromatin condensation. Changes in nuclear morphology were evaluated by fluorescence microscopy, using the Hoechst 33258 dye that specifically binds to DNA. Nuclei in control cells exhibited diffuse chromatin staining (Fig. 3A.a). However, after exposure to 25 nM staurosporine for 12 h, cells underwent typical morphologic changes of apoptosis, such as extreme nuclear shrinkage and intense bright round bodies of condensed chromatin (Fig. 3A.d). These characteristic nuclear images of apoptosis were absent in Epo-pretreated cells (Fig. 3A.e). The counting of positive apoptotic cells allowed us to describe different patterns. Whereas the mean value of spontaneous apoptosis in control cells was low (8.2±0.25%), a significant increase ($P<0.01$) was observed under staurosporine induction (30.3±0.39%, $P<0.01$), and this effect was prevented by the Epo pretreatment, being the percentage of apoptotic cells 11.1±0.45% (Fig. 3B). On the other hand, a 2-h preincubation period with wortmannin counteracted the action of Epo (25.9±0.88%) (Fig. 3A.f and B).

Cell treatments with Epo or wortmannin alone did not induce apoptotic cell changes in assays of either DNA fragmentation (Fig. 2: Lanes 2 and 3) or Hoechst nuclear staining (Fig. 3A.b, A.c and B).

3.3. Regulation of EpoR mRNA by Epo

In order to investigate whether the Epo protective effect observed on SH-SY5Y cells is mediated by regulation of its specific receptor expression, cell cultures were developed in the presence of different rHuEpo concentrations ranging from 1 to 25 U/ml. EpoR mRNA levels and EpoR protein expression were analyzed by RT-PCR and immunoprecipitation and Western blot respectively.

Results shown in Fig. 4A demonstrate that EpoR mRNA levels are dependent on Epo concentration within the range studied (Pearson coefficient, $r = 0.886$; $P < 0.001$). A basal EpoR mRNA level similar to that observed at 1 U/ml of rHuEpo was detected in this cell line (data not shown). The analysis of EpoR mRNA band intensity was expressed as arbitrary units in

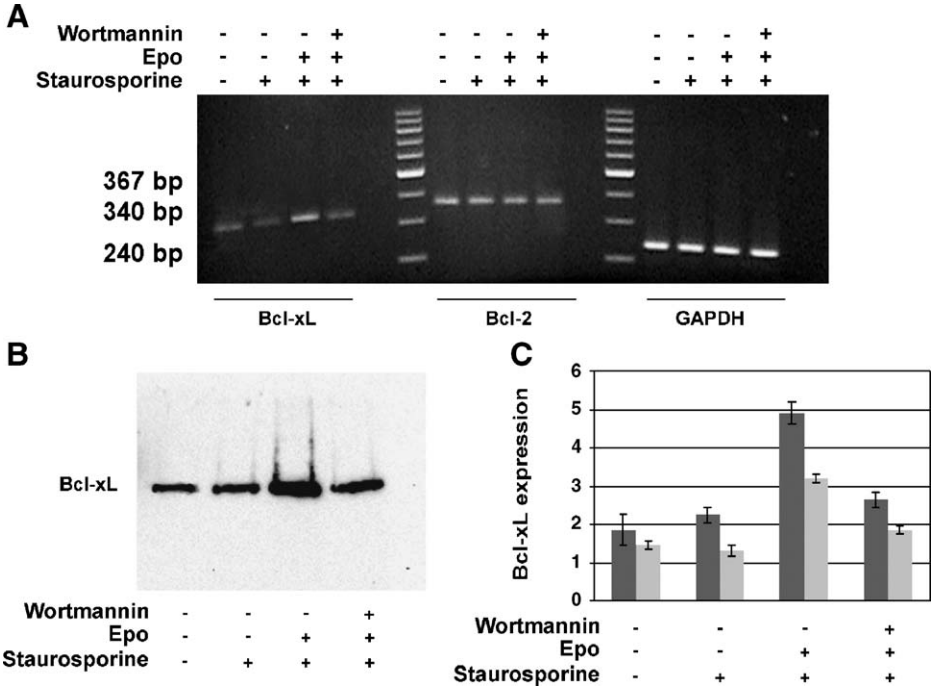

Fig. 5. Expression of members of the Bcl-2 family mediated by Epo. Cells were induced or not to apoptosis by staurosporine. In other assays, cells received either a previous treatment with rHuEpo or sequential previous treatments with wortmannin and rHuEpo. (A) At the end of the culture periods, total RNA was extracted, and Bcl-X, Bcl-2 and the internal standard GAPDH mRNA were amplified by RT-PCR. (B) Cell lysates were immunoprecipitated with anti-Bcl-xL monoclonal antibody, and immunoprecipitates analyzed by Western blotting with anti-Bcl-xL antibody and chemiluminescent detection. The patterns displayed are representative of four independent assays. (C) Results of the ratio of Bcl-xL/GAPDH (black bars) and signals of Bcl-xL protein levels (grey bars) were expressed as arbitrary units of band density in bars which indicate mean±S.E.M.

