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Research report

Neuroprotective erythropoietin attenuates microglial activation, including morphological changes, phagocytosis, and cytokine production

Tetsuya Tamura [a,b], Mineyoshi Aoyama [a,c,*], Seiko Ukai [a], Hiroki Kakita [a,d], Kazuya Sobue [b], Kiyofumi Asai [a]

[a] Department of Molecular Neurobiology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan
[b] Department of Anesthesiology and Intensive Care Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan
[c] Department of Pathobiology, Nagoya City University Graduate School of Pharmaceutical Sciences, Nagoya, Japan
[d] Department of Perinatal and Neonatal Medicine, Aichi Medical University, Nagakute, Japan

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ABSTRACT

Erythropoietin (EPO), a hematopoietic hormonal cytokine induced in response to hypoxia, has neuroprotective effects. EPO receptor (EPOR) is expressed in microglia, resident immune cells in the brain. However, the effect of EPO on microglial activation is not clear. In the present study, we demonstrated that the EPOR is highly expressed in microglia, rather than in neurons or astrocytes, in *in vitro* experiments. Therefore, we investigated whether EPO could attenuate lipopolysaccharide (LPS)-mediated activation of microglia *in vitro*. The BV-2 microglial cell line was treated with LPS in the absence or presence of EPO. In the presence of EPO, microglial expression of LPS-induced inflammatory cytokine genes was significantly decreased. In addition, EPO suppressed the LPS-induced phagocytic activity of BV-2 cells towards fluorescent beads, as well as induction of inducible nitric oxide synthase. In *in vivo* experiments, EPO significantly decreased the LPS-induced expression of inflammatory cytokine genes in mouse brains. Furthermore, morphological analysis of cortical microglia in the brains of mice stimulated with LPS revealed that combined treatment with EPO alleviated LPS-induced morphological changes in the microglia. These data indicate that EPO attenuates microglial activation, including morphological changes *in vivo*, phagocytosis *in vitro*, and the production of inflammatory cytokines *in vivo* and *in vitro*. Further investigation of EPO modulation of LPS-induced microglial activation may contribute to the development of novel neuroprotective therapies.

1. Introduction

Erythropoietin (EPO) is a 34 kDa glycoprotein hormone that is induced by hypoxia. The main site of EPO production is the fetal liver and the adult kidney. EPO supports the proliferation and differentiation of erythroid progenitor cells for survival (Lin et al., 1996; Wu et al., 1995). EPO is also expressed in the brain. At the cellular level, intrinsic EPO expression is detected in neurons and astrocytes, and primarily in astrocytes (Masuda et al., 1994; Ruscher et al., 2002). The EPO receptor (EPOR) is expressed in the brain, and provides a signaling mechanism for the neuroprotective role of EPO in ischemic diseases and neurodegenerative diseases (Juul et al., 1998; Marti et al., 1996; Masuda et al., 1993; Morishita et al., 1997). At the cellular level, the EPOR is expressed in human and rodent neurons, astrocytes, and microglia (Chin et al., 2000; Nagai et al., 2001; Noguchi et al., 2007), and we reported previously that EPOR was expressed in mouse cultured oligodendrocyte precursor cells (OPC; Kato et al., 2011). EPO signaling from astrocytes to OPCs could prevent OPC damage under conditions of hypoxia/reoxygenation injury (Kato et al., 2011).

Microglia are the resident macrophages in the brain and have innate immune functions in the central nervous system (CNS; Lawson et al., 1990). Microglial activation is associated with morphological changes, proliferation, motility, phagocytosis, and cytokine release (Boscia et al., 2009; Harrigan et al., 2008). Microglial activation states can be divided into classical and alternative activation states (Colton, 2009). Classically activated microglia are cytotoxic because they secrete proinflammatory cytokines and reactive oxygen species (ROS). In contrast, alternatively activated microglia induce anti-inflammatory cytokines and are involved in wound repair and debris clearance (Gordon, 2003). Phagocytosis

* Corresponding author at: Department of Pathobiology, Nagoya City University Graduate School of Pharmaceutical Sciences, 3-1, Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan.

