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RESEARCH PAPER

JAK2/STAT5/Bcl-xL signalling is essential for erythropoietin-mediated protection against apoptosis induced in PC12 cells by the amyloid β–peptide $A\beta_{25–35}$

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BACKGROUND AND PURPOSE

Erythropoietin (EPO) exerts neuroprotective actions in the CNS, including protection against apoptosis induced by the amyloid β–peptide $A\beta_{25–35}$. However, it remains unclear which signalling pathway activated by EPO is involved in this neuroprotection. Here, we have investigated whether JAK2/STAT5/Bcl-xL and ERK1/2 signalling pathways are essential for EPO-mediated protection against apoptosis induced by $A\beta_{25–35}$.

EXPERIMENTAL APPROACH

EPO was added to cultures of PC12 cells, 1 h before $A\beta_{25–35}$. For kinase inhibitor studies, AG490 and PD98059 were added to PC12 cells, 0.5 h before the addition of EPO. Transfection with siRNA was used to knockdown STAT5. Activation of JAK2/STAT5/Bcl-xL and ERK1/2 signalling pathways were investigated by Western blotting. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide assay and apoptosis was detected by TUNEL and acridine orange–ethidium bromide double staining.

KEY RESULTS

EPO increased phosphorylation of JAK2 and STAT5 in PC12 cells treated with $A\beta_{25–35}$. Furthermore, EPO modulated the nuclear translocation of phospho-STAT5, which increased expression of Bcl-xL and decreased levels of caspase-3. These beneficial effects were blocked by the JAK2 inhibitor, AG490 or STAT5 knockdown. However, the ERK1/2 pathway did not play a crucial role in our model.

CONCLUSIONS AND IMPLICATIONS

EPO protected PC12 cells against $A\beta_{25–35}$-induced neurotoxicity. Activation of JAK2/STAT5/Bcl-xL pathway was important in EPO-mediated neuroprotection. EPO may serve as a novel protective agent against $A\beta_{25–35}$-induced cytotoxicity in, for instance, Alzheimer's disease.

Abbreviations

Aβ, amyloid β; EPO, erythropoietin; MTT, 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide

Introduction

The characteristic feature of Alzheimer's disease is the neuronal degeneration associated with senile plaques (Harkany *et al.*, 2000). Such plaques are composed of the amyloid β-peptide (Aβ), a 40–43 amino acid peptide (Selkoe, 2006). The deposition of soluble Aβ produces the aggregation of the peptide-forming amyloid fibrils, which are neurotoxic *in vitro* (Yankner, 1996; Muñoz and Inestrosa, 1999) and *in vivo* (Miranda *et al.*, 2000). The amyloid-derived peptide $A\beta_{25–35}$ contains the hydrophobic amino acids 25–35 of the amyloid beta protein and aggregates as insoluble fibrils that retain the toxic effect of the larger Aβ peptides (Harkany *et al.*, 2000).

Erythropoietin (EPO) is a haematopoietic growth factor that controls erythrocyte production through regulating proliferation, differentiation, and survival of erythroid progenitors (Jelkmann, 1992; Youssoufian *et al.*, 1993). EPO exerts these effects by interaction with its cell surface receptor (Koury and Bondurant, 1992), a member of the cytokine receptor superfamily (Alexander *et al.*, 2013a). Once EPO binds to the EPO receptor, a ligand-induced receptor homodimer conformational change leads to trans-phosphorylation and activation of JAK2 (Bergelson *et al.*, 1998; Miller *et al.*, 1999). Activated JAK2 phosphorylates key tyrosine residues in the cytoplasmic domain of the EPO receptor, thereby providing docking sites for SH2 domain-containing downstream-signalling molecules including STAT5, PI3K/Akt and ERK1/2 (Wojchowski *et al.*, 1999; Alexander *et al.* 2013b).

In addition to haematopoietic cells, EPO receptors are also found in other cells responding to EPO (Anagnostou *et al.*, 1994), such as muscle (Morakkabati *et al.*, 1996) and neural cells (Tabira *et al.*, 1995; Morishita *et al.*, 1996). Expression of EPO receptors in brain particularly in the embryonic stage suggests a role for EPO in brain development and/or tissue maintenance (Wu *et al.*, 1999; Yu *et al.*, 2002). As EPO can stimulate the survival and proliferation of neural progenitor cells, it is regarded as one of the most promising neuroprotective agents under investigation and EPO showed neuronal protective action *in vivo* (Bernaudin *et al.*, 1999; Genc *et al.*, 2001) and *in vitro* (Signore *et al.*, 2006). Although the neuroprotective mechanisms of EPO are still not clearly understood, the signal transduction mechanism of EPO in brain may be similar to that in erythropoiesis. Our previous studies have showed that EPO could protect PC12 cells against $A\beta_{25–35}$-induced oxidative stress, mitochondrial dysfunction, and neurotoxicity by activating the PI3K/Akt signalling pathway (Ma *et al.*, 2009). However, to date, whether the JAK2/STAT5 and/or the ERK1/2 pathway participate in the molecular mechanisms that underlie the protective effect of EPO against the neurotoxicity of $A\beta_{25–35}$ has not been established.

The JAK2/STAT5 signalling pathway plays an important role in a variety of physiological processes, including cell growth, differentiation, immune function, and haematopoiesis (Liu *et al.*, 1999). The JAK2/STAT5 pathway is also the most frequently used signal transduction system involved in EPO-induced protection. EPO-induced phospho-STAT5 plays an important anti-apoptotic role in the heart, following cardiac ischaemia (Yamaura *et al.*, 2003), in the death of hippocampal neurons, induced by cerebral ischaemia (Zhang *et al.*, 2007), and in the survival of neuroblastoma SH-SY5Y cells (Um and Lodish, 2006). EPO also activates the ERK1/2 pathway which is also important for neuronal survival. However, the relative contribution of ERK1/2 to EPO-induced neuroprotection varies, depending on the model system examined.

The overall objective of the present study was to investigate the role of JAK2/STAT5 and ERK1/2 signalling pathways in EPO-induced protection against $A\beta_{25–35}$ neurotoxicity in PC12 cells. We found that EPO increased the phosphorylation and activation of JAK2 and STAT5 in Aβ-treated PC12 cells. Furthermore, EPO modulated the nuclear translocation of phospho-STAT5, increased the expression of Bcl-xL and decreased the levels of caspase-3. These beneficial effects were prevented by the JAK2 inhibitor, AG490 or by STAT5 knockdown. EPO also induced a transient increase of phospho-ERK1/2, but ERK1/2 activation was less important for the protective effect of EPO.

Methods

Preparation of aggregated β-amyloid

The peptide $A\beta_{25–35}$ was reconstituted in sterile water at a concentration of 400 μM. Aliquots were incubated at 37°C for 72 h to form aggregated amyloid. During the experiments we added aliquots of the peptide stock solution directly to the solution bathing the cell to achieve a final concentration of 20 μM.

Cell culture

The rat pheochromocytoma cell line PC12 cells were originally obtained from Chinese Type Culture Collection. All cells were plated in various size poly-L-lysine coated culture dishes at 37°C with a humid 5% CO_2 in DMEM supplemented with heat-inactivated fetal bovine serum (10% v/v) and 2 mM glutamine and the density of cells was not more than 80% confluence. Twenty-four hours after plating, the growth medium was replaced with fresh normal medium (control cultures) or with medium supplemented with 20 μM $A\beta_{25–35}$. In experiments involving EPO, it was added to cell cultures 1 h before $A\beta_{25–35}$ application. For kinase inhibitor studies, AG490 (10 μM) or PD98059 (20 μM) was applied to PC12 cells 30 min before the addition of EPO. The inhibitors were freshly prepared in DMSO.

siRNA transfection

STAT5-siRNA was used to inhibit endogenous STAT5 expression. A scrambled siRNA was used as a control. PC12 cells were seeded at 60% confluency into 6-well plates. 5 μL of STAT5-siRNA and 5 μL lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were diluted into 250 μL Opti-MEM I (Invitrogen) respectively, gently mixed and incubated at room temperature for 5 min, then lipofectamine 2000 mixture was added to the STAT5-siRNA mixture at a final concentration of 100 nM siRNA. Transfection of PC12 cells with scrambled siRNA served as a negative control. After incubation for 6 h at 37°C, 2 mL of complete medium with 10% FBS was added to the transfected cells to replace transfection solution and the cells were exposed to $A\beta_{25–35}$ at the same time. The knockdown of endogenous STAT5 by siRNA was confirmed by

Western blot. The transfected cells were cultured for 48 h then harvested for further analysis.

