Endogenous Erythropoietin as Part of the Cytokine Network in the Pathogenesis of Experimental Autoimmune Encephalomyelitis

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Erythropoietin (EPO) is of great interest as a therapy for many of the central nervous system (CNS) diseases and its administration is protective in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS). Endogenous EPO is induced by hypoxic/ischemic injury, but little is known about its expression in other CNS diseases. We report here that EPO expression in the spinal cord is induced in mouse models of chronic or relapsing-remitting EAE, and is prominently localized to motoneurons. We found a parallel increase of hypoxia-inducible transcription factor (HIF)-1α, but not HIF-2α, at the mRNA level, suggesting a possible role of non-hypoxic factors in EPO induction. EPO mRNA in the spinal cord was co-expressed with interferon (IFN)–γ and tumor necrosis factor (TNF), and these cytokines inhibited EPO production in vitro in both neuronal and glial cells. Given the known inhibitory effect of EPO on neuroinflammation, our study indicates that EPO should be viewed as part of the inflammatory/anti-inflammatory network in MS.

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using two models of EAE: a chronic model induced in C57BL/6 mice by immunization against a peptide of myelin oligodendrocyte glycoprotein (MOG) (6), and a relapsing-remitting model induced in SJL mice by immunizing against myelin proteolipid protein (PLP) peptide (16). Since we found that EPO is co-induced along with interferon (IFN)-γ and tumor necrosis factor (TNF), pro-inflammatory cytokines with a pathogenic role in EAE, we investigated the effect of IFN-γ and TNF on EPO expression in neuronal and glial cells. The results indicate that EPO is induced during EAE and is negatively regulated by IFN-γ and TNF.

**MATERIALS AND METHODS**

**Animal Experiments**

Procedures involving animals and their care were conducted in conformity with the institutional guidelines in compliance with national and international laws and policies (17–19). The protocols for the proposed investigation were reviewed and approved by the Animal Care and Use Committees (IACUC) of the Mario Negri Institute for Pharmacological Research.

Chronic EAE was induced in female C57BL/6 mice (6–8 wks; Harlan, Bresso, MI, Italy) by subcutaneous (s.c.) immunization with 200 μg of MOG peptide 35–55 (Multiple Peptide Systems, San Diego, CA, USA) per mouse in incomplete Freund’s adjuvant (Sigma, St Louis, MO, USA) supplemented with 8 mg/mL of *M. tuberculosis* (H37Rv; Difco Laboratories, Detroit, MI, USA). Mice received 500 ng of pertussis toxin (Sigma) intravenously (i.v.) at the time of immunization and 48 h later. Clinical score was recorded daily as described elsewhere (6).

Relapsing-remitting EAE was induced in female 8- to 12-wk-old SJL mice (Charles River, Calco, LC, Italy) by s.c. immunization with PLP peptide 139–151 (100 μg/mouse, in incomplete Freund’s adjuvant, Sigma, containing 2 mg/mL of heat-killed *M. tuberculosis*, Difco) as described (16).

**Real-Time RT-PCR**

Total RNA was extracted from spinal cords or cell cultures using the TRizol reagent (Invitrogen, Carlsbad, CA, USA), retrotranscribed according to standard procedures, and amplified by real-time reverse-transcription (RT)-PCR. All procedures were performed on the ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For quantification, we used the comparative threshold cycle (ΔCt) method, according to the manufacturer’s guidelines. EPO mRNA was analyzed by the TaqMan method, with gene expression assays for EPO and GAPDH (housekeeping gene) commercially available from Applied Biosystems. Data were expressed as EPO mRNA units, with a cDNA from mouse kidney used as a calibrator (or rat kidney when EPO mRNA was measured in rat glial cells). IFN-γ and TNF mRNA levels were analyzed by the SYBR Green method, with primer sequences for TNF, IFN-γ, and GAPDH as reported (20). Data were expressed as fold induction versus control mice, using one of the controls as a calibrator.

**Immunohistochemistry**

Animals were sacrificed by intracardial perfusion (21). EPO localization was evaluated along the whole length of the spinal cord. Sections (40 μm-thick) were washed in phosphate-buffered saline (PBS), incubated in 10% blocking serum in PBS at room temperature and exposed to polyclonal rabbit anti-EPO (1:100, Aves Labs, Logan, UT, USA), with or without recombinant human IFN-γ (Hy-Cult Biotechnology, Uden, The Netherlands) or recombinant human TNF-α (R&D Systems, Minneapolis, MN, USA). After 24 h of incubation, EPO was measured in the supernatant by Enzyme-Linked ImmunoSorbent Assay (ELISA) (Quantikine EPO, R&D Systems, sensitivity 0.6 mU/mL). All samples were assayed in duplicate. Results were expressed as mU/mL.

