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Erythropoietin protects primary cultures of rat cortical neurons from hypoxia-induced toxicity through attenuating both glutamate release and NMDA receptor evoked neurotoxicity pathway

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Aim: To investigate the neuroprotective effect of EPO against hypoxia, and determine the mechanism with respect to the EPO-induced neuroprotection. *Methods:* Experiments were conducted using primary neuron cultures. Neuron survival and glutamate release were measured after bein insulted by hypoxia. Glutamate concentrations were determined by an HPLC-ECD system. *Results:* Neurons were significantly damaged in hypoxia. Application of recombinant human EPO (10^{-11} M) within 24 h before hypoxia significantly increased neuronal survival compared with no EPO treatment. Moreover, the enhancing of glutamate release stimulated by hypoxia was inhibited by pretreatment with EPO at a concentration of 10^{-11} M. Further studies demonstrated that EPO also prevented glutamate and NMDA insulted neurotoxicity. *Conclusion:* These findings suggest that EPO prevents neuronal death in the cultured cortical neuron, possibly through attenuating both glutamate release and NMDA receptor evoked neurotoxicity pathway.

1. Introduction

Glutamate and related excitatory amino acids are well-known neurotoxins, and their extracellular levels increase dramatically in the course of brain ischemia. The excitotoxicity hypothesis suggests that, in neuronal hypoxia/ischemia, neuron degeneration can be triggered by cytoplasmic Ca^{2+} overload, which occurs when N-methyl-(D)-aspartate receptors are overstimulated by excessive glutamate (Lee et al. (1999). Numerous pharmacological approaches have been explored to prevent or attenuate neuronal cell death in ischemia; however, no satisfactory methods have been developed (Muir and Lees 1995).

The hematopoietic growth factor erythropoietin (EPO) is a primary regulator of mammalian erythropoiesis and is produced by kidney and liver in an oxygen-dependent manner (Jelkmann 2005). In the brain, EPO gene expression is regulated by the transcription factor hypoxia-inducible factor-1, which is activated by a variety of stressors, including hypoxia (Semenza 2000). EPO-induced neuroprotection is mediated by interaction with the cognate receptor EPO receptor (EPOR) (Marti 2004). EPO binding to EPOR induces receptor oligomerization and subsequent activation by autophosphorylation of Janus kinase 2 (JAK2), a protein-tyrosine kinase that associated with EPOR (Wojchowski et al. 1999). Many studies have identified a major protective role for EPO in the brain, where it has been also shown that locally administered recombinant human EPO prevents ischemia-induced cell death (Calvillo et al. 2003).

It was also shown that the activation of EPOR attenuates Ca^{2+}-induced glutamate release from cultured cerebellar granule cells and hippocampal neurons (Kawakami et al. 2001). Furthermore, EPO has also been shown to protect primary cultured neurons from N-methyl-(D)-aspartate (NMDA) receptor-mediated glutamate toxicity (Morishita et al. 1997). However, it has not yet been deduced whether EPO suppresses hypoxia-induced cell death in cultured cortical neurons by inhibiting the release of glutamate or attenuating NMDA receptor-mediated neurotoxicity. In the present study, we demonstrated that EPO protects primary cultures of rat cortical neurons from hypoxia-induced toxicity through attenuating both glutamate release and NMDA-induced neurotoxicity pathway.

2. Investigations and results

2.1. EPO protects neurons from hypoxia injury

When rat cortical neurons were exposed to hypoxia for 24 h, the mean survival rate significantly decreased to $73.5 \pm 10.7\%$ (Fig. 1). After pretreatment with EPO at final concentrations of $10^{-14} \sim 10^{-9}$ M for 24 h, concentrations of EPO at 10^{-11} M achieved the maximal neuronal survival ($97.7 \pm 10.5\%$), compared with cultures exposed to hypoxia only ($p < 0.05$). However, EPO at concentrations lower than 10^{-13} M or higher than 10^{-10} M did not improve neuronal survival during hypoxia ($p > 0.05$, Fig. 1).

