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Erythropoietin Requires NF- κ B and its Nuclear Translocation to Prevent Early and Late Apoptotic Neuronal Injury During β -Amyloid Toxicity

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Abstract

No longer considered exclusive for the function of the hematopoietic system, erythropoietin (EPO) is now considered as a viable agent to address central nervous system injury in a variety of cellular systems that involve neuronal, vascular, and inflammatory cells. Yet, it remains unclear whether the protective capacity of EPO may be effective for chronic neurodegenerative disorders such as Alzheimer's disease (AD) that involve β -amyloid (A β) apoptotic injury to hippocampal neurons. We therefore investigated whether EPO could prevent both early and late apoptotic injury during A β exposure in primary hippocampal neurons and assessed potential cellular pathways responsible for this protection. Primary hippocampal neuronal injury was evaluated by trypan blue dye exclusion, DNA fragmentation, membrane phosphatidylserine (PS) exposure, and nuclear factor- κ B (NF- κ B) expression with subcellular translocation. We show that EPO, in a concentration specific manner, is able to prevent the loss of both apoptotic genomic DNA integrity and cellular membrane asymmetry during A β exposure. This blockade of A β generated neuronal apoptosis by EPO is both necessary and sufficient, since protection by EPO is completely abolished by co-treatment with an anti-EPO neutralizing antibody. Furthermore, neuroprotection by EPO is closely linked to the expression of NF- κ B p65 by preventing the degradation of this protein by A β and fostering the subcellular translocation of NF- κ B p65 from the cytoplasm to the nucleus to allow the initiation of an anti-apoptotic program. In addition, EPO intimately relies upon NF- κ B p65 to promote neuronal survival, since gene silencing of NF- κ B p65 by RNA interference removes the protective capacity of EPO during A β exposure. Our work illustrates that EPO is an effective entity at the neuronal cellular level against A β toxicity and requires the close modulation of the NF- κ B p65 pathway, suggesting that either EPO or NF- κ B may be used as future potential therapeutic strategies for the management of chronic neurodegenerative disorders, such as AD.

Keywords

A β ; amyloid; apoptosis; Alzheimer's disease; erythropoietin; gene silencing; hippocampal; neurons; nuclear factor- κ B; phosphatidylserine; programmed cell death; RelA; siRNA

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INTRODUCTION

Alzheimer's disease (AD) leads to a progressive deterioration of cognitive function with memory loss and injury to hippocampal neurons. The generation of extracellular plaques of amyloid- β peptide aggregates composed of a 39-42 amino acid peptide ($A\beta$) are considered to be one of the pathological mechanisms that may promote the development of AD (Chong, ZZ *et al.*, 2005d). $A\beta$ is generated from the cleavage of amyloid precursor protein (APP) into products that include a 40-residue peptide ($A\beta_{40}$) and a 42-residue peptide ($A\beta_{42}$). Although $A\beta_{42}$ is produced in much smaller quantities than $A\beta_{40}$, it more readily forms fibrils and cerebral plaque than $A\beta_{40}$. As a result, $A\beta_{42}$ is considered to be the β -amyloid product that most directly contributes to the pathogenesis of AD and apoptotic injury.

Experimental evidence has shown that $A\beta$ accumulation can lead to apoptotic injury with chromatin condensation, DNA fragmentation, and cellular membrane phosphatidylserine (PS) exposure (Maiese, K and Chong, ZZ, 2004a). $A\beta$ may lead to the induction of caspase mediated pathways (Nakagawa, T *et al.*, 2000, Troy, CM *et al.*, 2001) that work in concert with oxidative stress (Tamagno, E *et al.*, 2003). Furthermore, $A\beta$ has been found to induce hydrogen peroxide generation through metal ion reduction and to lead to oxidative injury in neurons (Huang, X *et al.*, 1999). As a result, therapeutic strategies that address the toxicity of $A\beta$ may foster novel developments for the treatment of AD.

Erythropoietin (EPO) may be an agent that closely fits this desired profile to reduce or eliminate $A\beta$ toxicity during AD. The premise that EPO is required only for erythropoiesis has been advanced by studies demonstrating the existence of EPO and its receptor in the brain (Maiese, K *et al.*, 2005a, Maiese, K *et al.*, 2005b) and that protection by EPO in the central nervous system can be quite robust (Maiese, K *et al.*, 2004b). For example, EPO has been demonstrated to reduce or prevent vascular injury and cellular inflammation (Chong, ZZ *et al.*, 2002, Chong, ZZ *et al.*, 2003b). In addition, during glutamate exposure or free radical generation, EPO can block apoptotic cellular injury (Chong, ZZ *et al.*, 2003b, Morishita, E *et al.*, 1997). EPO also is rare in its ability to prevent both the exposure of membrane PS residues and inhibit the committed stages of genomic DNA destruction in several experimental models (Chong, ZZ *et al.*, 2002, Chong, ZZ *et al.*, 2003b, Chong, ZZ *et al.*, 2003c, Grimm, C *et al.*, 2002, Parsa, CJ *et al.*, 2003, Sun, Y *et al.*, 2004), offering vital cellular protection for several injury paradigms.

