Erythropoietin (EPO) Increases Myelin Gene Expression in CG4 Oligodendrocyte Cells through the Classical EPO Receptor

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Erythropoietin (EPO) has protective effects in neurodegenerative and neuroinflammatory diseases, including in animal models of multiple sclerosis, where EPO decreases disease severity. EPO also promotes neurogenesis and is protective in models of toxic demyelination. In this study, we asked whether EPO could promote neurorepair by also inducing remyelination. In addition, we investigated whether the effect of EPO could be mediated by the classical erythropoietic EPO receptor (EPOR), since it is still questioned if EPOR is functional in nonhematopoietic cells. Using CG4 cells, a line of rat oligodendrocyte precursor cells, we found that EPO increases the expression of myelin genes (myelin oligodendrocyte glycoprotein [MOG] and myelin basic protein [MBP]). EPO had no effect in wild-type CG4 cells, which do not express EPOR, whereas it increased MOG and MBP expression in cells engineered to overexpress EPO (CG4-EPOR). This was reflected in a marked increase in MOG protein levels, as detected by Western blot. In these cells, EPO induced by 10-fold the early growth response gene 2 (Egr2), which is required for peripheral myelination. However, Egr2 silencing with a siRNA did not reverse the effect of EPO, indicating that EPO acts through other pathways. In conclusion, EPO induces the expression of myelin genes in oligodendrocytes and this effect requires the presence of EPOR. This study demonstrates that EPOR can mediate neuroreparative effects.

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INTRODUCTION

Erythropoietin (EPO) has protective effects and decreases neuroinflammation in various models of neurological diseases, including traumatic and ischemic injury of the brain and the spinal cord and multiple sclerosis (MS) (1,2). Inhibition of neuronal death and neuroinflammation are important for the protective effects (3). However, many studies have pointed out that EPO also promotes neurorepair, in terms of neurogenesis, angiogenesis and promotion of synaptic plasticity (4–6).

In the context of MS, EPO has antiinflammatory (7,8) and immunoregulatory properties (9,10). In addition, it inhibits demyelination and axonal damage (11,12), but it is unclear whether this effect is secondary to its antiinflammatory and immunoregulatory action. However, there are evidences that EPO is effective also in nonimmune models of demyelination. EPO is protective in vivo in a model of chemically induced demyelination (13) and induces myelin repair in an ex vivo model of demyelination induced by lysolecithin (14). Interestingly, EPO increases the number of myelin basic protein (MBP)-positive cells in primary oligodendrocytes (15).

The role of the EPO receptor (EPOR) in the neuroprotective actions of EPO is a debated issue (16). EPO mediates erythropoiesis by homodimerizing EPOR (17) but derivatives of EPO that do not bind the homodimeric EPOR, and are therefore not erythropoietic, are still neuroprotective (18,19), and EPO can reduce brain damage in mice lacking neural EPOR (20). On the other hand, EPOR is required for normal brain development (21) and for inhibition of apoptosis in neuronal cells (22). Also, the observation that brain EPOR expression is increased during pathological conditions in humans, including ischemic infarcts and hypoxic brain damage, suggests a poten-
tial protective role of the classical receptor (23). Recent studies have indicated that the spectrum of actions of EPOR can go beyond those induced by its homodimerization, and the tissue-protective activities of EPO might be due, at least in part, to heterodimerization of EPOR with the common β chain (βc) of interleukin (IL)-3/IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF).

EPO variants (for example, carbamylated EPO, CEPO) that can bind the heterodimeric EPOR/bc but not the EPOR dimer have tissue-protective effects equivalent to EPO in multiple animal models of disease (24).

Here, we studied the effect of EPO on myelination, specifically investigating the role of EPOR. For this purpose, we measured the expression of two major myelin genes, myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP), in the differentiated CG4 oligodendrocytic cell line, with or without EPOR expression. CG4 cells are considered a good tool to study myelination in vitro (25,26). We also investigated the role of early growth response gene 2 (Egr2), a gene required for myelination in the peripheral nervous system (27), whose expression is induced by EPO in the brain (6). The results of this study clearly indicate that EPOR is required for EPO-induced myelination.