2.2. EPO suppresses hypoxia-induced glutamate release

In the present study, after hypoxia insult for 1 h or longer, the glutamate release was markedly elevated (Fig. 2). The

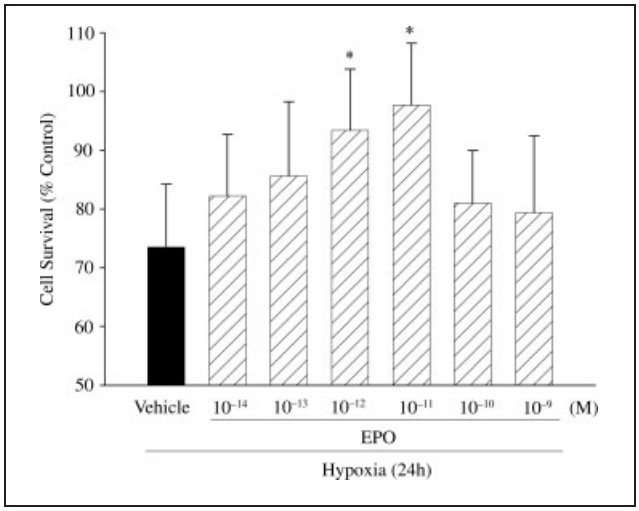

Fig. 1: Effects of EPO on hypoxia-induced survival rate. Cortical neurons, after 12–14 days in primary culture, were exposed to vehicle or EPO for 24 h and then hypoxia for 24 h. Neuronal viability was determined by MTT assay. Values are expressed as percentage of control values and are from 4–6 independent experiments with 3 replicates for each condition. $^{*}P < 0.05$, compared with vehicle treated group (ANOVA followed by Student-Newman-Keuls test)

maximum glutamate release was about 178% at 1 h, compared with vehicle-treated samples. When the cultures pretreated with 10^{-11} M EPO for 24 h, glutamate release was significantly decreased after exposed to hypoxia compared with non-treated cultures ($P < 0.05$, Fig. 2).

2.3. Effects of EPO on glutamate and NMDA induced neurotoxicity

Perfusion with 10^{-11} M EPO alone for 24 h did not alter the neuron survival (Fig. 3). When neurons were exposed to 1 mM glutamate or 100 μM NMDA for 24 h, the mean survival rate significantly decreased to 72.4 ± 6.8% and 71.3 ± 7.2% respectively, which were significantly reversed by pretreatment with EPO ($P < 0.05$, Fig. 3).

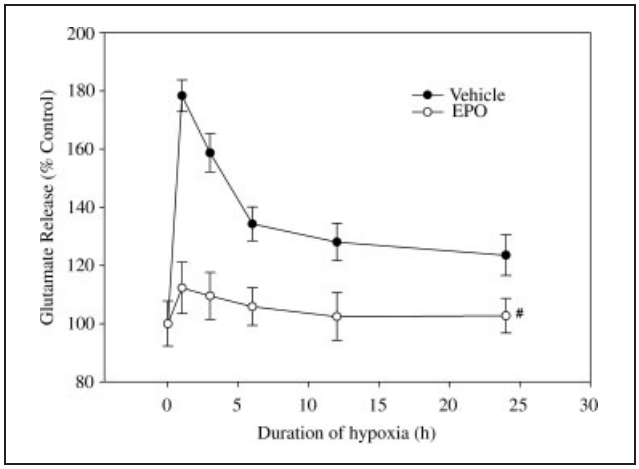

Fig. 2: Time course analysis for glutamate release evoked by hypoxia. Cortical neurons, after 12–14 days in primary culture, were exposed to vehicle or EPO for 24 h and then hypoxia for 1, 3, 6, 12 or 24 h. The amount of glutamate was quantified by HPLC at the indicated time points. Values are from 4–6 independent experiments with 3 replicates for each condition. A two-way ANOVA assessed a significant difference between groups ($^{\#}P < 0.05$ compared with vehicle treated group)