Recent work for EPO has begun to focus on a number of cellular mechanisms that may mediate protection for this agent. In particular, both the expression and cytoprotection of EPO has been associated with nuclear factor- κ B (NF- κ B). NF- κ B has been shown to be cytoprotective through the inhibitors of apoptotic protein (IAPs) which can block caspase activity (Reed, JC, 2001), through suppression of tumor necrosis factor- α (TNF- α) generated apoptosis (Wang, CY *et al.*, 1998), through the modulation of growth arrest and DNA damage protein 45 (Gadd45 β) (De Smaele, E *et al.*, 2001), and through the direct activation of Bcl- x_L (Chen, C *et al.*, 2000). Interestingly, NF- κ B can enhance the expression of EPO (Figueroa, YG *et al.*, 2002). On the reverse end, EPO uses NF- κ B for the production of neural stem cells (Shingo, T *et al.*, 2001) and to differentiate neuronal stem cells into astrocytes (Lee, SM *et al.*, 2004). In addition, cellular protection by EPO has been proposed to be dependent upon the expression and nuclear translocation of NF- κ B (Sae-Ung, N *et al.*, 2005). EPO also may require NF- κ B to remove cellular reactive oxygen species (Nakata, S *et al.*, 2004) and to ultimately prevent apoptotic tissue injury (Bittorf, T *et al.*, 2001, Matsushita, H *et al.*, 2003).

Here we demonstrate that EPO provides robust cellular protection against $A\beta$ toxicity in primary hippocampal neuronal cell cultures that involves the prevention of early apoptotic cellular membrane PS externalization and subsequent genomic DNA degradation. Furthermore, the capacity of EPO to foster neuronal survival and modulate early and late

pathways of apoptotic injury during A β injury is intimately tied to the maintenance of the expression and integrity of NF- κ B p65. Loss of NF- κ B p65 through siRNA gene silencing eliminates the ability of EPO to offer cellular protection and to block apoptotic demise during A β exposure.

MATERIALS AND METHODS

Primary Hippocampal Neuronal Cultures

The hippocampi were obtained from E-19 Sprague-Dawley rat pups and incubated in dissociation medium (90 mM Na₂SO₄, 30 mM K₂SO₄, 5.8 mM MgCl₂, 0.25 mM CaCl₂, 10 mM kynurenic acid, and 1 mM HEPES with the pH adjusted to 7.4) containing papain (10 U/ml) and cysteine (3 mM/l) for two 20-minute periods. The hippocampi were then rinsed in dissociation medium and incubated in dissociation medium containing trypsin inhibitor (10–20 U/ml) for three 5-minute periods. The cells were washed in growth medium (Leibovitz's L-15 medium, Invitrogen, Carlsbad, CA) containing 6% sterile rat serum (ICN, Aurora, OH), 150 mM NaHCO₃, 2.25 mg/ml of transferrin, 2.5 μ g/ml of insulin, 10 nM progesterone, 90 μ M putrescine, 15 nM selenium, 35 mM glucose, 1 mM L-glutamine, penicillin and streptomycin (50 μ g/ml), and vitamins. The dissociated cells were plated at a density of $\sim 1.5 \times 10^3$ cells/mm² in 35 mm polylysine/laminin-coated plates (Falcon Labware, Lincoln Park, NJ). Neurons were maintained in growth medium at 37 °C in a humidified atmosphere of 5% CO₂ and 95% room air for 10–14 days.

Experimental Treatments

β -Amyloid (A β) Treatment—A β (1–42) (American Peptide Company, Sunnyvale, CA) was dissolved in PBS at a concentration of 100 μ M. To allow for A β aggregation, A β was incubated at 37°C for a 7 day period and then directly applied to neuronal cell cultures per the experimental protocols. During both pre-paradigm applications, EPO or the EPO antibody (R&D Systems, Minneapolis, MN) application was continuous.

Assessment of Neuronal Survival

Hippocampal neuronal injury was determined by bright field microscopy using a 0.4% trypan blue dye exclusion method 24 hours following A β exposure per our previous protocols (Chong, ZZ *et al.*, 2005a, Lin, SH *et al.*, 2000, Maiese, K and Vincent, AM, 2000b). Neurons were identified by morphology and the mean survival was determined by counting eight randomly selected non-overlapping fields with each containing approximately 10–20 neurons (viable + non-viable) in each 35 mm² Petri dish. Each experiment was replicated 4–6 times independently with different cultures.

Assessment of DNA Fragmentation

Genomic DNA fragmentation was determined by the terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay (Chong, ZZ *et al.*, 2003a, Lin, SH *et al.*, 2000, Maiese, K *et al.*, 2000b). Briefly, neuronal cells were fixed in 4% paraformaldehyde/0.2% picric acid/0.05% glutaraldehyde and the 3'-hydroxy ends of cut DNA were labeled with biotinylated dUTP using the enzyme terminal deoxytransferase (Promega, Madison, WI) followed by streptavidin-peroxidase and visualized with 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA).

