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Erythropoietin Decreases Cytotoxicity and Nitric Oxide Formation Induced by Inflammatory Stimuli in Rat Oligodendrocytes

K. GENC[1], S. GENC[1], H. BASKIN[2], I. SEMIN[3]

[1]Learning Resources Center Research Laboratory, [2]Department of Microbiology, [3]Department of Physiology, School of Medicine, Dokuz Eylul University, Izmir, Turkey

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Summary

In the present study, we investigated whether erythropoietin (Epo) has a protective effect against cytotoxicity induced by interferon-gamma (IFN-γ) and lipopolysaccharide (LPS) in primary rat oligodendrocyte cultures. The possible modulatory effect of erythropoietin on inducible nitric oxide synthase (iNOS) mRNA expression and nitrite production were also analyzed. Erythropoietin exerted a significant protective effect against IFN-γ and LPS-induced oligodendrocyte injury as determined by lactate dehydrogenase assay. Treatment with erythropoietin inhibited the expression of iNOS mRNA and nitrite production resulting from proinflammatory stimulation by IFN-γ and LPS. These results suggest that erythropoietin has protective effects against inflammatory oligodendrocyte injury *in vitro* and may play a protective role in neurological disorders characterized by oligodendrocyte death, such as multiple sclerosis.

Key words

Erythropoietin • Oligodendrocyte • Cytotoxicity • Lipopolysaccharide • Interferon-gamma • Nitric oxide

Introduction

Erythropoietin (Epo) is a hematopoietic cytokine and growth factor that stimulates erythropoiesis (Genc *et al.* 2004). Recently, the presence of Epo and its receptor (EpoR) in the central nervous system (CNS) and neuronal cells has been verified. Many *in vitro* and *in vivo* studies have demonstrated that Epo has neuroprotective effects against various insults such as hypoxia, ischemia, trauma, and inflammation (for review see Maiese *et al.* 2004, Genc *et al.* 2004). However, the primary pathology in some acquired disorders of CNS with different etiologies (inflammatory, toxic, autoimmune, etc.) concerns myelin injury and oligodendrocyte death (Benn *et al.* 2001). Additionally, in acute and chronic neurodegenerative disorders of the CNS such as stroke, hypoxic-ischemic and traumatic injury, white matter involvement, myelin loss and oligodendrocyte death also occur with axonal and neuronal injury (Benn *et al.* 2001). Therefore, the determination of the agents that may prevent oligodendroglial death in various injury models *in vitro* might contribute to establish effective treatment strategies for these neurological diseases. Erythropoietin might be one of these candidate agents.

Various events such as inflammatory processes, glutamate excitotoxicity, the formation of reactive oxygen and nitrogen species, and apoptotic cell death might be involved in the pathogenesis of oligodendrocyte death in CNS diseases (Benn *et al.* 2001). Since Epo exerts its neuroprotective effect *via* action on these mechanisms (Maiese *et al.* 2004, Genc *et al.* 2004), it is likely that it also has oligodendroglioprotective action. Recently, it has also been shown that rat oligodendrocytes express EpoR, and Epo promotes the maturation of oligodendrocytes (Sugawa *et al.* 2002). Epo prevents secondary oligodendrocyte death due to trauma in spinal cord white matter *in vivo* (Gorio *et al.* 2002).

In the present study, we aimed to investigate the possible protective effect of Epo against lipopolysaccharide (LPS) and interferon-gamma (IFNγ)-induced inflammatory cytotoxicity in neonatal rat oligodendrocyte cultures. The possible modulatory effect of Epo on inducible nitric oxide synthase (iNOS) mRNA expression and nitrite production were analyzed as a possible cytoprotective mechanism.