MATERIALS AND METHODS

Cell Culture and Generation of CG4 Cells Expressing EPOR (CG4-EPOR)
The oligodendrocyte progenitor cell line CG4 was cultured in poly-L-ornithine–coated tissue culture plates (Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained at the precursor stage using growth medium (GM) consisting of Dulbecco’s modified Eagle medium (DMEM) (PAA Laboratories, Yevil, Sommerset, UK) supplemented with bFGF (5 ng/mL), PDGF (1 ng/mL), N1 supplement (all from Sigma-Aldrich) and 30% B104-conditioned medium. Rat neuroblastoma B104 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen/Life Technologies, Carlsbad, CA, USA). For the preparation of the B104-conditioned medium, B104 confluent cells were cultured for 4 d in DMEM, without FBS, but with the addition of N1 supplement. CG4 cells overexpressing EPO receptor (CG4-EPOR) were obtained by transduction of CG4 cells with the mouse EPO gene in a constitutive lentiviral vector (28), modified to include the V5 epitope, the mouse encephalomyocarditis internal ribosome entry site (IRES) and the enhanced green fluorescent protein (EGFP) reporter (6,25). Clones were isolated by limiting dilution, by plating transduced cells at the concentration of 0.3 cells/well in 96 well plates in GM, and then screened for EPO expression by quantitative polymerase chain reaction (qPCR), as described below. Control CG4 cells (CG4-EGFP) were obtained by transduction of CG4 cells with a lentiviral vector containing EGFP only.

CG4 cells were induced to differentiate to oligodendrocytes by switching to differentiation medium (DM) consisting of DMEM-F12 (PAA) supplemented with progesterone (3 ng/mL), putrescine (5 μg/mL), sodium selenite (4 ng/mL), insulin (12.5 μg/mL), transferrin (50 μg/mL), biotin (10 ng/mL), thyroxine (0.4 μg/mL) and glucose (3 g/L) (all from Sigma-Aldrich). Cells were treated with recombinant human erythropoietin (rhEPO) (Creative Dynamics, New York, NY, USA) at the doses indicated. Carbamylated EPO (CEPO), prepared as described (18), was kindly supplied by Warren Pharmaceuticals, Ossining, NY, USA.

**EPO Expression in CG4-EPOR Cells**
The expression of recombinant V5-tagged EPO in transduced CG4 cells was verified by measuring by flow cytometry the EGFP reporter expression, as well as by immunoblotting with the anti-V5-tag mouse monoclonal antibody (Invitrogen/Life Technologies), as described (6,25). EGFP, whose translation is linked to EPO via the internal ribosome entry site (IRES), is a very reliable marker of the gene of interest expression when bicistronic vectors are used (25).

**Quantitative PCR**
Total RNA was extracted from cultured cells using TRIzol (Invitrogen/Life Technologies). RNA quality and concentration were determined using a NanoDrop ND-1000 (NanoDrop Technologies/Thermo Fisher Scientific, Wilmington, DE, USA). Reverse transcription and real-time qPCR were carried out as reported (6), using TaqMan gene expression assays for rat MOG, rat MBP, rat Egr2, mouse EPO and rat hypoxanthine phosphoribosyltransferase 1 (HPRT1, housekeeping gene), commercially available from Applied Biosystems/Life Technologies. For quantification, we used the comparative threshold cycle (ΔΔCt) method, following Applied Biosystems/Life Technologies guidelines. Results were normalized to HPRT1 expression and expressed as arbitrary units, using as a calibrator one of the control samples, as specified in the figure legends. Gene expression was considered undetectable when the threshold cycle for fluorescence detection was >38. Statistical significance was determined using the unpaired two-tailed Student t test or, for multiple comparisons, the Dunnett or Tukey-Kramer test.

**MOG Western Blot**
CG4 and CG4-EPOR cells were plated in poly-L-ornithine–coated petri dishes in GM, differentiated by switching to DM and treated with or without EPO. Cells were collected and centrifuged, supernatants were discarded and pellets lysed in 50 μL of lysis buffer without β-mercaptoethanol, as described (25). Protein concentration was measured using the BCA reagent kit (Pierce Biotec, Rockford, IL, USA), and 100 μg of cellular proteins were analyzed by polyacrylamide gel electrophoresis. Separated proteins were blotted using Trans-Blot Turbo Blotting System (Bio-Rad, Hemel Hempstead, Hertfordshire, UK) onto a polyvinylidenefluoride (PVDF) membrane. Membranes were blocked overnight with 2%...
casein (Fisher Scientific, Loughborough, Leicestershire, UK) in PBS, as reported (25). Antibodies used were: mouse monoclonal anti-MOG Z12 (kindly donated by Gareth Pryce, ICMS, Queen Mary University of London, UK), mouse anti-β-actin (clone AC-15, #A54541) (Sigma-Aldrich), goat anti-mouse IgG-HRP (#3697) (Santa Cruz Biotechnology, Heidelberg, Germany). Protein bands were visualized by exposing membranes developed with the ECL reagent (Amer sham/GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) to chemiluminescence film (Hyperfilm ECL) (Amersham/GE Healthcare Life Sciences).