Assessment of Membrane Phosphatidylserine (PS) Residue Externalization

Phosphatidylserine (PS) exposure was assessed through the established use of annexin V. Per our prior protocols (Chong, ZZ *et al.*, 2004a, Kang, JQ *et al.*, 2003b, Maiese, K *et al.*, 2000a), a 30 μ g/ml stock solution of annexin V conjugated to phycoerythrin (PE) (R&D Systems, Minneapolis, MN) was diluted to 3 μ g/ml in warmed calcium containing binding buffer (10

mmol/L Hepes, pH 7.5, 150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 1.8 mmol/L CaCl₂). Plates were incubated with 500 µl of diluted annexin V for 10 minutes. Images were acquired with “blinded” assessment with a Leitz DMIRB microscope (Leica, McHenry, IL) and a Fuji/Nikon Super CCD (6.1 megapixels) using transmitted light and fluorescent single excitation light at 490 nm and detected emission at 585 nm.

Gene Silencing of NF-κB p65

NF-κB p65 siRNA was selected by targeting the sequence 5'-AACATCCCTCAGCACCATCAA -3' and was designed by using *Silencer*® siRNA construction kit synthesized by Ambion (Austin, TX). Primary rat hippocampal neurons were seeded into 35 mm dishes. Transfection of siRNA duplexes was performed in cells using the siPORT™ *Amine* transfection agent (Ambion, Austin, TX) according to the guidelines provided by the manufacturer. NF-κB p65 siRNA was maintained in primary neuronal cultures for 72 hours.

Western Blot Analysis for the NF-κB p65 Family Member Expression

Cells were homogenized and following protein determination, each sample (50 µg/lane) was then subjected to 7.5% SDS-polyacrylamide gel electrophoresis. Following transfer, the membranes were incubated with primary rabbit antibody against NF-κB p65 (1:200) (Santa Cruz Biotechnologies, Santa Cruz, CA). After washing, the membranes were incubated with a horseradish peroxidase conjugated secondary antibody (goat anti-rabbit IgG, 1:15000) (Pierce, Rockford, IL). The antibody-reactive bands were revealed by chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

The cytoplasmic and nuclear proteins were prepared by using NE-PER nuclear and cytoplasmic extraction reagents according to manufacture's instruction (purchased from Pierce, Rockford, IL). The expression of NF-κB p65 in nucleus and cytoplasm was determined by Western blot performed as described as above.

Statistical Analysis

For each experiment involving assessment of neuronal cell survival, DNA degradation, membrane PS exposure, the mean and standard error were determined from 4 to 6 replicate experiments. Statistical differences between groups were assessed by means of analysis of variance (ANOVA) with the post-hoc Student's t-test. Results are expressed as the mean ± the standard error. Statistical significance was considered at $P < 0.05$.

RESULTS

Increasing Concentrations of Aβ Leads to Progressive Neuronal Injury

Aβ was applied to neuronal cultures in a series of concentration (1, 5, 10, 20 µM) and neuronal survival was assessed 24 hours later by the trypan blue exclusion method. As shown in Fig. 1A, cell survival in neurons was significantly reduced from a survival of $88 \pm 4\%$ in untreated control cells to $78 \pm 4\%$, $53 \pm 3\%$ ($P < 0.01$), $45 \pm 4\%$ ($P < 0.01$), and $31 \pm 3\%$ ($P < 0.01$) during exposure to Aβ in the serial concentrations of 1 µM, 5 µM, 10 µM, and 20 µM respectively. Since 5 µM of Aβ was the minimum concentration to result in a significant decrease in neuronal survival to approximately 50% (50% neuronal cell loss), this concentration of Aβ was employed for the remainder of the experimental paradigms.

EPO Provides Neuroprotection During Aβ Toxicity that is Concentration Dependent

Increasing concentrations of EPO (0.01 to 1000 ng/ml) were administered directly to neuronal cultures and cell survival was evaluated by a trypan blue dye exclusion method 24 hours later

to examine the possible toxicity of EPO in neurons. No significant toxicity over a 24 hours period was present in the cultures exposed to EPO (0.01 to 1000 ng/ml, data not shown).

We next investigated the capacity of EPO to protect neurons during A β exposure. In Fig. **1B**, representative pictures demonstrate that A β leads to neuronal injury and intracellular trypan blue staining. In contrast, pretreatment with EPO (10 ng/ml) 1 hour prior to A β administration significantly prevent trypan blue uptake and neuronal cell injury. Furthermore, neuronal survival was significantly reduced to $52 \pm 6\%$ following exposure to A β (5 μ M) alone when compared with untreated control cultures ($88 \pm 3\%$, $P < 0.01$) (Fig. **1C**). In contrast, application of EPO with the concentrations of 1.0 ng/ml to 50 ng/ml significantly increased neuronal survival. A concentration of EPO of 10 ng/ml achieved the maximum neuronal survival $74 \pm 5\%$, but concentrations lower than 0.1 ng/ml or higher than 100 ng/ml did not improve neuronal survival during A β exposure.