Methods

Cell culture

This study was approved by the Local Ethical Committee for Experimental Research at Dokuz Eylul University. Rat oligodendrocyte cultures were prepared from brains of newborn (1 to 2-day-old) Wistar rats as previously described (Genc *et al.* 2003). The cells were plated in 25-cm^2 poly-D-lysine (PDL)-coated flasks for the reverse transcriptase-polymerase chain reaction (RT-PCR), 96-well PDL-coated plates for cytotoxicity and cell viability assays, and nitrite measurement and PDL-coated glass coverslips in 35 mm Petri dishes for immunocytochemistry at a density of 5 x 10^4 cells/cm^2. Cells were grown in a medium containing Dulbecco's Minimum Essential Medium / Ham's F12 (DMEM/F12), 0.5 % fetal bovine serum (FBS), and insulin-transferrin-selenite (Roche) for another seven days. The cells plated onto PDL-coated glass coverslips were used for galactocerebroside C (GalC) immunocytochemistry as previously described (Genc *et al.* 2003). In these cultures more than 98 % of cells were GalC stained oligodendrocytes (not shown). For experiments, cells were incubated with Epo for 24 h before the addition of toxic agents. Then, 1 μg/ml LPS (derived from *Escherichia coli* O26:B6; Sigma) and 100 U/ml recombinant rat IFNγ (GIBCO) at various concentrations were added to cultures and incubated for 72 h.

RT-PCR analysis

For RT-PCR analysis of EpoR mRNA, cells were treated for 24 h with 1 μg/ml LPS and 100 U/ml IFNγ. After incubation, cells were rinsed with PBS and total RNA was isolated using Nucleospin RNA isolation kit (Macherey Nagel). RNA concentration was quantified spectrophotometrically. Isolated RNA was treated with DNase to digest any contaminant genomic DNA. RT-PCR amplification was carried out with 5 μg RNA using primers for rat EpoR (sense 5'-CTATGGCTGTTG CAACGCGA-3'; antisense 5'-CCGAGGGCACAG GAGCTTAG-3') (Morishita *et al.* 1997), iNOS (sense 5'-CCACAATAGTACAATACTACTTGG-3'; antisense 5'-ACG AGGTGTTCAGCGTGCTCCACG-3') (Genc *et al.* 2003). Glyceraldehyde 3 phosphate dehydrogenase (GADPH) gene was used as an internal standard (sense 5'-ACCACAGTCCATGCCATCAC-3'; antisense 5'-TCCACCACCCTGTTGCTGTA-3'). PCR reactions were carried out in a thermal cycler (Perkin Elmer Cetus). The steps of amplification were 95 °C for 3 min, 95 °C for 30 s, 55 °C 30 s, 72 °C 1 min, 72 °C 5 min during 35 cycles for EpoR, 32 cycles for iNOS and 28 cycles for GAPDH. PCR products were resolved on 2 % agarose gel. Optic density of each band was calculated using the Multianalyst software (1.1. version) and Densitometer GS700 (BioRad). The results were expressed by density ratios to GAPDH.

Cytotoxicity assay

Cytotoxicity was quantified by spectrophotometrically measuring release of lactate dehydrogenase (LDH) from cells into medium utilizing a commercially available kit (Cytotoxicity Detection Kit; Roche). To determine the percentage of cytotoxicity, the average absorbance values of the triplicates was calculated and the absorbance value obtained in the background control (media alone) was subtracted from each of these. The resulting values were substituted in the following equation: *cytotoxicity (%) = (experimental value – spontaneous release)/(maximum release – spontaneous release) X 100*, where 'spontaneous release' means the LDH activity released from untreated normal cells. The maximum amount of releasable LDH enzyme activity was determined by lysing the cells with Triton X-100 (final concentration 1 % Triton X-100). 50 μl supernatant was removed from each well and transferred into an optically clear 96-well flat bottom microtiter plate. To

determine LDH activity in these supernatants, a 50 µl reaction mixture was prepared according to the manufacturer's instructions and added to each well. The absorbance of the samples was measured at 490 nm using an ELISA plate reader. The reference wavelength was 620 nm.