Assay of cell viability with 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT)

Cell viability was assessed by the MTT assay. PC12 cells were exposed to $A\beta_{25-35}$ (20 μM), with or without EPO (2 U·mL^{-1}) or AG490 or PD98059 for 24 h. After incubation, cells were treated with MTT (10 μL of 5 mg·mL^{-1}) for 4 h at 37°C. After this, the medium was removed and the cells were dissolved in 200 μL DMSO. The formazan reduction product was measured by reading absorbance at 570 nm in a plate reader. Results were expressed as the percentage of MTT reduction, setting that the absorbance of control cells to 100%.

Detection of apoptosis by TUNEL and acridine orange–ethidium bromide (AO/EB) double staining

Apoptotic cells were determined by the TUNEL method. Briefly, PC12 cells were seeded on coverslips coated with poly-L-lysine. After washing with PBS, the cells were fixed in 4% paraformaldehyde for 30 min. Endogenous peroxidase was quenched for 30 min at room temperature and the cells further permeabilized with 0.1% Triton X-100 in 0.1% sodium acetate for 5 min at 4°C. Thereafter, the cells were labelled by incubation with the TUNEL reaction mixture for 1 h at 25°C followed by labelling with peroxidase-conjugated anti-fluorescein anti-goat antibody for an additional 0.5 h. Subsequently, cells were incubated with diaminobenzidine substrate to produce a dark brown precipitate. TUNEL-positive and -negative cells were counted using five independent microscopic fields.

Morphological signs of apoptosis were also detected using AO/EB staining in PC12 cells. The cells were incubated with $A\beta_{25-35}$ for 24 h. The procedure to perform AO/EB staining is described as follows: 10 μL of AO (1 μg·μL^{-1}), and EB (1 μg·μL^{-1}) were added to 1 mL of cell suspension and incubated for 20 min at 37°C. The apoptotic cells were counted under an inverted fluorescence microscope (Olympus, Tokyo, Japan). Viable cells have uniform bright green nuclei with intact structure; early apoptotic cells have green nuclei, but chromatin condensation; late apoptotic cells stained by AO and EB were red-orange with chromatin condensation and small size.

Nuclear protein extraction

Nuclear protein was isolated according the procedure described by Davis *et al.*, (2001). Briefly, PC12 cells were washed in PBS and collected by centrifugation. The cell pellet was resuspended in 0.5 mL of 10 mM Tris·HCl (pH 7.5)/5 mM $MgCl_2$/0.05% (v/v) Triton X-100 and lysed with 20 strokes in a homogenizer. The homogenate was centrifuged at 10 000× *g* for 15 min at 4°C. The pellet was obtained. The nuclei pellet volume was estimated and the pellet was resuspended in an equal volume of $MgCl_2$ (5mM) in Tris·HCl buffer (10 mM; pH 7.4) The lysed nucleus was left on ice for 30 min and then centrifuged at 10 000× *g* for 15 min at 4°C. The supernatant (nuclear extract) was removed and 80% glycerol was added so that the final glycerol concentration was 20% (v/v). The concentration of the nuclear protein was determined.

Western blot analysis

Western blotting was performed using standard methods. After exposure to $A\beta_{25-35}$ and/or EPO, cells were rinsed twice with cold PBS and lysed in buffer (50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 100 mg·mL^{-1} PMSF). After incubation on ice for 30 min, cell lysates were then clarified by centrifugation at 16 000× *g* for 10 min at 4°C and the supernatant was saved for protein analysis and Western blot. Total protein concentration was determined by BCA kit. Equal amounts of proteins were fractionated by SDS-PAGE (7.5% standard gel), transferred to nitrocellulose membrane, and incubated with primary antibodies against JAK2, p-JAK2 ($Tyr^{1007/1008}$), STAT5, p-STAT5 (Tyr^{694}), ERK1/2, p-ERK ($Thr^{202/204}$) and Bcl-xL at 4°C overnight. The membranes were then washed twice with Tris-buffered saline with Tween 20 (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) and probed with the corresponding secondary antibodies conjugated with HRP at room temperature for 1 h. The signals were enhanced chemiluminescence, then exposed to X-ray films (Fuji, Japan). Blots were quantified using the analysis system (GDS8000, Ultra-Violet Products, Cambridge, UK). All data from three independent experiments were expressed as the ratio to OD values of the corresponding controls for the statistical analyses.

Assay for caspase-3 activity

The activity of caspase-3 was detected with the caspase-3 fluorometric assay kit. After appropriate treatments, 1×10^5 cells were harvested by centrifugation at 1000× *g* for 5 min at 4°. Cell pellets were washed with 1 mL of PBS, then suspended in 100 μL of lysis buffer and lysed on ice for 10 min. The protein concentrations of the supernatant fluids were ascertained with the BCA kit. Samples containing 200 μg protein were mixed with the reaction buffer and DEVD-AFC substrate, followed by 2 h incubation at 37°C. The fluorescence was measured at an excitation wavelength of 400 nm and emission wavelength of 505 nm with fluorometric reader. Experiments were run three times separately. Levels of caspase-3-like activity were expressed relative to the content in the cells incubated in the medium containing serum without Aβ.

Data analysis

Data in text and figures are expressed as mean ± SD. Two group comparisons were evaluated by paired or unpaired *t*-tests. Multiple comparisons were analysed by ANOVA and Tukey's or Newman–Keul's *post hoc* tests. All *P*-values were two-sided and a value of $P < 0.05$ was considered statistically significant.

Materials

EPO was purchased from Shenyang Sunshine Pharmaceutical Co., Ltd. (Shenyang, China), $A\beta_{25-}$35, DMSO, PMSF, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and mouse β-actin monoclonal antibody were provided by Sigma-Aldrich (St. Louis, MO, USA). DMEM,

penicillin, streptomycin, and FBS were purchased from Gibco (Carlsbad, CA, USA). Antibodies to JAK2, p-JAK2 (Tyr$^{1007/1008}$), STAT5, p-STAT5 (Tyr^{694}), ERK1/2, p-ERK (Thr$^{202/204}$) Bcl-xL, antimouse-HRP IgG, anti-rabbit-HRP IgG, and AG490 were obtained from Cell Signalling Technology (Beverly, MA, USA). All other reagents were of the highest grade and obtained from Sigma, unless otherwise indicated.

Results

EPO induces tyrosine phosphorylation of JAK2 in Aβ-treated PC12 cells

EPO increases the phosphorylation and activation of JAK2, which is one of the kinases promoting cell survival and preventing apoptosis (Yamaura *et al.*, 2003). To measure phosphorylation of JAK2 in EPO-treated PC12 cells, serum-starved PC12 cells were treated with 2 U·mL^{-1} of EPO and the samples were collected at 0, 0.5, 1, 3 and 6 h. As shown in Figure 1A, treatment of PC12 cells with EPO increased expression of p-JAK2 as early as 0.5 h and this was maintained for 6 h. Interestingly, pretreatment of cells with EPO for 0.5 h followed by exposure to $A\beta_{25-35}$ for 3 and 6 h, respectively, also increased p-JAK2 (Figure 1B).

To further determine the role of JAK2 signalling pathway in the protective effect of EPO against Aβ-induced neurotoxicity, we next sought to examine whether tyrphostin AG490, a high selective specific inhibitor of JAK2 could block EPO-mediated protection. PC12 cells were pretreated with AG490, for 0.5 h before addition of EPO, and this treatment decreased EPO-induced tyrosine phosphorylation of JAK2 in PC12 cells (Figure 1C). In these experiments, treatment with AG490 alone also decreased p-JAK levels.

EPO induces tyrosine phosphorylation and promotes nuclear translocation of STAT5 during Aβ toxicity

STAT5 is well recognized as an important substrate of JAK2 signalling with a key role in neuronal survival and proliferation (Sato *et al.*, 1993). As shown in Figure 2A, phosphorylation of STAT5 was induced by EPO in a time-dependent manner, and the time course of enhanced phosphorylation of STAT5 is in parallel with the activation of JAK2. In order to determine the contribution of STAT5 in EPO-mediated neuroprotection, PC12 cells treated with EPO and/or Aβ for 3 or 6 h were harvested and subjected to Western blot analysis. Intriguingly, treatment with EPO and Aβ for either 3 or 6 h resulted in activation of STAT5 (Figure 2B), whereas pretreatment with the JAK2 inhibitor AG490 inhibited the tyrosine phosphorylation of STAT5 (Figure 2C). In these experiments, total STAT5 levels did not change.