EPO is Necessary and Sufficient for Neuronal Protection Against A β

Since EPO in a concentration manner increases neuronal survival during A β application, we next examined whether specific antagonism against exogenous EPO application with an antibody to EPO (EPO Ab) could neutralize the protective capacity of EPO. Application of EPO Ab in a series of concentrations of 0.01-1.0 μ g/ml alone did not significantly alter neuronal survival when compared to untreated control cultures (data not shown). Next, administration of EPO Ab in a series of concentrations of 0.01-1.0 μ g/ml during application of A β also did not significantly alter neuronal survival when compared to cultures of A β treated alone (Fig. **2A**).

When the EPO Ab (0.01-1.0 μ g/ml) was applied with EPO (10 ng/ml) during A β (5 μ M) application, the EPO Ab progressively attenuated the protective capacity of EPO on neuronal survival (Fig. **2B**). EPO Ab (0.01-1.0 μ g/ml) combined with EPO (10 ng/ml) was applied directly to the neuronal cultures 1 hour prior to A β administration and neuronal survival was determined 24 hours later. At the concentrations of 0.01-0.10 μ g/ml, the EPO Ab did not alter protection by EPO when compared to cultures treated with EPO alone ($73 \pm 6\%$). In contrast, the EPO Ab concentrations of 0.50 μ g/ml and 1.0 μ g/ml significantly neutralized the protective capacity of EPO, yielding neuronal survivals of $50 \pm 4\%$ ($P < 0.01$) and $48 \pm 7\%$ ($P < 0.01$) respectively (Fig. **2B**).

EPO Blocks Apoptotic Genomic DNA Fragmentation and Membrane Phosphatidylserine (PS) Externalization During A β Toxicity

Hippocampal neurons were exposed to A β (5 μ M) and either cellular genomic DNA fragmentation was assessed with TUNEL or cellular membrane PS exposure was determined by annexin V labeling method 24 hours later. In Fig. **3A**, representative pictures demonstrate that A β lead to both DNA fragmentation and membrane PS externalization in neurons. In neurons exposed to A β , cell injury is evident by chromatin condensation and significant induction of annexin V labeling. In contrast, pretreatment with EPO (10 ng/ml) 1 hour prior to A β significantly reduced nuclear condensation and membrane PS externalization.

To quantitatively determine the ability of EPO to prevent A β generated DNA fragmentation and membrane PS externalization, EPO (10 ng/ml) was administered 1 hour prior to A β (5 μ M) administration and assessment was performed 24 hours later. As shown in Fig. **3B**, A β application alone resulted in a significant increase in percent DNA fragmentation ($54 \pm 5\%$) in neurons when compared to untreated control cultures ($6 \pm 3\%$). DNA fragmentation was reduced to $27 \pm 4\%$ in cells treated with EPO during A β exposure. Similarly, an increase in membrane PS exposure was observed in neurons 24 hours following A β exposure that reached a level of $56 \pm 5\%$ when compared to untreated control cultures of $10 \pm 3\%$ (Fig.

3B). Neurons treated with EPO displayed a significant reduction in membrane PS externalization to $28 \pm 5\%$ 24 hours following A β administration.

EPO Prevents the Degradation of NF- κ B p65 and Promotes its Nuclear Translocation During A β Toxicity

NF- κ B has three family members known as p65 (RelA), RelB, and c-Rel. Each NF- κ B member contains C-terminal transactivation domains that can lead to gene transcription. Yet, the most prominent gene activation is triggered by p65 that consists of two potent transactivation domains within its C terminus (Schmitz, ML and Baeuerle, PA, 1991). For this reason, we chose to examine the expression of NF- κ B p65.

To investigate changes in the expression of NF- κ B p65 during A β toxicity, we determined the expression of NF- κ B p65 by using Western blot in neurons prepared at 6 and 24 hours following application of A β (5 μ M). As shown in Fig. **4A**, no change in the expression of NF- κ B p65 was seen in neurons at 6 hours following A β exposure. In contrast, the NF- κ B p65 expression was significantly decreased in neurons after exposure to A β for 24 hours. When EPO (10 ng/ml) was applied 1 hour prior to A β administration, the NF- κ B p65 expression was maintained without evidence of degradation at 24 hours following A β exposure (Fig. **4A**). EPO (10 ng/ml) administered alone did not significantly change the expression of NF- κ B p65 in neurons over a 24 hour period when compared to control (Fig. **4A**).

We next investigated whether EPO could modulate the nuclear translocation of NF- κ B p65 in neurons during A β exposure. Upon activation, NF- κ B p65 translocates to the nucleus and increases the expression of many of its target genes, which function to prevent apoptosis (Chen, C *et al.*, 2000, Chong, ZZ *et al.*, 2005c, Chong, ZZ *et al.*, 2005e, Li, ZW *et al.*, 1999). We determined the expression of NF- κ B p65 in the cytoplasmic and nuclear fractions separately by Western blot analysis. EPO (10 ng/ml) was applied 1 hour prior to A β (5 μ M) exposure and cytoplasmic and nuclear protein was extracted at 6 hours following A β administration. No significant change in NF- κ B p65 expression in the neuronal cytoplasmic fraction was observed at 6 hours following A β exposure, EPO (10 ng/ml) administration, or combined EPO/A β application (Fig. **4B**). Yet, the nuclear expression of NF- κ B p65 was significantly increased by EPO (10 ng/ml) application alone as well as during A β exposure (Fig. **4C**), suggesting that EPO can maintain the integrity and translocation of NF- κ B p65 to the cell nucleus.