Nitrite measurement

The activity of iNOS was evaluated by determination of nitrite levels in the supernatants as previously described (Genc *et al.* 2003). Nitrite is a stable product of NO and generated by the rapid oxidation of NO. Aliquots of 100 µl culture supernatants were mixed with equal volumes of Griess reagent (0.1 % naphthyl-ethylenediamine dihydrochloride, 1 % sulphanilamide and 2.5 % phosphoric acid) mixture in a 96-well microtitre plate (Maxisorb Immunoplate, NUNC). After 10 min of incubation at room temperature, the absorbance at a wavelength of 540 nm was measured in a microplate reader (Model 230S; Organon Technica). A range of twofold dilutions of sodium nitrite (0-128 µM) in PBS were run in each assay to generate a standard curve.

Statistical analysis

Results are presented as mean ± S.E.M. of three different separate experiments performed with separate cell cultures. Each condition was triplicated in each experiment. Multiple group comparisons of the differences in quantitative measurements were made by ANOVA and followed by t test for statistical analysis. $P<0.05$ was considered to be significant.

Results

RT-PCR analysis revealed that differentiated rat oligodendroglial cells express baseline EpoR mRNA (Fig. 1). PC12 cells were used as positive controls for EpoR mRNA expression. Inflammatory stimuli did not significantly change EpoR mRNA expression in rat oligodendrocytes as revealed by semiquantitative evaluation, densitometric ratios being 1.01±0.12 in baseline condition and 1.30±0.09 in LPS plus IFNγ-induced condition ($p>0.05$).

The cytotoxicity assay confirmed that the pro-inflammatory cytokine IFNγ and LPS are toxic for oligodendrocytes in primary cell culture (Fig. 2). Erythropoietin demonstrated significant protection at all doses in oligodendrocyte cultures subjected to LPS + IFNγ-induced toxicity (Fig. 2). The magnitude of protection by Epo appears to be dose-dependent. Heat-inactivated Epo did not show any effect on cell survival (data not shown).

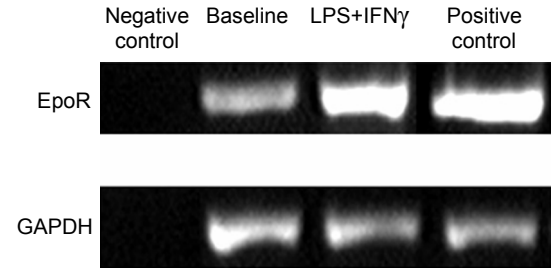

Fig. 1. Qualitative evaluation of EpoR mRNA expression in rat oligodendrocyte cultures. Cells were exposed to 100 U/ml rat IFNγ plus 1 µg/ml LPS for 24 h and total RNA was extracted, and subjected to RT-PCR. Concomitantly, analysis of GAPDH mRNA was carried out. The agarose gels were photographed and scanned. Rat oligodendrocytes express EpoR in baseline conditions. Rat PC12 pheochromocytoma cell line was used as positive control for EpoR mRNA expression. RT enzyme was not used in negative control samples.