Activation of STAT5 can be evaluated by its phosphorylation but also by its translocation to the nucleus (Penta and Sawyer, 1995), where it increases the expression of its target genes, thus preventing apoptosis. In the present experiments, we assessed the effects of EPO on the nuclear translocation of p-STAT5 in PC12 cells during $A\beta_{25-35}$ exposure. Treatment

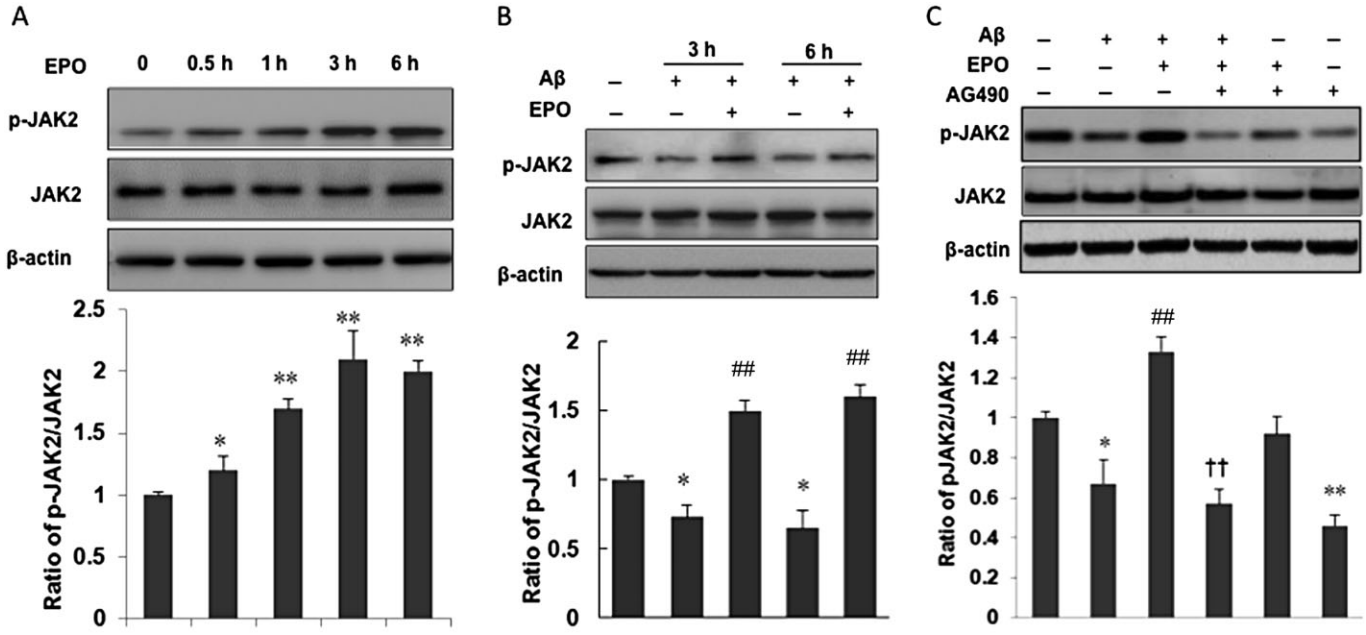

Figure 1

EPO enhances phosphorylation of JAK2 in Aβ-treated PC12 cells. (A) EPO increases phospho-JAK2 expression in a time-dependent manner. PC12 cells were exposed to EPO (2 U/mL) for 0.5, 1, 3 and 6 h, and subjected to Western blot analysis. (B) EPO increases p-JAK2 in Aβ-treated PC12 cells at 3 and 6 h. PC12 cells were pretreated with EPO for 0.5 h, and then exposed to Aβ for 1 and 3 h. The levels of phosphorylated JAK2 were determined by Western blot. (C) AG490 inhibited the effect of EPO on JAK2 phosphorylation. PC12 cells were treated with EPO and/or Aβ in the absence or presence of JAK2 inhibitor AG490 followed by detection of JAK2 phosphorylation by Western blot. Data are means ± SD, $n = 3$. *$P <$ 0.05, **$P < 0.01$ versus control. $^{\#\#}P < 0.01$ compared with $A\beta_{25-35}$. $^{\dagger\dagger}P < 0.01$ versus EPO + $A\beta_{25-35}$.

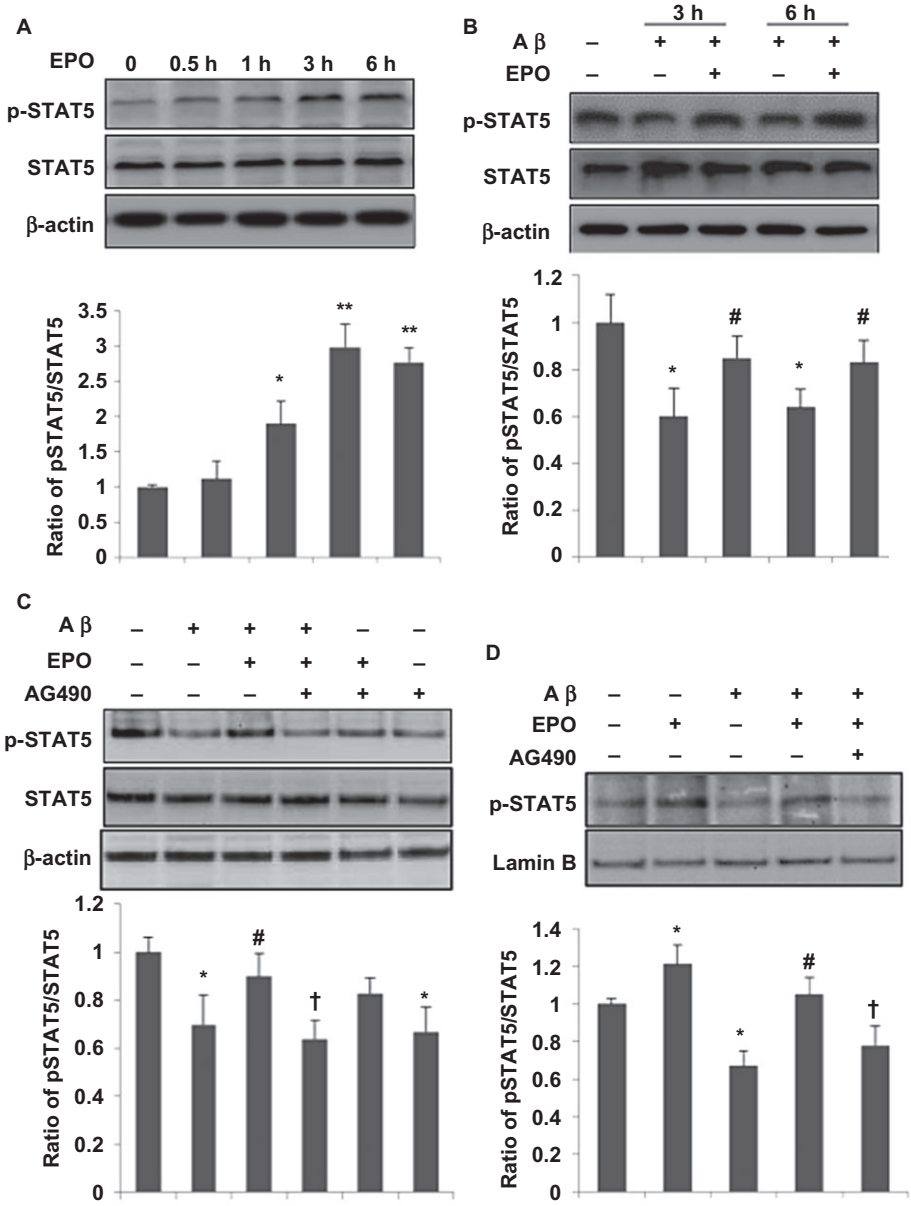

Figure 2

EPO enhances phosphorylation and nuclear transcription of STAT5 in Aβ-treated PC12 cells. (A) EPO increases phospho-STAT5 expression in a time-dependent manner. PC12 cells were exposed to EPO for 0.5, 1, 3 and 6 h, and subjected to Western blot analysis. (B) EPO increases p-STAT5 in Aβ-treated PC12 cells at 3 and 6 h. PC12 cells were pretreated with EPO for 0.5 h, and then exposed to Aβ for 3 and 6 h. The levels of phosphorylated STAT5 were determined by Western blot. (C) AG490 inhibited the effect of EPO on STAT5 phosphorylation. PC12 cells were treated with EPO and/or Aβ in the absence or presence of JAK2 inhibitor AG490 followed by detection of STAT5 phosphorylation by Western blot. (D) PC12 cells were pretreated with EPO for 1 h, exposed to Aβ for 6 h. Nuclear proteins for phospho-STAT5 expression were extracted for Western blot analysis. EPO promotes p-STAT5 translocation and AG490 could inhibit it. Data are means ± SD, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. $^{\#}P < 0.05$ compared with $A\beta_{25-35}$. $^{\dagger}P < 0.05$ versus EPO + $A\beta_{25-35}$.