E-mail addresses: tetsuharuhina1226@yahoo.co.jp (T. Tamura), aomine@phar.nagoya-cu.ac.jp (M. Aoyama), s.ukai@med.nagoya-cu.ac.jp (S. Ukai), hkaki1019@yahoo.co.jp (H. Kakita), kazsobue@gmail.com (K. Sobue), kiyoasai@med.nagoya-cu.ac.jp (K. Asai).

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of microbes is associated with inflammation, whereas the phagocytosis of apoptotic cells occurs in the absences of inflammation. Recognition of microbes induces a microglial phagocytic response, which is associated with the release of pro-inflammatory cytokines (Neumann et al., 2009). Accordingly, phagocytosis with inflammation could be considered the result of classic microglial activation. Activated microglia are observed in nearly all kinds of neurological diseases, including neurodegenerative diseases, such as Alzheimer's disease (AD; Prokop et al., 2013), Parkinson's disease (PD; Long-Smith et al., 2009), and amyotrophic lateral sclerosis (ALS; Sargsyan et al., 2005), infectious and inflammatory diseases, such as multiple sclerosis (MS; Brown, 2001; Napoli and Neumann, 2010), stroke (Yenari et al., 2010), and traumatic (Loane and Byrnes, 2010) and radiation-induced (Chiang et al., 1993) brain injury. Because classical microglial activation is considered to be the major cause of these diseases, from a therapeutic viewpoint it is important to control classical microglial activation. In the present study, we chose to use a lipopolysaccharide (LPS)-stimulated model because LPS is known to induce classical microglial activation (Kobayashi et al., 2013).

In the present study, we investigated whether the neuroprotective effects of EPO could attenuate microglial activation. To determine the effects of EPO on microglia, we examined the effects of EPO on: (i) the expression of inflammatory cytokines and the phagocytic activity of the BV-2 murine microglial cell line after LPS treatment *in vitro*, and (ii) *in vivo* cytokine production and morphological changes to microglia in the brains of mice after LPS treatment.

2. Results

2.1. Expression of EPOR in rat primary cultured cells

The quantitative analyses of EPOR expression were performed on neurons, astrocytes, and microglia from primary cultures of rat cells in the present study. EPOR expression was found to be higher in microglia than in neurons or astrocytes (Fig. 1A; $P < 0.01$; n = 6). Immunocytochemical analysis revealed higher expression of EPOR on neurons and microglia (Fig. 1B).