comparison with that of the corresponding internal standard GAPDH (Fig. 4C). In addition, increasing EpoR expression at protein level was observed in cultures stimulated by growing Epo concentrations (Fig. 4B).

3.4. Expression of factors of the Bcl-2 family mediated by Epo

Taking into consideration that some members of the protein family Bcl-2 afford resistance to cells facing apoptotic stimuli, it was interesting to investigate whether the Epo effect was associated to enhancement of the expression of antiapoptotic factors of this family. Fig. 5 shows that the regulatory factors Bcl-xL and Bcl-2 are expressed in SH-SY5Y cells and that the pretreatment with Epo induces upregulation of Bcl-xL but not of Bcl-2 mRNA levels (Fig. 5A and C). In agreement, increased Bcl-xL protein expression was detected by immunoprecipitation and Western blot in cells which had been protected with Epo before being treated with staurosporine (Fig. 5B and C). The upregulation of Bcl-xL expression induced by Epo was counteracted by cell pretreatment with the inhibitor wortmannin (Fig. 5).

A 2-h treatment of SH-SY5Y cells with wortmannin alone had no effect on Bcl-xL expression (data not shown).

3.5. Effect of Epo on staurosporine-induced cell differentiation

SH-SY5Y cells treated with staurosporine exhibited a differential neuronal morphology [20–22,35]. Phase contrast inverted microscopy and photograms were used to monitor neuritogenesis in cell cultures developed in the presence of staurosporine with or without Epo-pretreatment for 12 h. The morphological changes characteristic of cell differentiation observed after the different experimental protocols are shown in Fig. 6. The length of neurite outgrowth was measured by an appropriate software as was indicated in Materials and methods, and the results, expressed as arbitrary units, are displayed in Fig. 6B. Neuritogenesis induced by staurosporine (25 or 100 nM) was observed as an increase in neurite processes combined with a prominent branching and the subsequent reduction in cell body size (Fig. 6.d and f with respect to Fig. 6.a; $P<0.05$). Cells pretreated with 25 U/ml Epo for 12 h failed to differentiate due to 25 nM staurosporine (Fig. 6.e). However, cellular treatment with Epo and higher staurosporine concentration (100 nM) resulted in some neurite extension, proving that the inducer effect was partially reverted ($P<0.05$) but could not be completely blocked by the same Epo dosis (Fig. 6.g). No significant differences were observed