**siRNA Transfection**

CG4-EPOR cells were plated in GM without penicillin and streptomycin. The next day cells were switched to DM and transfected with a Silencer Select Pre-designed Egr2-siRNA (s137448) (Ambion/Life Technologies) or control siRNA (Ambion/Life Technologies) using lipofectamine RNAiMAX (Invitrogen/Life Technologies), according to the manufacturer’s instructions. After a further 24 h, the cells were treated with or without EPO as indicated.

**RESULTS**

**EPO Does Not Induce Myelin Gene Expression in Wild-type CG4 Cells Not Expressing EPOR**

We first studied the effect of EPO on MOG and MBP gene expression in wild-type CG4 cells. Cells were differentiated for 5 d in differentiation medium (DM) with or without 80 ng/mL EPO. Differentiation alone induced both MOG and MBP gene expression by about nine-fold compared with undifferentiated cells (Figure 1). The addition of 80 ng/mL EPO during the differentiation period did not increase MOG or MBP gene expression compared with untreated cells (see Figure 1). However, EPO receptor (EPOR) was not detected in these cells by qPCR analysis (fluorescence threshold cycle for EPOR amplification was >38).

**EPO Induces Myelin Gene Expression in EPOR-Expressing CG4 Cells**

EPOR expression in genetically modified CG4-EPOR cells was verified by measuring the EGFP reporter expression by flow cytometry, which showed that 90.2% of cells expressed the transgene (Figure 2A). We then characterized the expression of EPOR in CG4-EPOR cells differentiated for 3, 6 and 9 d with or without EPO by Western blot, using anti-V5-tag antibodies. EPOR was detected in CG4-EPOR cells at all time points (Figure 2B), and its expression level was not affected by EPO.

Having established that EPOR was expressed, we then treated CG4-EPOR cells with different concentrations of EPO for 6 d during cell differentiation and analyzed MOG and MBP mRNA. As shown in Figure 3A–B, EPO, at concentrations of 8 ng/mL and higher, increased MOG and MBP gene expression by six- and three-fold respectively, compared with untreated cells. Although no further induction was found with doses of 80 and 400 ng/mL, the intermediate dose of 80 ng/mL was chosen for further experiments since this dose is the most used in neuroprotection studies (6). A time-course experiment investigating the effect of EPO at 80 ng/mL on MOG and MBP mRNA expression in CG4-EPOR cells is shown in Figure 3C–D. EPO increased MOG (see Figure 3C) and MBP (see Figure 3D) expression in differentiated cells by two- to four-fold at all time points analyzed.

**Induction of MOG Protein Expression by EPO**

As all the above experiments were performed analyzing the mRNA expression for myelin genes, we sought confirmation of the effect of EPO at the protein level. In preliminary experiments, we set up a reliable detection of MOG protein by Western blot. In the experiments shown in Figure 4A, CG4-EPOR and wild-type cells were differentiated with DM and treated with or without 80 ng/mL EPO. MOG protein expression was analyzed by Western blot at 3, 6 and 9 d of differentiation in CG4-EPOR cells and at d 9 in wild-type CG4 cells. Western blot for β-actin was used as a loading control (Figure 4).

The inducing effect of EPO on MOG protein was very clear at 6 and 9 d in CG4-EPOR cells. No induction was observed in wild-type CG4 cells, confirming the results obtained by qPCR. We then confirmed EPO-induced MOG protein increase in CG4-EPOR cells in an independent experiment (Figure 4B). MOG protein was detectable at d 9 of differentiation in the absence of EPO, but was induced at higher levels upon incubation with EPO for the whole differentiation period (d 0–9), confirming the results of the experiment shown in Figure 4A. This
Effect also was observed when the cells were incubated with EPO on d 0–7 of differentiation, whereas treatment with EPO only on d 0–3 was less effective (see Figure 4B). Therefore, EPO increased MOG expression also at the protein level in CG4-EPOR cells.

EPOR is Specifically Required by EPO to Induce Myelin Genes

Because in the experiments reported above we compared EPOR-expressing CG4 cells obtained using a lentiviral vector with wild-type cells, we wanted to ascertain that the observed differences were actually due to expression of EPOR and not to the vector itself.