with EPO alone could significantly promote p-STAT5 translocation, which is consistent with a previous report (Zhang *et al.*, 2007). Additionally, when PC12 cells treated with EPO and/or Aβ for 6 h were fractionated into cytoplasmic and nuclear compartments, we found an increased nuclear accumulation of p-STAT5 after EPO, compared with that in $A\beta_{25-35}$-treated cells (Figure 2D). These effects of EPO were reversed by the JAK2 inhibitor AG490.

EPO increases the expression of the anti-apoptotic protein Bcl-xL and decreases the level of caspase-3

As Bcl-xL, a member of the Bcl-2 family, is a response gene of active STAT5 protein, we investigated whether the effects of EPO effect were associated with enhancement of the expression of the anti-apoptotic factor Bcl-xL. As shown in

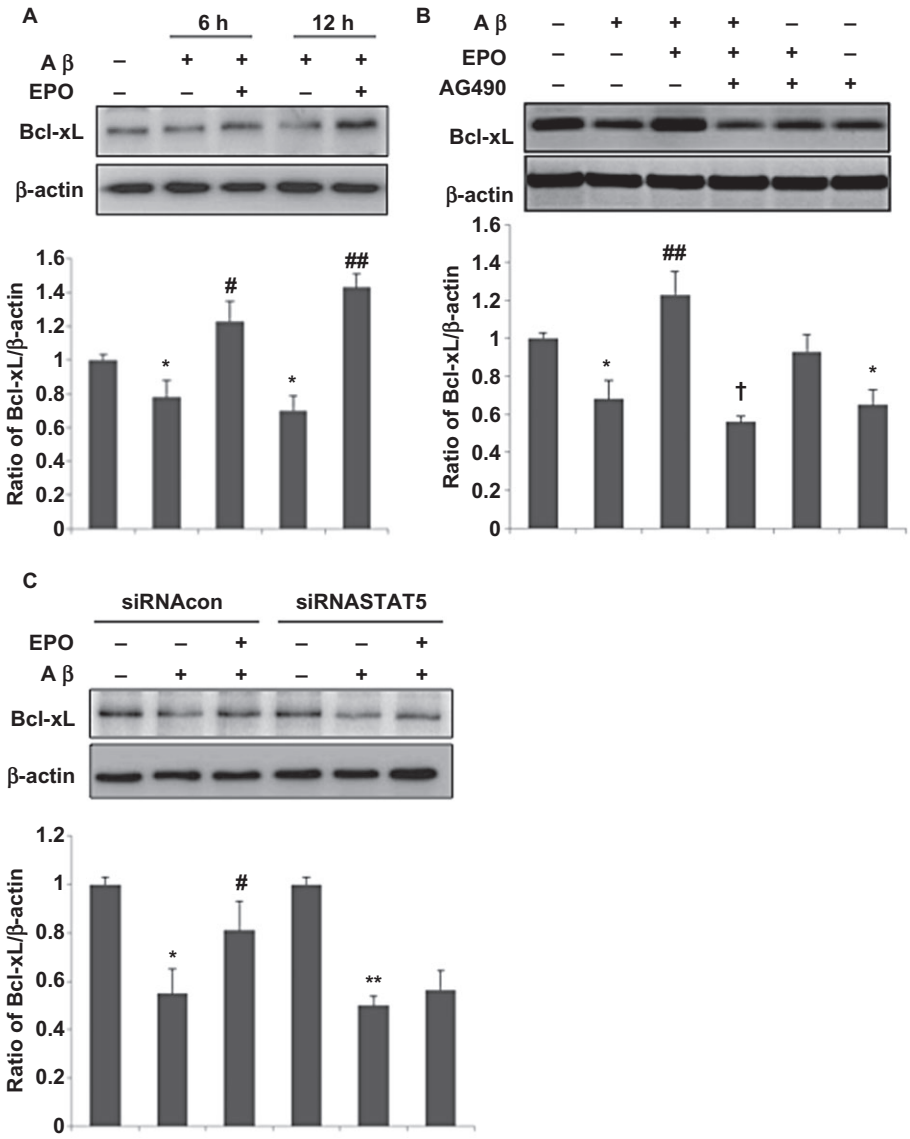

Figure 3

Effects of EPO on Aβ-induced decrease in Bcl-xL level in PC12 cells. (A) PC12 cells were pretreated with EPO for 1 h, and then exposed to Aβ for 6 and 12 h. The levels of Bcl-xL were determined by Western blot. (B) JAK2 inhibitor AG490 attenuated EPO-induced up-regulation of- Bcl-xL. (C) STAT5 knockdown attenuated EPO-induced up-regulation of Bcl-xL. STAT5-siRNA or scramble siRNA was transfected into PC12 cells and after 2 days of transfection Aβ was applied to PC12 cells for 12 h with or without pretreatment of EPO. Data are means ± SD, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with $A\beta_{25-35}$. $^{\dagger}P < 0.05$ versus EPO + $A\beta_{25-35}$.

Figure 3A, the expression of Bcl-xL after EPO pretreatment was up-regulated, compared with that in the group treated with $A\beta_{25-35,}$ at 6 h. We also detected a significant increase of Bcl-xL at 12 h following $A\beta_{25-35}$ and EPO co-incubation. Furthermore, the up-regulation of Bcl-xL expression induced by EPO was blocked by pretreatment with the JAK2 inhibitor AG490 or STAT5-siRNA (Figure 3B and C).

Down-regulation of Bcl-xL and subsequent release of cytochrome c from mitochondria and activation of caspase-3 could be responsible for cell apoptosis. We found that, in PC12 cells treated with $A\beta_{25-35}$, caspase-3 was increased, and this response was depressed by EPO pretreatment. However, pretreatment with the AG490 or STAT5-siRNA inhibited EPO-mediated cell protection compared with EPO and $A\beta_{25-35}$ co-incubation (Figure 4).

Effect of JAK2 inhibitor AG490 or siRNA targeting STAT5 on the protection of EPO against Aβ-induced apoptosis

To further understand the mechanisms underlying the protective effect of EPO on $A\beta_{25-35}$-induced PC12 cell death, we measured cell death with two different assays. First, the TUNEL technique was used to show the typical morphological features of apoptosis such as chromatin condensation, cytoplasmic budding and apoptotic bodies. Cells co-incubated with EPO (2 U·mL^{-1}) and $A\beta_{25-35}$ (20 μM) for 24 h

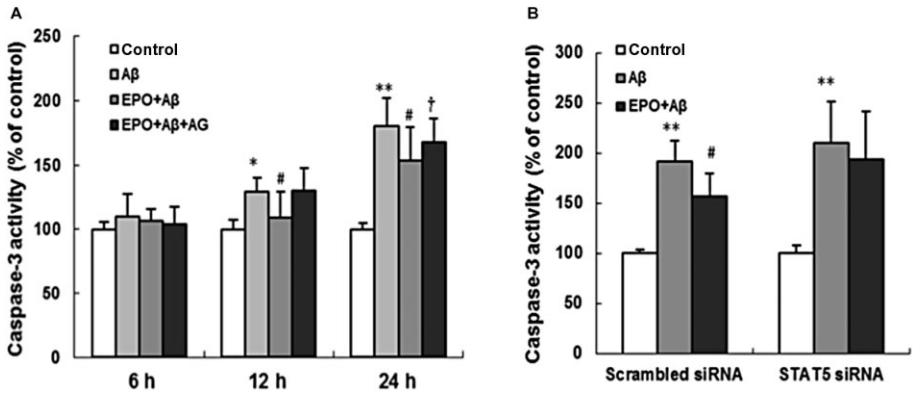

Figure 4

EPO inhibited $A\beta_{25-35}$-induced increase in caspase-3 activity. (A) PC12 cells were pretreated with EPO for 1 h, and then exposed to Aβ for 6, 12 and 24 h with or without JAK2 inhibitor AG490. Caspase-3 activity was measured by fluorescence assay. (B) STAT5 knockdown attenuated EPO-induced down-regulation of caspase-3. STAT5-siRNA or scrambled siRNA was transfected into PC12 cells and after two days of transfection, Aβ was applied to PC12 cells for 24 h with or without pretreatment of EPO. The data expressed as described in the Methods section. Each value is the mean ± SD of five wells per experimental group. Results were confirmed by three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. $^{\#}P < 0.05$ versus $A\beta_{25-35}$. $^{\dagger}P < 0.05$ versus EPO + $A\beta_{25-35}$.