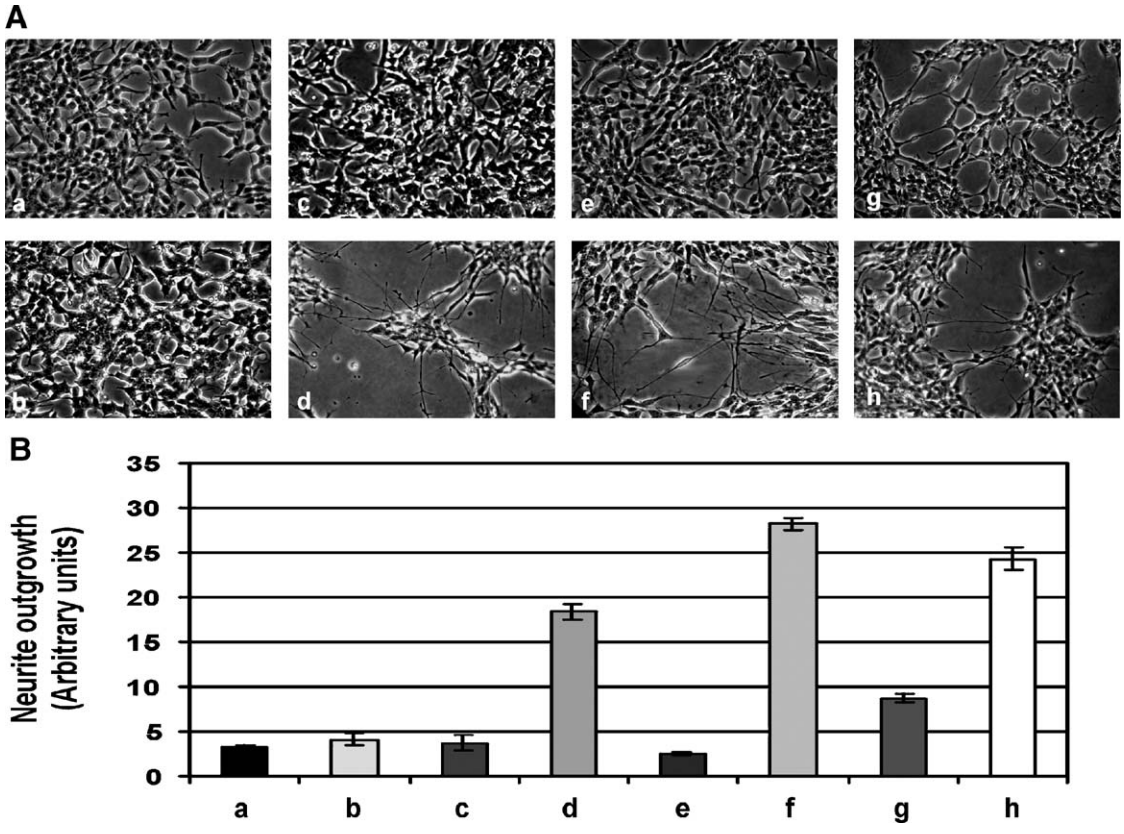

Fig. 6. Effect of Epo upon staurosporine-induced cell differentiation. (A) Cell growth was observed under an inverted microscope and photographed after receiving the following treatments: 25 nM staurosporine for 12 h (d); 25 U/ml rHuEpo for 12 h + 25 nM staurosporine for 12 h (e); 100 nM staurosporine for 12 h (f); 25 U/ml rHuEpo for 12 h+100 nM staurosporine for 12 h (g); 200 nM wortmannin for 2 h+25 U/ml rHuEpo for 12 h+25 nM staurosporine for 12 h (h). Untreated control cells (a) and cells treated with Epo (b) or wortmannin (c) alone were also assayed. Magnification 400×. Long neurites emerging and interconnecting cell bodies can clearly be seen in (d) and (f). (B) The lengths of neurite outgrowth were measured and results (mean ± S.E.M.) of ten independent assays are expressed as arbitrary units. Significantly statistical differences ($P < 0.05$) were found in cultures induced to apoptosis by staurosporine between cells protected or not by Epo (d vs. e and f vs. g).

between untreated cells and cells treated with Epo or wortmannin alone.

The action of Epo upon cell differentiation was counteracted by previous treatment with the inhibitor wortmannin (Fig. 6h).

4. Discussion

Apoptosis is a mechanism of programmed cell death involved in the homeostasis of nervous tissues and its dysregulation has been associated to pathology of neurodegenerative diseases. Therefore, neuroprotection has been critically involved in the investigation of therapeutic approaches that focuses upon growth factors as pharmacological agents to prevent neuronal cell apoptosis. In this regard, Epo is considered to have a major neuroprotective role [2,15,16,27,36]. It is well known that Epo is related to mechanisms of survival, proliferation and differentiation of erythroid precursors in order to increase the circulating erythrocyte mass. However, it has recently become clear that Epo and its specific receptor EpoR mediate diverse functions in nonhematopoietic tissues as well.

Taking into account that staurosporine not only induces maturation but also triggers the apoptotic phenomenon upon the human neuroblastoma SH-SY5Y cell line [20], this model was used to study the response of differentiating cells to the antiapoptotic effect of Epo. It has been suggested that the behavior of neurodegenerative tissues is a particularly relevant field of application of this model, where mechanisms of cell death can be investigated in a differentiated neuronal cell line [20]. It is known that apoptosis is induced by various intra- or extracellular stimuli. In this regard, the effects of staurosporine, in terms of its ability to induce transient apoptosis during cell differentiation, might reflect conditions in vivo [20].