Primary cultures of glial cells were prepared from 1- to 2-d-old newborn rats (Sprague-Dawley, Charles River) as described (3). Cells were treated for 24 h with the HIF-inducer dimethyloxalylglycine (DMOG, Frontier Scientific Inc., Logan, UT, USA), with or without recombinant rat IFN-γ (R&D Systems) or recombinant rat TNF-α (R&D Systems), then total RNA was extracted and analyzed for EPO expression by real-time RT-PCR.

Coronal sectioning was preferred to better evaluate the localization of the different markers in specific areas of the grey matter. EPO was detected with polyclonal rabbit anti-EPO and biotinylated goat anti-rabbit antibodies as described above, and the immunofluorescence revealed with Alexa 488 goat anti-rabbit secondary antibody (1:1000, Molecular Probes, Eugene, OR, USA). Neurons were stained with 530-615 NeuroTrace Fluorescent Nissl reagent (1:5000, Molecular Probes). Astrocytes were identified by a mouse anti-GFAP monoclonal antibody (1:1000, Immunological Science, Rome, Italy) followed by an Alexa 546 goat antimouse secondary antibody (1:1000, Molecular Probes). Sections were observed with an Olympus Fluoview microscope BX61 with confocal system FV500. To confirm the specificity of the signal, we checked that incubating with either a primary or a secondary antibody alone gave no staining (not shown).

**Cell Culture**

Human Kelly neuroblastoma cells were cultured and stimulated with the HIF-inducer deferoxamine mesylate (DFX, Sigma) as reported (22), with or without recombinant human IFN-γ (Hy-Cult Biotechnology, Uden, The Netherlands) or recombinant human TNF-α (R&D Systems, Minneapolis, MN, USA). After 24 h of incubation, EPO was measured in the supernatant by Enzyme-Linked ImmunoSorbent Assay (ELISA) (Quantikine EPO, R&D Systems, sensitivity 0.6 mU/mL). All samples were assayed in duplicate. Results were expressed as mU/mL.

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RESULTS

EPO Expression Is Increased in the Spinal Cord of Mice with EAE

In chronic EAE induced by immunization of C57BL/6 mice with MOG35-55, clinical signs of disease have an onset at d 10-15 after immunization, peak at d 20, and then stabilize (6). By quantitative real-time RT-PCR, we found a marked increase in EPO mRNA expression in the spinal cord of mice with EAE at d 20 as compared with control naive mice (Figure 1A). EPO mRNA levels remained elevated at d 40, when the clinical signs of the disease are stabilized.

In SJL mice, PLP139-155-induced EAE is characterized by a relapsing-remitting type of disease (16). In this model, EPO expression was increased in the spinal cord at the peak of the disease, and remained elevated in the remission phase (Figure 1B).

Immunohistochemical Localization of EPO in the Spinal Cord

Immunohistochemistry on d 20 after immunization of C57BL/6 mice revealed an almost selective immunoreactivity in the gray matter (central layer), with a spot pattern, and a very low staining in the white matter (external layers) (Figure 2A). Higher magnification of the area corresponding to the ventral horns showed that EPO is localized in cell bodies of neurons with different sizes, likely motoneurons and interneurons (Figure 2B). Although DAB immunohistochemistry does not allow a quantitative evaluation, staining intensity of large-sized neurons appears higher in EAE mice (Figure 2B) compared with healthy controls (Figure 2C). Incubation of sections without the primary antibody allowed us to exclude the presence of a non-specific immunoreactivity due to the biotinylated secondary antibody (not shown).

When Nissl was used to identify neurons (Figure 2D, red), EPO staining (Figure 2E, green) co-localized with it (Figure 2F, merge), showing a cytoplasmic localization. On the other hand, co-localization experiments using GFAP to stain astrocytes (Figure 2G, red) and EPO (Figure 2H, green) confirmed the lack of expression of EPO by astrocytes (Figure 2I, merge).

HIF-1α Expression Is Induced in EAE

We investigated the mRNA expression of HIF-1α and HIF-2α in the spinal cord of mice with chronic EAE (C57BL/6) or relapsing-remitting EAE (SJL), and correlated it with the increased expression of EPO mRNA. For purpose of comparison, we also measured HIFs and EPO mRNA in the spinal cord of mice treated with phenylhydrazine (PhH), which also is known to induce EPO expression in the CNS (23). In both models of EAE, a significant increase of HIF-1α, but not HIF-2α, expression was observed (Figure 3), accompanied by a two- to three-fold induction in EPO mRNA (see above and Figure 3). On the contrary, PhH did not increase spinal cord HIF-1α and HIF-2α mRNA, even though it strongly induced EPO expression (controls, 0.11 ± 0.02; PhH, 1.01 ± 0.15; P < 0.001, n = 4), acting through stabilization of the HIF protein, as reported (24).