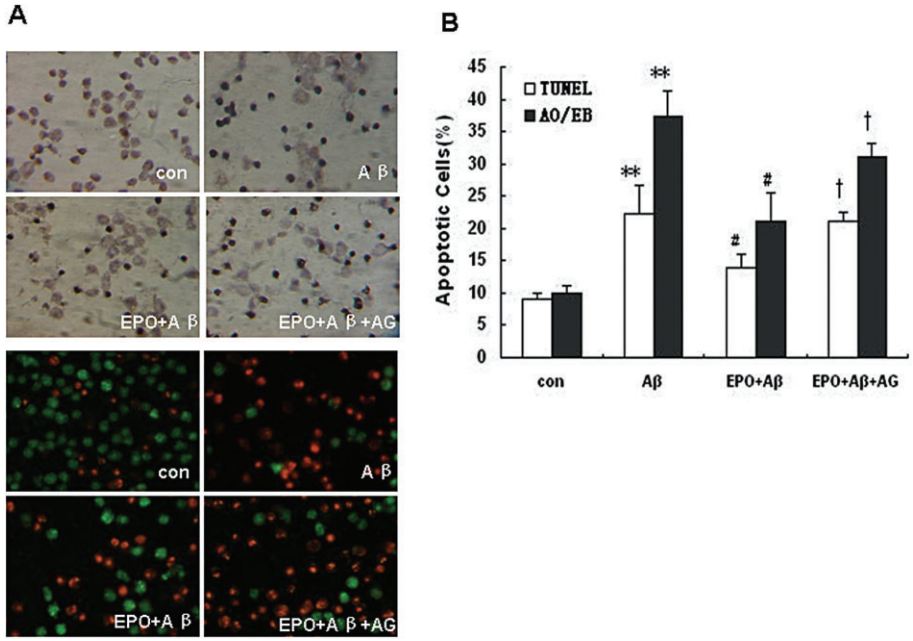

Figure 5

EPO prevented $A\beta_{25-35}$-induced apoptosis in PC12 cells. (A) Apoptosis as stained by TUNEL and AO/EB staining. (B) Quantification of percent apoptotic PC12 cells after exposure to $A\beta_{25-35}$. Results are shown as the mean ± SD. and represent four independent experiments. $^{**}P < 0.01$ versus control. $^{\#}P < 0.01$ compared with $A\beta_{25-35}$. $^{\dagger}P < 0.01$ versus EPO + $A\beta_{25-35}$.

showed greater viability than cells treated only with $A\beta_{25-35}$. However, this neuroprotective effect was lost when the cells were pretreated with the JAK2 inhibitor AG490 or STAT5-siRNA (data not shown), followed with EPO and $A\beta_{25-35}$ co-incubation (Figure 5).

Next, we examined nuclear morphology by staining the cells with AO/EB after treatment with AG490. PC12 cells stained with AO/EB showed typical morphological features of apoptosis, including nuclear condensation and/or fragmentation after treatment with 20 μM $A\beta_{25-35}$. When PC12 cells were treated with AG490 0.5 h before being treated with EPO, the neuroprotective effects of EPO were significantly antagonized (Figure 5).

Effect of AG490 or STAT5- siRNA on the viability of PC12 cells

Loss of cell viability in culture is usually measured by a decrease in MTT activity. As shown in Figure 6A,

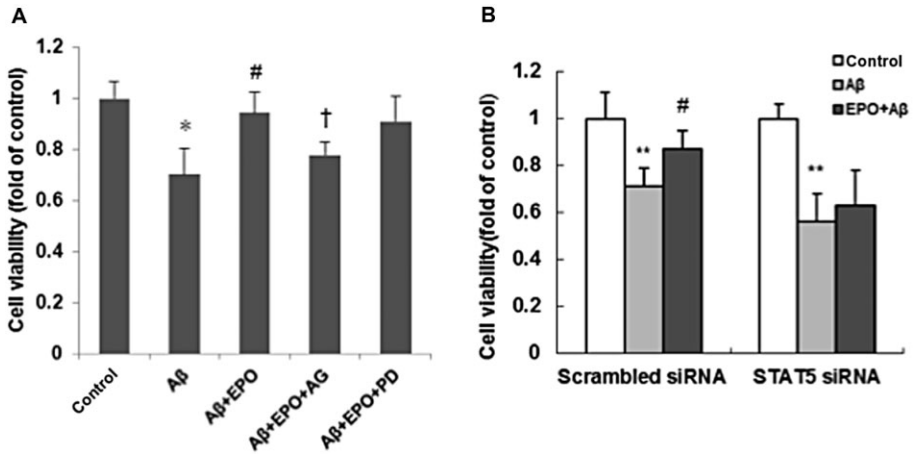

Figure 6

Protective effects of EPO on cell viability against $A\beta_{25-35}$-induced cytotoxicity in PC12 cells. (A) PC12 cells were pretreated with EPO for 1 h, and then exposed to Aβ for 24 h with or without JAK2 inhibitor AG490 or PD98059. Cell viability was measured by MTT assay. (B) STAT5 knockdown attenuated EPO-induced up-regulation of PC12 cell viability. STAT5-siRNA or scrambled siRNA was transfected into PC12 cells and after 2 days of transfection, Aβ was applied to PC12 cells for 24 h with or without pretreatment of EPO. The data are the mean ± SD expressed as percentage of control values obtained from five replicate values in three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. $^{\#}P < 0.05$ compared with $A\beta_{25-35}$. $^{\dagger}P < 0.05$ versus EPO + $A\beta_{25-35}$.

pretreatment with 20 μM $A\beta_{25-35}$ alone decreased the viability of PC12 cells, compared with the control. After PC12 cells were treated with EPO at 2 $U{\cdot}mL^{-1}$ for 24 h, cell viability was clearly increased. However, when the cells were pre-treated with AG490 for 0.5 h or transfected with STAT5-siRNA, and subsequently exposed to EPO and $A\beta_{25-35}$ for 24 h, cell viability was again considerably decreased, (Figure 6A and B).

EPO induced transient phosphorylation of ERK, but ERK was not involved in EPO-induced neuroprotection in Aβ-treated PC12 cells

The ERK pathway is another major mediator for the pro-survival effects of EPO in haematopoietic cells. Therefore, we investigated whether protection of EPO against $A\beta_{25-35}$ neurotoxicity involved activation of ERK. EPO treatment increased phosphorylation of ERK1/2 at 1 h, which then decreased markedly by 6 h. Interestingly, pretreatment of cells with EPO for 0.5 h followed by exposure to $A\beta_{25-35}$ for 3 and 6 h, respectively, increased p-ERK1/2 at 3 h (Figure 7B), but had no effect by 6 h. This phosphorylation of ERK was completely inhibited by the ERK inhibitor, PD98059 at 3 h (Figure 7C) but, when PC12 cells were pretreated with PD98059, there was no significant effect on the protection conferred by EPO (Figure 6A) Similar results were obtained using TUNEL and AO/EB staining assay (data not shown).