Protection of Neuronal Survival by EPO During A β Toxicity Requires the Presence of NF- κ B p65

Since EPO could prevent the degradation of NF- κ B p65 and enhance its nuclear translocation during A β toxicity, we next evaluated whether NF- κ B was required for EPO protection against A β toxicity. In the initial set of experiments, wildtype neurons (absent of siRNA transfection) or neurons transfected with NF- κ B p65 siRNA for 72 hours prior were then exposed to EPO (10 ng/ml). One hour after EPO application to the neuronal cultures, A β (5 μ M) was applied to the cultures. Expression of NF- κ B p65 through Western blot analysis was determined 24 hours after A β application. In Fig. **5A**, EPO (10 ng/ml) administered in wildtype neurons blocked the degradation of NF- κ B p65 during A β exposure. Yet, neurons with gene silencing for NF- κ B p65 (NF- κ B p65 siRNA) lacked NF- κ B p65 expression either without exposure to EPO or A β and during combined EPO and A β administration.

Following our determination that NF- κ B p65 siRNA could eliminate NF- κ B p65 expression, we investigated whether EPO required NF- κ B p65 for protection against A β . In Fig. **5B**, representative figures illustrate that EPO (10 ng/ml) prevents trypan blue uptake 24 hours following exposure to A β (5 μ M). To quantitatively determine whether NF- κ B p65 is necessary for EPO protection, EPO (10 ng/ml) was administered 1 hour prior to A β (5 μ M) administration

in wildtype neurons (absent of siRNA transfection) or in neurons transfected with NF- κ B p65 siRNA for 72 hours and trypan blue assessment was performed 24 hours after A β application. As shown in Fig. 5C, A β application alone resulted in a significant decrease in neuronal survival ($51 \pm 5\%$) in neurons when compared to untreated control cultures ($89 \pm 3\%$). As expected, EPO (10 ng/ml) increased neuronal survival to $76 \pm 4\%$ during A β exposure, but this protection was lost with gene silencing of NF- κ B p65 decreasing neuronal survival to $54 \pm 4\%$ 24 hours following EPO treatment and A β exposure (Fig. 5C).

Prevention of Apoptotic Injury by EPO During A β Toxicity is Mediated by NF- κ B p65

Since increased survival afforded by EPO during A β exposure requires NF- κ B expression, we next investigated whether the ability of EPO to prevent apoptotic DNA degradation and cellular membrane PS exposure would be compromised during gene silencing of NF- κ B. Neurons were transfected with NF- κ B p65 siRNA for 72 hours prior to EPO and A β application. DNA fragmentation was determined by TUNEL and membrane PS exposure was assessed by annexin V phycoerythrin labeling 24 hours following A β administration. Representative images in Fig. 6A illustrate DNA fragmentation in neurons and membrane PS externalization in neurons using transmitted (T) light and fluorescence (F) light of the same microscopy field. EPO (10 ng/ml) significantly decreased TUNEL and annexin V staining following A β (5 μ M) exposure. In contrast, gene silencing of NF- κ B p65 with siRNA abrogates protection by EPO in neurons and yields increased TUNEL labeling and increased membrane PS labeling.

As depicted in Fig. 6B, A β application alone resulted in a significant increase in percent DNA fragmentation ($57 \pm 5\%$) in neurons when compared to untreated control cultures ($7 \pm 3\%$). DNA fragmentation was reduced to $28 \pm 3\%$ with EPO during A β administration. An increase in PS externalization also was observed following A β exposure to $54 \pm 4\%$ when compared to untreated control cultures of $9 \pm 4\%$ (Fig. 6B). EPO significantly reduced membrane PS externalization to $27 \pm 4\%$ 24 hours during A β exposure. Yet, during gene silencing of NF- κ B p65, administration of EPO (10 ng/ml) did not significantly reduce DNA degradation and membrane PS exposure during A β application, suggesting that NF- κ B p65 expression mediates the ability of EPO to control both early and late programs of apoptotic neuronal injury.

DISCUSSION

One of the hallmarks of AD is the presence of β -amyloid (A β) deposition that is composed of a 39-42 amino acid peptide (A β), which is the proteolytic product of the APP (Chong, ZZ *et al.*, 2005d, Chong, ZZ *et al.*, 2005e). A β can lead to the generation of reactive oxygen species, such as hydrogen peroxide, and lead to toxicity in neurons (Huang, X *et al.*, 1999). Free radical generation by A β is strongly influenced by the aggregational state of the peptides (Monji, A *et al.*, 2001). In addition, A β can not only precipitate a significant inflammatory response with microglial activation and the secretion of TNF- α (Bornemann, KD *et al.*, 2001), but also A β can elicit the neuronal expression of inducible nitric oxide synthase, peroxynitrite production, and neuronal apoptosis during an acute inflammatory response (Chong, ZZ *et al.*, 2005c, Combs, CK *et al.*, 2001). Given the link between A β deposition and oxidative stress, agents that modulate the toxicity of A β may be potentially useful in the therapy of AD.