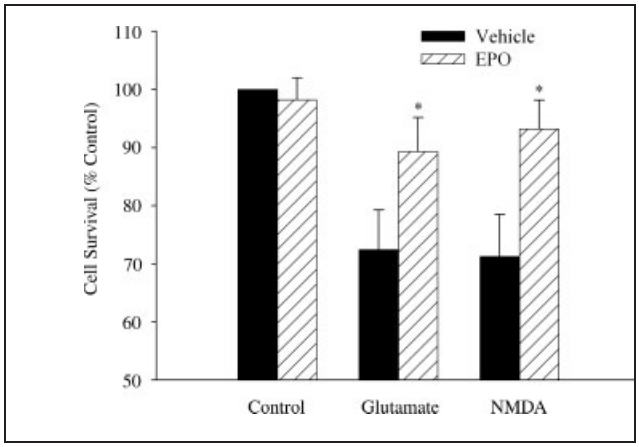

Fig. 3: Effects of EPO on glutamate and NMDA induced survival rate. Cortical neurons, after 12–14 days in primary culture, were exposed to vehicle or EPO for 24 h and then insulted with glutamate or NMDA for 24 h. Neuronal viability was determined by MTT assay. Values are expressed as percentage of control values and are from 4–6 independent experiments with 3 replicates for each condition. $^{*}P < 0.05$, compared with vehicle treated group (ANOVA followed by Student-Newman-Keuls test)

3. Discussion

Oxygen deficiency, which results from hypoxic insults, triggers a host of intrinsic adaptive processes designed to promote tissue protection and regeneration (Bunn and Poyton (1996). Perhaps the best example of this process is the hypoxia-induced expression of EPO, which acts at the EPO receptor to promote proliferation and differentiation of erythroid progenitors and the survival of maturing erythroid cells (Youssoufian et al. 1993). The expression of the EPO receptor in the developing mouse and human CNSs (Lin et al. 1997; Juul et al. 1998, 1999) supports a possible role for EPO in CNS development. Furthermore, persistent expression of EPO and EPO receptors in the adult CNS and the upregulation of EPO in the CNS after hypoxia (Morishita et al. 1997; Digicaylioglu et al. 1995; Marti et al. 1996; Chihuma et al. 2000), support a role for EPO in the brain's response to injury. It was further reported that the expression of EPO receptor contributes to the neuroprotection in heat acclimation (Shein et al. 2005). In line with this hypothesis, previous studies provide evidence for EPO as a neuroprotectant in the CNS. *In vitro* studies of cultured CNS neurons have shown that EPO protects against cell death induced by hypoxia or glutamate (Morishita et al. 1997; Juul et al. 1998). The present studies also provide clear evidence that hypoxia-induced injury to rat cortical cultures can be prevented by EPO pre-treatment (Fig. 1).

The exact mechanisms responsible for the neuroprotective effects of EPO remain to be defined. Numerous mechanisms such as reducing NO overproduction that mediates glutamate neurotoxicity and preventing free radical formation potentially exist, whereby EPO protects the immature brain from hypoxic-ischemic damage. It was also shown that excitatory amino acids (EAAs) such as glutamate and aspartate may be important for the development of hypoxic-ischemic brain injury (Delivoria-Papadopoulos and Mishra 1998). Our study further demonstrates the mode of action of EPO in preventing hypoxia injury to cortical neurons likely occurred through its potential to prevent glutamate release (Fig. 2). On the other hand, EPO dose-dependently prevents glutamate-induced damage of cultured neurons (Morishita et al. 1997), which may be the

mechanism by which EPO protects hippocampal and cerebral cortical neurons from hypoxia-induced death (Kawakami et al. 2001). But it was also reported that EPO can reduce neuronal cell death from hypoxia with glucose deprivation, but not prevent the neurotoxic effect of NMDA (Sinor and Greenberg 200). In the present study, we also show the protection of EPO in hypoxia injury to cortical neurons likely occurred through attenuating NMDA receptor evoked exitotoxicity (Fig. 3).

In conclusion, our data suggest that pretreatment with EPO can attenuate glutamate release during hypoxia and reverse both glutamate and NMDA induced neurotoxicity. So EPO protects the cortical culture neuron against hypoxia-ischemia by attenuating both glutamate release and NMDA receptor evoked neurotoxicity pathways.

4. Experimental

4.1. Materials

Cell culture plates were obtained from Corning Inc. (Corning, NY, USA). Clinical grade recombinant human EPO was obtained from Kirin (Tokyo, Japan). L-Glutamine, B-27 supplement, penicillin, trypsin, streptomycin, poly-L-lysine, and Dulbecco's modified Eagle's medium (DMEM) were bought from GIBCO-BRL (Grand Island, NY, USA).