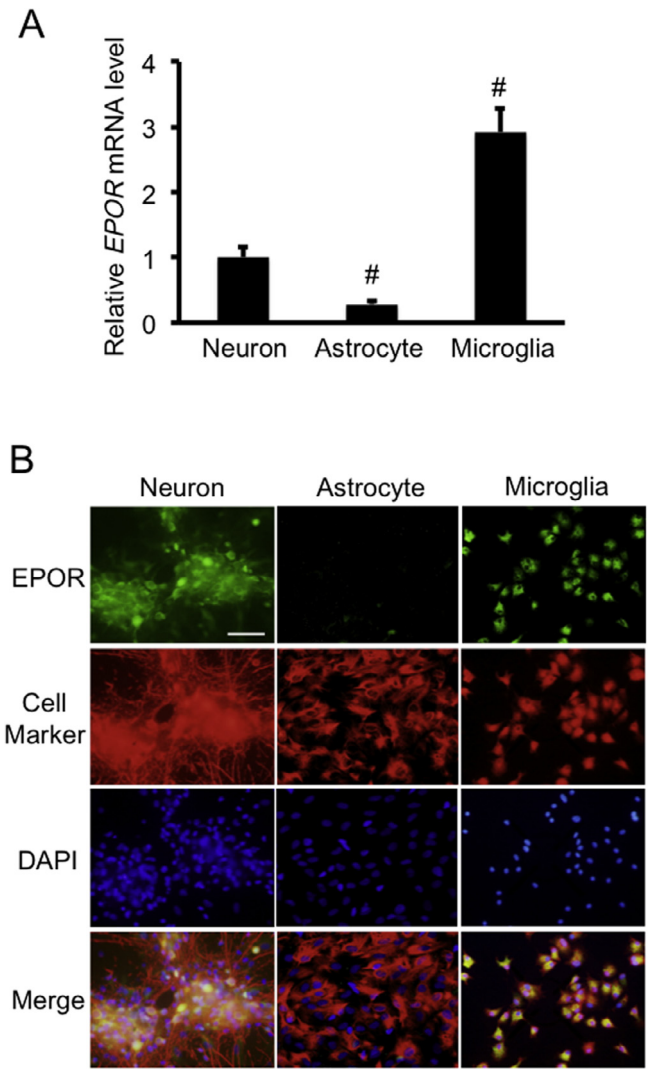

Fig. 1. Expression of erythropoietin (EPO) receptor (EPOR) in primary cultures of rat neurons, astrocytes, and microglia. (A) *EPOR* mRNA levels were evaluated by quantitative RT-PCR and expression was normalized against that of the β-actin (*Actb*) gene. *EPOR* mRNA was highly expressed in microglia compared with neurons and astrocytes. Data are the mean ± SEM (n = 6 in each group). $^{\#}P < 0.01$ compared with neurons (one-way ANOVA with Bonferroni correction for all comparison pairs). (B) Neurons, astrocytes, and microglia were stained with antibodies against cell-specific markers (red) and EPOR (green). Neurons were stained with antibodies against the neuron cell marker β-tubulin (red), astrocytes were stained with antibodies against the astrocyte cell marker glial fibrillary acidic protein (GFAP; red), and microglia were stained with antibodies against the microglial cell marker ED1 (red). Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI; blue). Bar, 100 μm. Representative photomicrographs show that EPOR was highly expressed in microglia.

2.2. Inflammatory cytokine expression in BV-2 cells after LPS and EPO treatment

The effects of EPO on LPS-induced activation of microglia were assessed by measuring the expression of inflammatory cytokines in the murine microglial BV-2 cell line. We analyzed the time course of LPS-induced cytokine expression under control conditions and in the presence of EPO. Tumor necrosis factor-α (*Tnfα*) expression peaked 1 h after LPS treatment (Fig. 2A), whereas interleukin-1β (*Il1b*) and interleukin-6 (*Il6*) expression peaked 3 h (Fig. 2B) and 6 h (Fig. 2C) after LPS treatment, respectively. In addition, we examined the effect of EPO treatment on peak cytokine mRNA expression and found that EPO significantly reduced peak mRNA expression for all three inflammatory cytokines (Fig. 2A–C; $P < 0.01$; n = 6).

2.3. Effects of EPO on LPS-induced inducible nitric oxide synthase (iNOS) expression in BV-2 cells

The effects of EPO on LPS-induced *inducible nitric oxide synthase* (*iNOS*) mRNA expression in BV-2 cells were evaluated. In both untreated control and EPO-treated BV-2 cells, *iNOS* expression was very low or below the threshold of the assay detection. However, LPS induced *iNOS* gene expression in BV-2 cells, which peaked 6 h after LPS treatment, and this increase in *iNOS* expression was significantly reduced by EPO (Fig. 3A; $P < 0.01$; n = 6).

Western blotting analysis revealed very faint iNOS protein expression in both the untreated control and EPO-treated groups. In contrast, significantly higher iNOS protein expression was seen in the LPS-treated group, which was reduced by EPO (Fig. 3B). Quantitative analysis revealed an approximate 3.3-fold increase in iNOS protein in LPS-treated BV-2 cells, which was significantly decreased by EPO (Fig. 3B; $P < 0.01$; n = 3).

In addition, nitric oxide (NO) levels in the conditioned medium of untreated and treated BV-2 cells were evaluated as nitrite concentrations using the Griess assay. However, NO levels were very low and could not be reliably measured (data not shown).