We show that EPO is neuroprotective against A β in primary hippocampal neuronal cultures. EPO in a concentration range of 0.001 ng/ml to 1000 ng/ml was not toxic to neurons. Yet, protection with EPO is achieved in a small concentration range. Concentrations of EPO less than 1 ng/ml or greater than 50 ng/ml did not enhance neuronal survival during A β administration. Additional studies in a number of cell models also illustrate a tight therapeutic concentration range for EPO (Bernaudin, M *et al.*, 1999, Chong, ZZ *et al.*, 2002, Chong, ZZ *et al.*, 2003a, Chong, ZZ *et al.*, 2003b, Kawakami, M *et al.*, 2001).

It appears that EPO is both necessary and sufficient to protect neurons during A β toxicity. Application of the EPO Ab, which can bind to EPO and block its biological activities in cells (Koshimura, K *et al.*, 1999), did not alter neuronal survival when compared to untreated control cultures. Yet, administration of EPO provided significant protection that was blocked only with co-application of the EPO Ab. These results illustrate that EPO provides necessary and sufficient protection against neuronal injury.

Interestingly, EPO enhances neuronal survival during A β exposure through apoptotic pathways that block the early induction of cellular membrane PS externalization and the later generation of genomic DNA fragmentation. Apoptotic injury is believed to contribute significantly to a variety of disease states of the nervous system (Chong, ZZ and Maiese, K, 2004b, Li, F *et al.*, 2004b). For example, both human and *in vitro* models of AD suggest that apoptosis contributes to the neuronal loss during the disease (Maiese, K *et al.*, 2004a). Data from *in situ* terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) assays of brain tissues from individuals with AD demonstrate neuronal demise consistent with apoptosis. A correlation between the incidence of TUNEL-positive cells and plaque density also exists (Colurso, GJ *et al.*, 2003). As an early event in the dynamics of cellular apoptosis, the biological role of membrane PS externalization can vary in different cell populations. In some cell systems, PS may be required for embryogenesis (Bose, J *et al.*, 2004). In the nervous system, cells expressing externalized PS may be removed by microglia (Chong, ZZ *et al.*, 2005b, Kang, JQ *et al.*, 2003a, Kang, JQ *et al.*, 2003b). In contrast to the early externalization of membrane PS residues, the cleavage of genomic DNA into fragments is considered to be a delayed event that occurs late during apoptosis (Dombroski, D *et al.*, 2000, Jessel, R *et al.*, 2002, Kang, JQ *et al.*, 2003b, Maiese, K *et al.*, 2000b). As a result, protection by EPO occurs through the maintenance of intact genomic DNA in neurons, but also involves more long-term protection through the inhibition of membrane PS residue exposure to block the phagocytosis of cells (Maiese, K *et al.*, 2000b, Verhoven, B *et al.*, 1999) and prevent cellular inflammation (Dombroski, D *et al.*, 2000).

Protection by EPO against A β is dependent upon the expression and nuclear translocation of NF- κ B. NF- κ B works in conjunction with protein kinase B (Akt) (Chong, ZZ *et al.*, 2005b), an essential pathway for EPO to enhance both neuronal and vascular survival during acute injury (Li, F *et al.*, 2004a). Akt uses I κ B kinase (IKK) and the IKK α catalytic subunit to stimulate the transactivation domain of the p65 subunit of NF- κ B. Once activated, NF- κ B results in the induction of several anti-apoptotic genes that include inhibitors of apoptotic protein (IAPs) c-IAP1, c-IAP2, and χ -chromosome-linked IAP. IAPs can specifically inhibit active forms of caspase 3, 7, and caspase-9 (Reed, JC, 2001), suppress TNF- α initiated apoptosis (Wang, CY *et al.*, 1998), and down-regulate c-Jun-amino terminal kinase (Tang, G *et al.*, 2001). NF- κ B also may prevent apoptosis through the direct activation of Bcl- χ_L (Chen, C *et al.*, 2000).

NF- κ B maintains a close association with the expression of the EPO gene during HIF-1 induction (Maiese, K *et al.*, 2004b, Maiese, K *et al.*, 2005b). Akt can significantly increase NF- κ B and HIF-1 activation resulting in the enhancement of EPO expression (Figueroa, YG *et al.*, 2002). Through a regulatory loop, EPO also can promote IKK activity, resulting in the degradation of I κ B and the subsequent liberation of NF- κ B. EPO employs NF- κ B activation to foster the production of neural stem cells (Shingo, T *et al.*, 2001) and differentiate neuronal stem cells into astrocytes (Lee, SM *et al.*, 2004). Although in contrast to scenarios that involve some EPO receptor-positive tumors (Carvalho, G *et al.*, 2005) or specific ischemia-reperfusion cardiac injury models (Xu, B *et al.*, 2005), the protective capacity of EPO to prevent apoptosis has been linked to the enhanced expression and nuclear translocation of NF- κ B as demonstrated in erythroid progenitor cells (Sae-Ung, N *et al.*, 2005). Additional studies support the premise that EPO requires NF- κ B for the removal of reactive oxygen species (Nakata, S *et al.*, 2004).