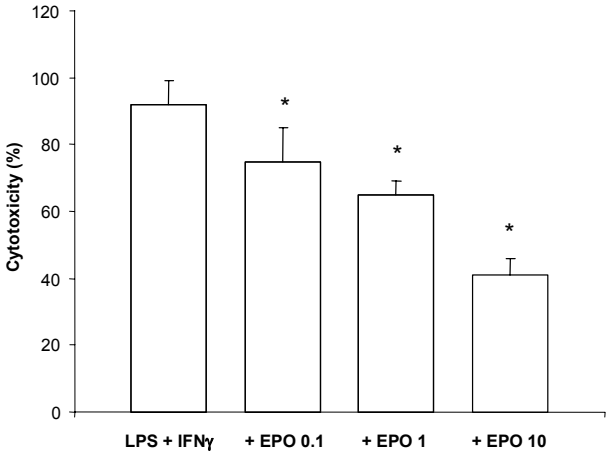

Fig. 2. Protective effect of Epo pretreatment on rat oligodendrocytes against cytotoxicity induced by LPS plus IFNγ. The results of the LDH assay reveal that Epo added to the cultures at various concentrations significantly decreases cytotoxicity induced by 1 µg/ml LPS and 100 U/ml IFNγ exposure for 72 h (* $p<0.05$). The results are means ± SEM of triplicate conditions obtained from three independent experiments.

When iNOS mRNA expression was evaluated by RT-PCR, iNOS mRNA expression was not found under control conditions, but inflammatory stimuli apparently induced iNOS mRNA expression (Fig. 3A). Erythropoietin pretreatment (1.0 U/ml) resulted in a significant decrease in increased iNOS mRNA expression. Densitometric ratios were 2.03±0.10 and 0.86±0.04 for with Epo and without Epo conditions, respectively ($p<0.05$). The activity of iNOS was also

evaluated by determination of nitrite levels in the supernatants. As shown in Figure 3B, 72 h exposure of rat oligodendroglial cells to 1 μg/ml LPS and 100 U/ml IFNγ significantly increased nitrite production. Pretreatment of cultures with Epo (1.0 U/ml) 24 h before LPS and IFNγ exposure produced significant reduction in induced-nitrite levels.

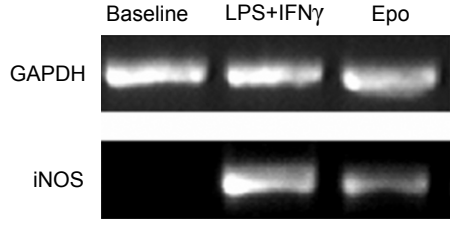

Fig. 3A. The effect of Epo pretreatment (1.0 U/ml) on iNOS mRNA expression upon 1 μg/ml LPS and 100 U/ml IFNγ exposure in rat differentiated oligodendrocyte cultures. Cells were exposed to Epo for 24 h and then, LPS and IFNγ were added to cultures. After 24 h incubation, cells were lysed and total RNA was extracted, and subjected to RT-PCR. Concomitantly, analysis of GAPDH mRNA was carried out. The agarose gels were photographed and scanned. Epo pretreatment decreases iNOS mRNA expression induced by inflammatory stimuli.

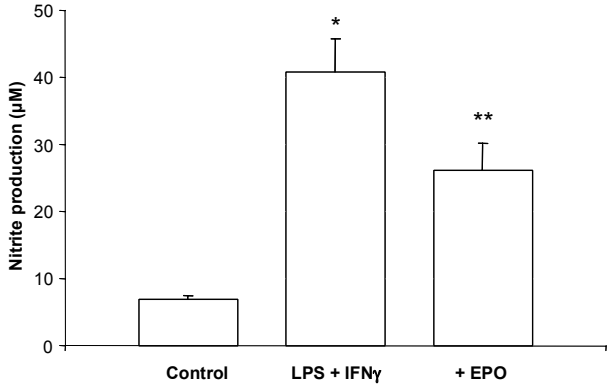

Fig. 3B. The effect of Epo pretreatment (1.0 U/ml) on endogenous nitrite production in differentiated rat oligodendrocytes. Cells were exposed to Epo for 24 h and then, 1 μg/ml LPS and 100 U/ml IFNγ were added to cultures. After another 72 h incubation, production of nitrite was determined in 100 μl aliquots of culture supernatant. The values represent the mean ± SEM of three independent experiments performed in triplicate. 72 h exposure of rat oligodendroglial cells to 1 μg/ml LPS and 100 U/ml IFNγ significantly induced nitrite production (* $p<0.05$). Epo pretreatment significantly decreases LPS plus IFNγ-induced nitrite production (** $p<0.05$).