4.2. Cell culture

Primary cultures of cerebrocortical neurons were obtained from rat pups (Sprague-Dawley) on the first postnatal day using previously described procedures (Dai et al. 2006). In brief, cortices were dissected from the brains under sterile conditions and were digested in 0.25% trypsin in Hank's balanced salt solution (HBSS) for 15 min at 37 °C, then mechanically dissociated. The cells were spun down for 5 min at 1000 × g and resuspended in DMEM containing 100 IU/ml penicillin, 100 mg/ml streptomycin, 0.5 mM L-glutamine, 33 mM (final concentration) glucose, 10% horse serum and 10% foetal calf serum. Cells were seeded to a density of 1.5×10^5 cells/cm^2 in 96-well plates previously coated with poly-L-lysine (0.1 mg/ml).

After 24 h *in vitro*, the culture medium was replaced with Neurobasal medium (Life Technologies, Grand Island, NY, USA) supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, 0.5 mM L-glutamine, 33 mM (final concentration) glucose and 2% B-27 supplement. The cultures were maintained in a humidified CO_2 incubator (5% CO_2, 95% air, 37 °C). Cytosine arabinoside (5 μM) was added 96 h after culture to inhibit the replication of non-neuronal cells. Cells were used for experiments after 12–14 days *in vitro*.

4.3. Hypoxia

Neuron rich cultures were pretreated with EPO at final concentrations of 10^{-14}∼10^{-9} M, respectively, for 24 h under normoxic condition and then placed in a preheated, humid anaerobic chamber. After the chamber flushed with 5% CO_2/balance N_2 for 30 min, pO_2 in the solutions decreased from 167.0 ± 4.2 to 28.5 ± 1.0 mmHg (n = 5). The chamber was then sealed and placed in an incubator at 37 °C. After 1, 3, 6, 12, or 24 h exposure, the glutamate content in the supernatant was assayed. Control neurons received the same procedure except hypoxia treatment.

4.4. Neurochemical analysis of glutamate

Glutamate concentrations were determined by an HPLC-ECD system consisting of a solvent delivery module (Model 582, ESA, Chelmsford, MA, USA), a 3 mm reversed-phase column (3.0 mm × 50 mm, CAPCELL PAK C18 MG, Shiseido, Japan), and an HPLC autosampler (Model 542, ESA, Chelmsford, MA, USA); a 4-channel CoulArray electrochemical detector (E1 = 250 mV, E2 = 550 mV) was used for the analysis of glutamate (Dai et al. 2006). The mobile phase (0.1 M Na_2HPO_4 in 22% methanol and 13% acetonitrile, pH 6.8 with H_3PO_4) was filtered through a 0.22 μm filter (Millipore, Bedford, MA, USA) and degassed before pumping at a flow rate of 0.75 ml/min. The derivatization stock reagent consisted of 27 mg of *o*-phthalaldehyde (OPA, Pickering, Mountain View, CA, USA) dissolved in 1ml of MeOH with 10 mg thioflour (Pickering, Mountain View, CA, USA) and 9 ml 0.1 M sodium tetraborate (pH 9.3). The working OPA-thioflour solution was prepared by diluting 1 ml OPA-thioflour stock solution with 4 ml 0.1 M sodium tetraborate, pH 9.3. Pre-column amino acid derivatization was performed by mixing 15 μl volumes of the standard glutamate or sample and 20 μl OPA-thioflour working solution in the autosampler before injection onto the analytical column. Data were collected and analyzed by CoulArray for Windows software (ESA, Chelmsford, MA, USA).

4.5. Glutamate and NMDA toxicity

As a general rule, drugs were dissolved in sterile purified water prior to dilution into Neurobasal medium. On days 12–14, *in vitro* cell cultures were exposed for 24 h to glutamate (1 mM) or NMDA (100 μM). Glycine (10 μM) was present in all external solutions used to elicit exitotoxicity responses. EPO was added 24 h before glutamate or NMDA insult.

4.6. Viability studies

Neurons were cultured on 96-well plates, with 3 wells in each group. The cells were incubated with MTT (final concentration 0.5 mg/ml) for 3 h at 37 °C. Then, the supernatant layer was removed, and 100 μL of dimethyl sulfoxide was added to each well. MTT metabolism was quantified spectrophotometrically at 490 nm in a Biorad microplate reader. Results were expressed as the percentage MTT reduction, assuming the absorbance of control cells was 100%.

4.7. Statistics

The data are given as mean ± S.D. of four to six experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test for multiple comparison tests (performed with SPSS software) or by two-way ANOVA (performed with GraphPad Prism software). P values lower than 0.05 were considered to be statistically significant.

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