and that EPO uses NF- κ B to prevent or reduce apoptotic cellular injury (Bittorf, T *et al.*, 2001, Matsushita, H *et al.*, 2003). Our present work demonstrates that EPO maintains the expression and integrity of NF- κ B p65 over a 24 hour period following the onset of A β application. Without the presence of EPO, administration of A β leads to a progressive loss in the expression of NF- κ B p65. In addition, EPO leads to the translocation of NF- κ B p65 from the cytoplasm to the nucleus consistent with prior work that demonstrates the need for this subcellular translocation for NF- κ B p65 to activate its anti-apoptotic program. Yet, we have extended this work further to address whether the expression of NF- κ B p65 is required for EPO to increase neuronal survival and prevent apoptotic neuronal injury. Using NF- κ B p65 siRNA to silence the expression of this gene, we show that protection by EPO during A β administration is significantly compromised and approaches neuronal cell survival levels comparable to cultures treated only with A β . In addition, NF- κ B p65 is necessary for EPO to prevent early apoptotic cellular membrane PS externalization and subsequent genomic DNA degradation, suggesting that NF- κ B p65 may be an upstream mediator of caspase activation that is known to control these apoptotic pathways (Chong, ZZ *et al.*, 2002, Li, P *et al.*, 1997, Maiese, K *et al.*, 2000b).

In conclusion, we show that EPO can function as a significant neuroprotectant against A β toxicity, a principal consideration for the development of treatments for AD. Through the use of a blocking antibody against EPO, we demonstrate that EPO is necessary and sufficient to protect neurons during A β exposure. Moreover, EPO prevents apoptotic injury from A β by blocking both the onset of cellular membrane PS externalization and subsequent genomic DNA degradation. Critical to the protective capacity of EPO against A β is the expression and translocation of NF- κ B p65. EPO not only prevents the cleavage of NF- κ B p65 during A β administration, but also promotes the nuclear translocation of NF- κ B p65 to allow it to activate its anti-apoptotic programs. Furthermore, the gene silencing of NF- κ B p65 by RNA interference removes the protective capacity of EPO and allows the induction of apoptotic cellular membrane PS exposure and genomic DNA degradation, illustrating the necessity of NF- κ B p65 for EPO to control these processes. These investigations point to the potential of either EPO or its down-stream partner of NF- κ B p65 as potential therapeutic targets for the treatment of neurodegenerative disorders, such as AD.

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Fig (1). Erythropoietin (EPO) increases neuronal survival during β-amyloid (Aβ) toxicity and is concentration specific

(A) Aβ was applied to primary hippocampal neuronal cultures at a concentration of 1, 5, 10, 20 μM and neuronal survival was determined 24 hours later by using the trypan blue exclusion method. Neuronal survival was progressively decreased with increased concentration of Aβ (* $P < 0.01$ vs. untreated control). (B) Representative images illustrate trypan blue staining. Aβ (5 μM) applied to neuronal cultures resulted in significant staining 24 hours following Aβ treatment. EPO (10 ng/ml) applied 1 hour prior to Aβ (5 μM) exposure significantly increased neuronal survival and prevented trypan blue uptake 24 hours following Aβ treatment. (C) Neurons were pretreated with EPO (0.01 to 1000 ng/ml) 1 hour prior to Aβ (5 μM) exposure and cell survival was assessed 24 hours later. Protection of EPO against Aβ toxicity was evident

in cultures with EPO (1 to 50 ng/ml) when compared with cultures exposed to A β alone (* P <0.01 vs. A β treated alone). In **A** and **C**, each data point represents the mean and SEM.

Fig (2). Erythropoietin (EPO) is necessary and sufficient for neuronal protection against β -amyloid ($A\beta$) toxicity

(A) The EPO blocking antibody (EPO Ab) (0.01-1 μ g/ml) was applied 1 hour prior to $A\beta$ (5 μ M) application. Neuronal survival was assessed 24 hours later. No significant changes in neuronal survival were observed following application of the EPO Ab when compared to cultures treated with $A\beta$ alone. **(B)** Increasing concentrations of the EPO Ab (0.01 - 1.00 μ g/ml) were applied to neuronal cultures in conjunction with EPO (10 ng/ml) for 1 hour prior to $A\beta$ (5 μ M) application. Neuronal survival was assessed 24 hours later. Protection by EPO against $A\beta$ toxicity was attenuated or abolished during application of the EPO Ab (0.50 and 1.00 μ g/ml) (* P <0.01 vs. $A\beta$ treated alone). In **A** and **B**, each data point represents the mean and SEM.