Discussion

In the present study, the protective effect of erythropoietin on oligodendrocyte viability has been evaluated in cells at only a single stage of oligodendrocyte lineage, i.e. in differentiated GalC-positive cells. Additional compromising factors such as cytotoxicity due to activation of contaminant microglial cells or protection due to contaminant astroglial cells were excluded by the high purity of cultures. This is important because of the differential sensitivity of oligodendroglial cells from different developmental stages to toxic factors and injury (Baerwald and Popko 1998, Benn *et al.* 2001). The response of oligodendrocytes from other developmental stages (progenitors or immature oligodendrocytes) to inflammatory stimuli and the effect of Epo treatment was not our focus in this study and further studies are needed to clarify this issue.

A recent study has shown EpoR on rat oligodendrocytes (Sugawa *et al.* 2002) and this finding was confirmed in the present study. Rodent oligodendrocytes are also known to express IFNγ receptor (Torres *et al.* 1995*).* The cytotoxic effect of pro-inflammatory cytokines and LPS in oligodendrocyte are well documented by *in vitro* studies (Baerwald and Popko 1998, Molina-Holgado *et al.* 2001). The presence of pro-inflammatory cytokines in multiple sclerosis (MS) lesions and the correlation between the levels of these and disease activity have been reported (Cannela and Raine 1995). The present study is the first *in vitro* demonstration of the protective effect of Epo on oligodendrocyte injury induced by IFNγ and LPS. Epo treatment following inflammatory injury did not show protection in rat oligodendrocytes (not shown). Although pretreatment with Epo might not fit to the clinical setting, Epo treatment may provide some benefit on ongoing oligodendroglial injury. Epo has ameliorating effects on the clinical status in the rat MS model (Agnello *et al.* 2002). In addition, anti-inflammatory effects of Epo have been determined in head injury model (Brines *et al.* 2000). A recent study using a rat spinal trauma model has shown the ameliorating effect of systemic Epo treatment on delayed white matter injury and apoptotic cell death (Gorio *et al.* 2002).

The present results suggest a mechanism of Epo protection, i.e. decrease of iNOS derived NO production induced by inflammatory stimuli. Recent evidence suggests that oligodendrocytes, apart from being a target, may be a source of cytokines and NO in inflammatory conditions (Merrill *et al.* 1997, Molina-Holgado *et al.* 2001). Among cytotoxic effector molecules evoked by the proinflammatory stimuli, increasing evidence supports a role of NO in MS and oligodendrocyte damage

(Bagasra *et al*. 1995, Mitrovic *et al*. 1995, Smith and Lassmann 2002). For this reason, we focused here on clarifying whether Epo has any effect on oligodendroglial inducible NO production and confirmed the suppressive effect of Epo on endogenous inducible NO production in rat oligodendrocytes. Epo has been shown to inhibit brain NO production in a global cerebral ischemia model (Calapai *et al*. 2000). However, no inhibiting effect of Epo on cultured microglial baseline NO production has been observed (Vairano *et al*. 2002). The effect of Epo on inducible NO production has not been evaluated in that study. The results of another recent study suggest that Epo protects neurons from NO-related neuronal apoptosis, but does not affect NO levels (Digicaylioglu and Lipton 2001). The effect of Epo on iNOS and inducible NO production may be cell type-specific.

The results of the present study suggest that Epo, in addition to its neuroprotective effect, might be a useful agent to protect oligodendrocytes in many clinical situations such as MS, characterized by primary oligodendrocyte injury, or trauma and hypoxic and ischemic injury of the CNS, which also affect white matter and oligodendrocyte survival.

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Reprint requests

Kursad Genc, Learning Resources Center Research Laboratory, School of Medicine, Dokuz Eylul University, Inciralti, 35340, Izmir, Turkey. E-mail: kkursadgenc@hotmail.com