Discussion

It has been proposed that EPO is a pleiotropic cytokine and has a neuroprotective effect in the CNS, but the detailed mechanisms for EPO have not been fully described. The present study elucidated some of the downstream neuroprotective signalling pathways initiated by EPO. We confirmed that treatment with EPO increased phosphorylation of JAK2 in Aβ-treated PC12 cells and that EPO induced tyrosine phosphorylation and increased nuclear translocation of STAT5 during $A\beta_{25-35}$ toxicity. Further, EPO-initiated STAT5 signalling to regulate neuronal death were executed by up-regulation of Bcl-xL and inhibition of caspase-3. Finally, we showed that the ERK1/2 pathway was not involved in the increased survival of PC12 cells exposed to $A\beta_{25-35}$-induced neurotoxicity, following treatment with EPO (Figure 8).

According to the 'Aβ toxicity' hypothesis, deposition of the Aβ peptide is a primary event in the pathological cascade of Alzheimer's disease (Masters *et al.*, 1985; Masters and Beyreuther, 2006). The neurotoxicity exerted by aggregated Aβ involves a number of cellular and molecular mechanisms, such as the generation of reactive oxygen species, increased intracellular Ca^{2}+ concentration and induction of apoptosis (Arispe *et al.*, 1993; Behl *et al.*, 1994). Inhibition of neuronal apoptosis may be considered as one of the effective therapeutic strategies to treat Aβ-induced neurotoxicity and thus to improve neurological outcome in Alzheimer's disease.

EPO, a potent cytokine that exerts pleiotropic functions related to proliferation, differentiation, and inhibition of apoptosis, is the prototypical member of a family of cytokines whose functions are still being identified. In neuronal injury models, rhEPO administration protected against ischaemia and free radical injury (Maiese *et al.*, 2004). Indeed, EPO acts by binding to the EPO receptor homodimer and thus changes the conformation of the EPO receptor, activating and phosphorylating JAK2, leading to the activation of several downstream-signalling pathways including STAT5, PI3K, ERK1/2 and other signalling molecules that promote cell survival (Wojchowski *et al.*, 1999). However, which of these

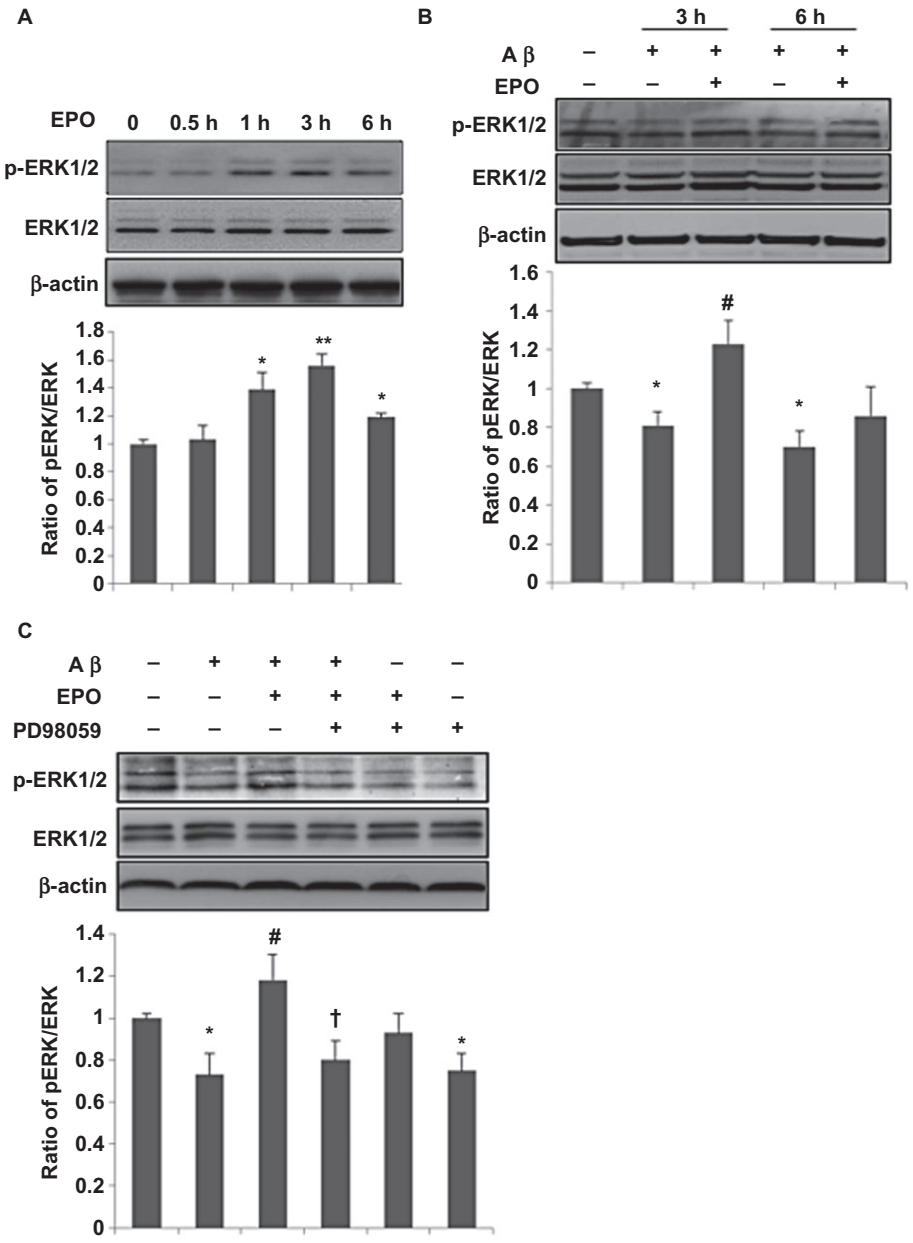

Figure 7

EPO-mediated phosphorylation of ERK1/2 in PC12 cells. (A) EPO increases p-ERK1/2 expression in a time-dependent manner. PC12 cells were treated with EPO for 0, 0.5, 1, 3 and 6 h, and the cytoplasm proteins for ERK1/2 and p-ERK1/2 were extracted for Western blot analysis. (B) EPO increases p-ERK1/2 in Aβ-treated PC12 cells at 3 and 6 h. PC12 cells were pretreated with EPO for 0.5 h, and then exposed to Aβ for 3 and 6 h. The levels of phosphorylated ERK1/2 were determined by Western blot. (C) Cell samples were respectively obtained from PC12 cells in different experimental groups: control, $A\beta_{25-35}$, EPO, $A\beta_{25-35}$ + EPO, $A\beta_{25-35}$ + EPO + PD98059, EPO + PD98059 and PD98059. The ERK1/2 inhibitors PD98059 decreased EPO-enhanced p-ERK1/2 levels when $A\beta_{25-35}$ applied to PC12 cells. Data are means ± SD for three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. $^{\#}P < 0.05$ compared with $A\beta_{25-35}$. $^{\dagger}P < 0.01$ versus EPO + $A\beta_{25-35}$.

pathways could mediate the protective effects of EPO on Aβ-treated PC12 cells was not known. In an earlier paper, we demonstrated that EPO protected against apoptosis induced in PC12 cells by Aβ not only by preventing the loss of the mitochondrial membrane potential and expression of Bcl-2 and increasing ROS levels, but also through the activation of the PI3K/Akt signalling pathway (Ma *et al.*, 2009). However, whether other pathways contributed to the protective effects of EPO in this model was still unknown.

From our earlier studies with a range of concentrations of $A\beta_{25-35}$ (0–40 μM) and of EPO (0.5–10 $U{\cdot}mL^{-1}$), we found that 20 μM $A\beta_{25-35}$ was clearly cytotoxic to PC12 cells and that EPO prevented Aβ-induced apoptosis concentration-dependently manner, with a maximal effect at 2 $U{\cdot}mL^{-1}$. We used these

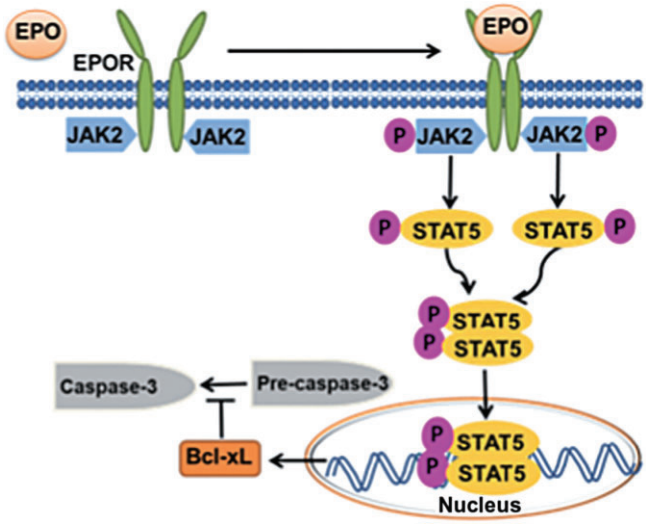

Figure 8

Diagram illustrating possible signalling pathways involved in EPO-mediated neuroprotection. EPO binds to the EPO receptor and induces receptor dimerization, leading to phosphorylation of JKA2. p-JAK2 activates STAT5 and promotes nuclear translocation of p-STAT5. In the nucleus, p-STAT5 binds to its cognate promoter, which results in the activation of transcription of genes that regulate cell proliferation and apoptosis.

experimental conditions to investigate the involvement of the JAK2/STAT5 and ERK signalling pathways to the protective effects of EPO.