Fig (3). Erythropoietin (EPO) prevents apoptotic genomic DNA fragmentation and membrane phosphatidylserine (PS) exposure during β -amyloid (A β) application
(A) Neurons were pretreated with EPO (10 ng/ml) 1 hour prior to A β application. DNA fragmentation was assessed using TUNEL and membrane PS exposure was determined by annexin V phycoerythrin labeling 24 hours following A β treatment. Representative images illustrate DNA fragmentation and membrane PS externalization in neurons using transmitted (T) light and fluorescence (F) light of the same microscopy field with 490 nm excitation and 585 nm emission wavelengths. EPO significantly prevented DNA fragmentation and membrane PS exposure during A β exposure. **(B)** Pretreatment with EPO (10 ng/ml) 1 hour prior to A β application decreased DNA fragmentation and membrane PS externalization significantly 24 hours following A β exposure (* P <0.01 vs. A β). In **B**, each data point represents the mean and SEM, control = untreated neurons.

Fig (4). Erythropoietin (EPO) prevents the degradation of NF-κ B and leads to its nuclear translocation during β-amyloid (Aβ) toxicity

(A) Equal amounts of neuronal protein extracts (50 μg/lane) were immunoblotted 6 and 24 hours following application of Aβ (5 μM), EPO (10 ng/ml), or with EPO (10 ng/ml) 1 hour pretreatment prior to Aβ with anti NF-κB p65 antibody. Exposure to Aβ decreased the expression NF-κB p65 at 24 hours following Aβ application, but EPO pretreatment prevented the decrease in the expression of NF-κB p65 during Aβ exposure (* $P < 0.01$ vs. control; † $P < 0.01$ vs. Aβ 24 hours). (B) and (C) EPO (10 ng/ml) was applied 1 hour prior to Aβ (5 μM) administration and cellular cytoplasm and nuclear proteins were prepared separately at 6 hours following Aβ application. The expression of NF-κB p65 in cytoplasmic extracts (B) and nuclear

extracts (**C**) was determined by Western blot analysis. No significant change in the expression of NF- κ B p65 was observed in the cytoplasmic extracts (**B**). Yet, EPO lead to a significant increase in nuclear NF-B p65 expression either alone or during A β application (**C**) (* P <0.01 vs. control; † P < 0.01 vs. A β). In **A**, **B**, and **C**, each data point represents the mean and SEM, control = untreated cells.

Fig (5). Erythropoietin requires NF- κ B p65 to increase neuronal survival during exposure to β -amyloid (A β)

(A) Neurons were transfected with NF- κ B p65 siRNA for 72 hours prior to EPO and A β application. Western blot analysis for NF- κ B p65 was performed 6 hours following A β administration. EPO (10 ng/ml) applied 1 hour prior to A β (5 μ M) prevented the degradation of NF- κ B p65. In contrast, NF- κ B p65 siRNA gene silencing significantly reduced the efficacy of EPO and decreased the expression of NF- κ B p65 in neurons during untreated cultures and during application of combined EPO and A β . (B) Representative images illustrate that A β (5 μ M) exposure leads to significant trypan blue uptake in neurons 24 hours following A β treatment. EPO (10 ng/ml) given 1 hour prior to A β application significantly reduced trypan

blue uptake in neurons indicative of the maintenance of intact cellular membranes. In contrast, NF- κ B p65 siRNA transfection for 72 hours prior to EPO application reduced the protective capacity of EPO resulting in neuronal injury with significant trypan blue uptake. (C) Neuronal survival is significantly increased by EPO (10 ng/ml) applied 1 hour prior to A β (5 μ M) when compared to A β treated neurons alone. In contrast, protection conferred by EPO against A β toxicity is lost during NF- κ B p65 gene silencing with targeted siRNA (* P <0.01 vs. A β treated alone; † P <0.01 vs. EPO/A β). Each data point represents the mean and SEM. Control = untreated cultures.

Fig (6). Prevention of apoptotic genomic DNA fragmentation and cellular membrane PS exposure by EPO during β -amyloid (A β) toxicity is mediated by NF- κ B p65

(A) Neurons were transfected with NF- κ B p65 siRNA for 72 hours prior to EPO and A β application. DNA fragmentation was assessed 24 hours after A β administration using TUNEL and PS exposure was determined by annexin V phycoerythrin labeling 24 hours following A β administration. Representative images illustrate DNA fragmentation and membrane PS externalization in neurons using transmitted (T) light and fluorescence (F) light of the same microscopy field with 490 nm excitation and 585 nm emission wavelengths. EPO (10 ng/ml) applied 1 hour prior to A β administration significantly prevented DNA fragmentation and membrane PS exposure following A β . In contrast, gene silencing of NF- κ B p65 with siRNA

abrogates protection of EPO in neurons and yields a significant increase in positive TUNEL and cellular membrane PS externalization. **(B)** NF- κ B p65 gene silencing with targeted siRNA eliminates protection conferred by EPO against A β , resulting in an increase in percent DNA fragmentation and cellular membrane PS exposure (* P <0.01 vs. A β treated alone; † P <0.01 vs. EPO/A β). Each data point represents the mean and SEM. Control = untreated cultures.