IFN-γ and TNF Are Co-Induced with EPO and Inhibit Its Expression in Neuronal and Glial Cells in vitro

We also measured the expression of the two inflammatory cytokines IFN-γ and TNF in the spinal cord. This was high at the peak of the disease and then, unlike EPO, declined both in chronic EAE in C57BL/6 mice (Figure 4A,4B) and in relapsing-remitting EAE in SJL mice (Figure 4C,4D).

The temporal co-induction of EPO, IFN-γ, and TNF in the spinal cord prompted us to investigate the effect of these cytokines on EPO expression in neuronal cells. To this end, we used Kelly human neuroblastoma cells, known to produce significant amounts of EPO protein, detectable by ELISA (22). IFN-γ or TNF, alone or in combination, did not induce EPO production in Kelly cells (not shown). On the contrary, when EPO was induced with the HIF-stabilizer DFX, IFN-γ dose-dependently inhibited DFX-induced EPO secretion (Figure 5A). TNF alone did not affect DFX-induced EPO production, but synergized with IFN-γ (Figure 5B). The observed synergy is consistent with the hypothesis that lack of inhibition by TNF alone could be due, at least partially, to low expression of TNF receptor 1 (TNFR1), which is known to be induced by IFN-γ. In fact, we found that, in our conditions, IFN-γ increased...
TNFR1 mRNA levels at 4 h (three-fold induction measured by real-time RT-PCR, data not shown).

Although immunolocalization of EPO in EAE suggests it is produced mainly by neurons, others reported that glial cells also express EPO (10,11,25,26). We thus studied the effect of IFN-γ and TNF on EPO expression in rat primary glial cells, where EPO was induced with the HIF-stabilizer DMOG. In these experiments, EPO was measured by RT-PCR because its levels in the culture supernatants were undetectable by ELISA. As shown in Figure 5, panels C and D, both IFN-γ and TNF markedly and dose-dependently inhibited EPO expression in glial cells.

**DISCUSSION**

While administration of EPO is protective in models of EAE (1,4–6) and possibly in MS patients (7), as well as in other CNS diseases (1,2), induction of endogenous EPO in the CNS has only been reported for hypoxic/ischemic pathologies, where EPO is induced as a result of the activation of HIF by hypoxia (10–12).

Our study shows that EPO is expressed in the CNS of mice with EAE, and immunohistochemistry indicated that it was associated mainly with neurons. The finding that neuronal cells are the main source of EPO expression in the spinal cord is in agreement with a previous study in the development of CNS (27).

In consideration of the anti-inflammatory action of exogenous EPO administration in EAE (6), the induction of endogenous EPO reported here suggests that EPO may be part of a protective response, along with anti-inflammatory cytokines such as interleukin-4 (IL-4), interleukin-10 (IL-10), and transforming growth factor (TGF)-β—whose expression correlates with recovery from EAE (28–30). Of note, considering the neuronal localization of EPO expression, others reported that neurons can express neurotrophic or protective cytokines: motoneurons can express brain-derived neurotrophic factor (BDNF) (31), and TGF-β is expressed by...
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The protective action of EPO in MS may be exerted also on other pathways than the inflammatory cytokine network. In fact, others have shown upregulation of VEGF, another target of HIF-1α, in the spinal cords of rats with EAE (32) and in MS active plaques from autopsy samples (14,32), and it was suggested that upregulated VEGF in MS might contribute to the increased permeability of the blood-brain barrier (32). In this context, EPO was reported to counteract the increase in permeability induced by VEGF in an in vitro model of blood-brain barrier (33).

The mechanisms that upregulate EPO expression in EAE remain to be elucidated. The hypoxia-induced upregulation of EPO is modulated at the transcriptional level by HIF. The predominant mode of HIF induction by hypoxia is at the level of protein stability. In particular, the α subunits of HIF (HIF-1α and HIF-2α) are stabilized and accumulate upon hypoxia (34). In addition, several in vivo studies showed increased levels of HIF-1α mRNA (35,36). We found that EPO induction in EAE is paralleled by an elevation of HIF-1α, but not HIF-2α, mRNA. Of note, PhH, that induces EPO expression in the CNS secondary to anemia (23), had no effect on HIF-1α mRNA even if EPO induction was more marked than in EAE, confirming previous studies showing that it acts by increasing HIF at the protein level (24). Indeed PhH even decreased HIF-2α expression, possibly through a negative feedback mechanism.

Recently, possible mechanisms for HIF-1α mRNA induction by hypoxia have been characterized (37), and it also has been reported that non-hypoxic inflammatory mediators, including reactive oxygen species, may activate HIF-1α at the transcriptional level, possibly through NF-κB induction (38). In particular, inflammatory stimuli can induce HIF-1α mRNA in macrophages (39) and microglial cells (40), and these mechanisms might play a role in the increase of EPO expression in EAE. Of note, the effect of inflammatory stimuli on HIF tran-
Endogenous EPO in the context of the cytokine network in MS.

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