JAKs, which are recruited to the membrane and phosphorylated following growth factor or cytokine stimulation, catalyse the selective activation and phosphorylation of STAT. To date, four mammalian JAKs (JAK1, JAK2, JAK3 or TYK2) and seven STATs have been identified [STAT1, 2, 3, 4, 5a, 5b (referred to as STAT5) and 6] (Ihle, 1995; Darnell, 1997). Each cytokine or growth factor receptor recruits different combinations of JAKs and STATs, thereby controlling, at least in part, the specificity of downstream transcriptional events. For EPO, the JAK2/STAT5 combination is the most important pro-survival signalling pathway.

To address whether acute EPO treatment could activate JAK2 in PC12 cells and to examine the time course of induction, PC12 cells were stimulated with EPO for different periods of time (1–6 h) and formation of p-JAK2 was assessed by Western blot analysis. There was a gradual clear increase in p-JAK2. Phosphorylated JAK2 leads to the activation of neighbouring JAK2, other receptor subunits, and several other substrates, which could be inhibited by pharmacological approaches. Tyrphostin AG490, a synthetic compound, is known to block the cytokine-induced phosphorylation and activation of JAK2 kinase in most cell types (Meydan *et al.*, 1996; Ishihara *et al.*, 2001). We also found that when AG490 was combined with EPO, phosphorylation of JAK2 was decreased in PC12 cells, with or without exposure to $A\beta_{25-35}$.

Phosphorylation of JAKs leads to their activation, and activated JAKs phosphorylate and thus activate STATs. The classical function of the STATs is to mediate the signalling pathways of cytokines and growth factors. Some of the STAT members can promote the processes of apoptosis (Chiba *et al.*, 2009), whereas STAT5 is closely involved in the processes of anti-apoptosis (Zhang *et al.*, 2007). Phosphorylation of STAT5 results in its dimerization and translocation to the nucleus where it activates the transcription of several anti-apoptotic genes. STAT5 was important in the anti-apoptotic effect of EPO in heart, following cardiac ischaemia, in the death of hippocampal neurons after transient global cerebral ischaemia and in the survival of neuroblastoma SH-SY5Y cells. However, it was not clear if EPO mediated its protective effect against different inducers of cell death through the same molecular mechanisms, nor was it clear if EPO protected PC12 cells against Aβ-induced cell death through the activation of STAT5. In our experiments, phosphorylation of STAT5 was induced within 1 h and then rapidly increased to peak levels. The time course of enhanced phosphorylation of STAT5 was in parallel with the activation of JAK2. We also found that AG490, a JAK2 inhibitor, diminished EPO-enhanced phosphorylation of STAT5.

Phosphorylated STAT5 homodimerizes and enters the cell nucleus where it activates the transcription of several genes including *Bcl-xL* (Kirito *et al.*, 2002), whose gene product, Bcl-xL, functions as an anti-apoptotic protein. Bcl-xL in turn prevents cytochrome c release from mitochondria into the cytosol, where it forms a complex, activating caspase-9. This process leads to the downstream activation of caspase-3 and, ultimately, cell death. In STAT5-deficient bone marrow mast cells, there is a marked decrease of detectable Bcl-xL protein and a corresponding marked increase in activation of caspase-3 (Shelburne *et al.*, 2002). We therefore investigated whether, in our model, EPO could change levels of Bcl-xL and caspase-3. We found that, after treatment of PC12 cells with EPO, Bcl-xL was up-regulated. However, the levels of caspase-3 in the cytosol were significantly lower than in the cells exposed only to Aβ peptide. Moreover, these effects of EPO were abolished by the JAK2-specific inhibitor AG490. These findings imply that EPO inhibited apoptosis by modulating of the expression of Bcl-xL and the release of caspase-3, through the JAK2/STAT5 pathway.

The JAK2/STAT5 transduction pathway has several components and STAT5 has biological activities other than transcriptional regulation. Particularly relevant in our context was the crosstalk between the PI3K and STAT5 pathways (Kirito *et al.*, 2002; Nyga *et al.*, 2005). Our previous results had demonstrated the protective effects of EPO against Aβ-induced neurotoxicity in PC12 cells were in part mediated by activation of the PI3K/Akt/GSK3β pathway. Other researchers have found that activated STAT5 interacts with the regulatory subunit of PI3K. Another possibility is that the PI3K/Akt pathway could synergize with STAT5 to enhance expression of Bcl-xL. More studies are needed to explore the interactions between the PI3K pathway and JAK2/STAT5 pathway, in the context of the protective effects of EPO during Aβ-induced apoptosis.

In addition to the STAT5-mediated pathway, EPO activates other parallel pathways, including ERK1/2, a signalling kinase downstream of MAPK, which is involved in the anti-apoptotic effects of EPO on erythroblasts (Sattler *et al.*, 2004). However, the importance of the ERK pathway in the neuroprotective capacity of EPO ranged from either unimportant or minor to significant. In the present study, EPO rapidly, but

only transiently, phosphorylated and activated ERK1/2. As shown in Figure 7B, in cells pretreated with EPO and then exposed to $A\beta_{25-35}$ for 3 and 6 h, respectively, enhanced-p-ERK1/2 was only observed at 3 h. Inhibition of the phosphorylation of ERK by PD98059 did not abolish the increased cell viability and decreased apoptosis, induced by EPO. These data suggested that ERK1/2 signal transduction cascade did not play a crucial role in EPO-mediated protection against Aβ-induced apoptosis. The reasons for this discrepancy are not clear, but differences in anti-apoptotic pathways in the different cell lines and other experimental differences have to be considered.

In conclusion, our results demonstrated that acute EPO treatment activated several signalling pathways in Aβ-treated PC12 cells, including JAK2/STAT5 and MAPK/ERK. The inhibition of EPO-induced protection of PC12 cells following the blockade of JAK2/STAT5 signalling pathway by pharmacological agents support the crucial role of this pathway in anti-apoptotic effects during $A\beta_{25-35}$ toxicity. The results from the present study may help in designing clinical studies of the potential benefits of the treatment of Aβ neurotoxicity in Alzheimer's disease.

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Author contributions

R. M. and G. L. conceived and designed the experiments. R. M., C. F. H., J. H. and M. W. performed the experiments. J. H., J. Z. X. and G. L. analysed the data. R. M. and G. L. wrote the paper.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013a). The Concise Guide to PHARMACOLOGY 2013/14: Catalytic Receptors. British Journal of Pharmacology, 170, 1676–1705

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013b). The Concise Guide to PHARMACOLOGY 2013/14: Enzymes. British Journal of Pharmacology, 170: 1797–1867

Anagnostou A, Liu Z, Steiner M, Chin K, Lee ES, Kessimian N *et al.* (1994). Erythropoietin receptor mRNA expression in human endothelial cells. Proc Natl Acad Sci 91: 3974–3978.

Arispe N, Rojas E, Pollard HB (1993). Alzheimer disease amyloid beta protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminium. Proc Natl Acad Sci U S A 90: 567–571.

Behl C, Davis JB, Lesley R, Schubert D (1994). Hydrogen peroxidemediates amyloid beta protein toxicity. Cell 77: 817–827.

Bergelson S, Klingmüller U, Socolovsky M, Hsiao JG, Lodish HF (1998). Tyrosine residues within the intracellular domain of the erythropoietin receptor mediate activation of AP-1 transcription factors. J Biol Chem 273: 2396–2401.