In agreement with other authors [20,21], we observed that staurosporine led to the development of different apoptotic signs on SH-SY5Y cells, such as DNA fragmentation − made evident by the typical pattern of electrophoretic ladder − and nuclear chromatin condensation − revealed by fluorescent microscopy after Hoechst staining. We also demonstrated that these signs can be blocked by cell pretreatment with rHuEpo for at least 12 h before apoptosis was induced by staurosporine and no protective effect is produced by coadministration of Epo and the apoptosis inducer.

The finding that rHuEpo exposure increased EpoR expression at mRNA and protein levels in a dose-dependent fashion lets us suggest that the effect of rising the level of Epo might be further amplified in this cell line due to an increase in the number of the specific receptors.

Given the clear role for Bcl-2 family members in regulating cell death during nervous system development, much interest has been focused on their role under neuropathological conditions. In this context, SH-SY5Y cells overexpressing antiapoptotic factors of this family were significantly protected from β-amyloid neurotoxicity [37] and staurosporine induced apoptosis [20]. On the other hand, the activation of genes of the *bcl-2* family by Epo treatment of retinal ganglion cells has been reported [38–40]. Based on this knowledge, we hypothesized that Epo might exert an antiapoptotic effect on SH-SY5Y neuronal cells through Bcl-2 regulatory factors of apoptosis, suggesting a mechanism similar to those described in hematopoietic cells [12–14].

Under the experimental conditions employed, upregulation of Bcl-xL but not of Bcl-2 was observed following SH-SY5Y cell activation by Epo. This Epo protective effect was counteracted in assays run in the presence of the enzyme inhibitor wortmannin, with appearance of apoptotic signs and decreased Bcl-xL mRNA and protein expression. A previous reduction of Bcl-xL induced by wortmannin before the Epo treatment can be excluded since a 2-h treatment with the inhibitor alone had no effect on the expression of the protective factor. Differential effects of Bcl-2 and Bcl-X in a single cell type with a given apoptotic stimulus have not been demonstrated conclusively yet. In erythroid lineages, both antiapoptotic factors are involved in the regulation of cell survival [12], although Bcl-xL appears to be the most important in the repression of apoptosis mediated by Epo [13]. Based on our results, it seems that Bcl-xL would be also more relevant than Bcl-2 in the neuroprotective effect of Epo on SH-SY5Y cells induced to apoptosis by staurosporine. This can be explained by the Bcl-xL higher efficiency with respect to Bcl-2 in preventing cytochrome *c* release from mitochondria found by other authors [20]. Even though Bcl-2 has been considered more related to regulation of neuronal differentiation [41,42], we did not find upregulation of this factor due to staurosporine-induced maturation of SH-SY5Y cells, at least at the drug concentration and incubation periods used in this study.

In agreement with previous reports [20,21,35], staurosporine proved not only to be an effective inducer of apoptosis but also a potent inducer of neuritogenesis in SH-SY5Y neuroblastoma cells. The Epo dosis leading to neuronal survival, which was associated to Bcl-xL expression, was also capable of blocking neuritogenesis. Under different conditions, other authors demonstrated that transfected SH-SY5Y cells, overexpressing Bcl-xL, acquire enough protection to resist the severe apoptosis that would be induced by very high staurosporine doses (1 μM) during long periods, thus allowing the cells to be induced to differentiation [20]. The comparison between this and our results supports the hypothesis that different signaling pathways, other than those activating *bcl-2* genes, may be triggered by Epo, thus affecting both cell apoptosis and differentiation.

In the present work, cell differentiation produced by low staurosporine dosis (25 nM) can be blocked by a previous treatment with 25 U/ml rHuEpo. However, this concentration was insufficient to overcome the effect of higher staurosporine levels (100 nM). It seems that Bcl-xL expression induced by Epo in SH-SY5Y cells increase the threshold for the apoptotic molecular mechanisms to be activated by staurosporine. However, it cannot be discarded that Epo counteracts the inhibitory effect of staurosporine upon PKC, given the role described for one PKC isoform in mediating Epo-induced erythroid differentiation of progenitor cells from human bone marrow [43].

Our results confirm and extend previous reports on EpoR expression in human neuronal cells [44] and demonstrate the EpoR regulation in a ligand dose-dependence. This may explain further evidence regarding the Epo classic effect of cell protection upon the human SH-SY5Y cell line. These findings may be related to mechanisms by which a growth factor inactivates and/or triggers critical components of the cell-intrinsic death machinery. The fact that both events induced by staurosporine, cell apoptosis and differentiation, were prevented in SH-SY5Y cells previously exposed to rHuEpo suggests interrelated signaling pathways triggered by the Epo/EpoR interaction.

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