Bernaudin M, Marti HH, Roussel S, Divoux D, Nouvelot A, MacKenzie ET *et al.* (1999). A potential role for erythropoietin in focal permanent cerebral ischemia in mice. J Cereb Blood Flow Metab 19: 643–651.

Chiba T, Yamada M, Sasabe J, Terashita K, Shimoda M, Matsuoka M *et al.* (2009). Amyloid-beta causes memory impairment by disturbing the JAK2/STAT3 axis in hippocampal neurons. Mol Psychiatry 14: 206–222.

Darnell JE (1997). STATs and gene regulation. Science 277: 1630–1635.

Davis JN, Kucuk O, Djuric Z, Sarkar FH (2001). Soy isoflavone supplementation in healthy men prevents NF-kappa B activation by TNF-alpha in blood lymphocytes. Free Radic Biol Med 30: 1293–1302.

Genc S, Kuralay F, Genc K, Akhisaroglu M, Fadiloglu S, Yorukoglu K *et al.* (2001). Erythropoietin exerts neuroprotection in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated C57/BL mice via increasing nitric oxide production. Neurosci Lett 298: 139–141.

Harkany T, Abrahám I, Kónya C, Nyakas C, Zarándi M, Penke B *et al.* (2000). Mechanisms of beta-amyloid neurotoxicity: perspectives of pharmacotherapy. Rev Neurosci 11: 329–382.

Ihle JN (1995). The Janus protein tyrosine kinase family and its role in cytokine signaling. Adv Immunol 60: 1–35.

Ishihara K, Satoh I, Mue S, Ohuchi K (2001). Possible participation of a JAK2 signaling pathway in recombinant rat interleukin-5-induced prolongation of rat eosinophil survival. Biochem Biophys Acta 1536: 73–84.

Jelkmann W (1992). Erythropoietin: structure, control of production, and function. Physiol Rev 72: 449–489.

Kirito K, Watanabe T, Sawada K, Endo H, Ozawa K, Komatsu N (2002). Thrombopoietin regulates Bcl-xL gene expression through Stat5 and phosphatidylinositol 3-kinase activation pathways. J Biol Chem 277: 8329–8337.

Koury MJ, Bondurant MC (1992). The molecular mechanism of erythropoietin action. Eur J Biochem 210: 649–663.

Liu RY, Fan C, Garcia R, Jove R, Zuckerman KS (1999). Constitutive activation of the JAK2/STAT5 signal transduction pathway correlates with growth factor independence of megakaryocytic leukemic cell lines. Blood 93: 2369–2379.

Ma R, Xiong N, Huang C, Tang Q, Hu B, Xiang J *et al.* (2009). Erythropoietin protects PC12 cells from beta-amyloid (25–35)-induced apoptosis via PI3K/Akt signaling pathway. Neuropharmacology 56: 1027–1034.

Maiese K, Li F, Chong ZZ (2004). Erythropoietin in the brain: can the promise to protect be fulfilled? Trends Pharmacol Sci 25: 577–583.

Masters CL, Beyreuther K (2006). Alzheimer's centennial legacy: prospects for rational therapeutic intervention targeting the Abeta amyloid pathway. Brain 129: 2823–2839.

Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K (1985). Amyloid plaque core protein in Alzheimer disease and Down syndrome. Proc Natl Acad Sci U S A 82: 4245–4249.

Meydan N, Grunberger T, Dadi H, Shahar M, Arpaia E, Lapidot Z *et al.* (1996). Inhibition of acute lymphoblastic leukemia by a JAK2 inhibitor. Nature 379: 645–648.

Miller CP, Liu ZY, Noguchi CT, Wojchowski DM (1999). A minimal cytoplasmic subdomain of the erythropoietin receptor mediates erythroid and megakaryocytic cell development. Blood 94: 3381–3387.

Miranda S, Opazo C, Larrondo LF, Muñoz FJ, Ruiz F, Leighton F *et al.* (2000). The role of oxidative stress in the toxicity induced by amyloid beta-peptide in Alzheimer's disease. Prog Neurobiol 62: 633–648.

Morakkabati N, Gollnick F, Meyer R, Fandrey J, Jelkmann W (1996). Erythropoietin induces Ca2 + mobilization and contraction in rat mesangial and aortic smooth muscle cultures. Exp Hematol 24: 392–397.

Morishita E, Narita H, Nishida M, Kawashima N, Yamagishi K, Masuda S *et al.* (1996). Anti-erythropoietin receptor monoclonal antibody: epitope mapping, quantification of the soluble receptor, and detection of the solubilized transmembrane receptor and the receptor-expressing cells. Blood 88: 465–471.

Muñoz FJ, Inestrosa NC (1999). Neurotoxicity of acetylcholinesterase amyloid beta-peptide aggregates is dependent on the type of Abeta peptide and the AChE concentration present in the complexes. FEBS Lett 450: 205–209.

Nyga R, Pecquet C, Harir N, Gu H, Dhennin-Duthille I, Régnier A *et al.* (2005). Activated STAT5 proteins induce activation of the PI 3-kinase/Akt and Ras/MAPK pathways via the Gab2 scaffolding adapter. Biochem J 390 (Pt 1): 359–366.

Penta K, Sawyer ST (1995). Erythropoietin induces the tyrosine phosphorylation, nuclear translocation, and DNA binding of STAT1 and STAT5 in erythroid cells. J Biol Chem 270: 31282–31287.

Sato N, Sakamaki K, Terada N, Arai K, Miyajima A (1993). Signal transduction by the high affinity GM-CSF receptor: two distinct regions of the common beta chain subunit responsible for different signaling. EMBO J 12: 4181–4189.

Sattler MB, Merkler D, Maier K, Stadelmann C, Ehrenreich H, Mbahr R (2004). Neuroprotective effects and intracellular signaling pathways of erythropoietin in a rat model of multiple sclerosis. Cell Death Differ 11: S181–S192.

Selkoe DJ (2006). Amyloid beta-peptide is produced by cultured cells during normal metabolism: a reprise. J Alzheimer Dis 9: 163–168.

Shelburne CP, McCoy ME, Piekorz R, Sexl VV, Gillespie SR, Bailey DP *et al.* (2002). Stat5: an essential regulator of mast cell biology. Mol Immunol 38: 1187–1191.

Signore AP, Weng Z, Hastings T, Van Laar AD, Liang Q, Lee YJ *et al.* (2006). Erythropoietin protects against 6-hydroxydopamine-induced dopaminergic cell death. J Neurochem 96: 428–443.

Tabira T, Konishi Y, Gallyas F (1995). Neurotrophic effect of hematopoietic cytokines on cholinergic and other neurons *in vitro*. Int J Dev Neurosci 13: 241–252.

Um M, Lodish HF (2006). Antiapoptotic effects of erythropoietin in differentiated neuroblastoma SH-SY5Y cells require activation of both the STAT5 and AKT signaling pathways. J Biol Chem 281: 5648–5656.

Wojchowski DM, Gregory RC, Miller CP, Pandit AK, Pircher TJ (1999). Signal transduction in the erythropoietin receptor system. Exp Cell Res 253: 143–156.

Wu H, Lee SH, Gao J, Liu X, Iruela-Arispe ML (1999). Inactivation of erythropoietin leads to defects in cardiac morphogenesis. Development 126: 3597–3605.

Yamaura G, Turoczi T, Yamamoto F, Siddqui MA, Maulik N, Das DK (2003). STAT signaling in ischemic heart: a role of STAT5A in ischemic preconditioning. Am J Physiol Heart Circ Physiol 285: H476–H482.

Yankner BA (1996). Mechanisms of neuronal degeneration in Alzheimer's disease. Neuron 16: 921–992.

Youssoufian H, Longmore G, Neumann D, Yoshimura A, Lodish HF (1993). Structure, function, and activation of the erythropoietin receptor. Blood 81: 2223–2236.

Yu X, Shacka JJ, Eells JB, Suarez-Quian C, Przygodzki RM, Beleslin-Cokic B *et al.* (2002). Erythropoietin receptor signaling is required for normal brain development. Development 129: 505–516.

Zhang F, Wang S, Cao G, Gao Y, Chen J (2007). Signal transducers and activators of transcription 5 contributes to erythropoietin-mediated neuroprotection against hippocampal neuronal death after transient global cerebral ischemia. Neurobiol Dis 25: 45–53.