We thus transduced CG4 cells with a vector containing only EGFP (CG4-EGFP), used as negative control. Cells were differentiated for 5 d with and without EPO and MOG gene expression was measured by qPCR. EPO did not have any effect in the low-EPOR clone R9, but induced MOG mRNA by 4.4-fold in the intermediate-EPOR clone R15 and 8-fold in the high-EPOR clone R21 and in the parental CG4-EPOR line (Figure 5C). Therefore, the MOG response to EPO was increased as the level of EPOR expression increased. Moreover, carbamylated EPO (CEPO), that does not bind to the homodimeric EPOR but is still cytoprotective and neuroprotective (18), did not have any effect on MOG gene expression in CG4-EPOR cells and in clone R21, showing that in this model induction of MOG likely occurs through the classical EPOR (see Figure 5C). In conclusion, MOG was not induced by EPO when EPOR expression was low (in CG4-EGFP cells [see Figure 5A] and in clone R9 [see Figure 5C]), and was induced by EPO, but not by CEPO (that does not bind the EPOR dimer), when EPOR expression was high (in CG4-EPOR cells and in clone R21 [see Figure 5C]). Therefore, in our in vitro system high EPOR expression was necessary and sufficient to induce MOG gene expression.

Egr2 is Induced by EPO but Does Not Mediate EPO-Induced Increase of Myelin Genes

We first investigated whether EPO induces Egr2 gene expression in CG4-EPOR cells. Based on our similar experiments where Egr2 expression was studied in EPOR-expressing neuronal B104 cells (6), we exposed differentiated CG4-EPOR cells to EPO for 1 h and Egr2 expression was measured by qPCR. EPO induced Egr2 by about 20-fold compared with untreated cells (Figure 6A).

To establish whether the observed upregulation of Egr2 has a role in the induction of myelin genes by EPO, Egr2 was silenced with an Egr2-siRNA, upon switching the cells to DM. After 1 d, when EPO-induced Egr2 expression was inhibited by 80% in Egr2-siRNA transfected cells (Figure 6B), cells were treated with or without EPO and MOG gene ex-
pression was measured at d 4 of differentiation. EPO significantly increased MOG mRNA in CG4-EPOR cells nontransfected (no siRNA) or transfected with a control siRNA (by 11-fold and 10-fold, respectively), as expected, and the EPO effect was not blocked by Egr2 silencing. In fact, in the presence of Egr2-siRNA, EPO still increased MOG expression by 8-fold (Figure 6C).

Of note, Egr2 silencing induced MOG by 2.7-fold as compared with nontransfected cells; no induction was observed using a control siRNA (see Figure 6C).

Therefore, EPO increased myelin gene expression in CG4-EPOR cells by a signaling pathway independent of Egr2. Moreover, knocking down Egr2 expression promoted expression of MOG with and without EPO stimulation, suggesting that Egr2 might be a negative regulator of MOG whose effect is overridden by EPOR activation.

**DISCUSSION**

This study shows that EPO increases the expression of myelin genes (MOG and MBP) in differentiated CG4-EPOR cells, and that this effect requires EPOR. The induction of both myelin genes was observed starting from an EPO concentration of 8 ng/mL, was already detectable at d 2 and still present at d 6 of differentiation. The requirement for EPOR was demonstrated by the lack of effect of EPO in non-EPOR-expressing wild-type cells or in cells transduced with EGFP only in a lentiviral vector, and by experiments showing that the effect of EPO is smaller in clones expressing lower EPOR levels. Interestingly, primary oligodendrocytes express low levels of EPOR under physiological conditions (15); however, EPOR is increased in the central nervous system in pathologies where EPO shows a protective effect (23,29).

To identify transcription factors implicated in the effect of EPO on myelination, we focused on Egr2. Egr2 is induced alongside with myelin genes in dorsal root ganglia cells treated with IL-6, which also stimulates myelination in vivo.
(30,31). Since EPO shares structural similarities with IL-6 (both are 4-α helical cytokines), and we observed that Egr2 is upregulated by EPO in models of cerebral ischemia (6), we asked whether Egr2 is involved in the induction of MOG and MBP by EPO in CG4 cells. However, silencing Egr2 gene expression in CG4-EPOR cells by an Egr2-siRNA did not inhibit the effect of EPO on myelin gene expression, indicating that Egr2 is not implicated in the action of EPO on oligodendrocytes. In fact, Egr2 silencing unexpectedly induced expression of myelin genes per se. Therefore, it may well be that Egr2 has an essential role in myelination only in the peripheral nervous system, where indeed its deficiency results in severe demyelination (27).

It is possible that other signaling pathways are implicated in EPO/EPOR-induced myelination. For instance, the survival kinase Akt has a key role in the neuroprotective action of EPO (32) and it has also been reported to promote myelination in the CNS (33).

CONCLUSION

The direct effect of EPO on myelination in oligodendrocytes reported here is further evidence that EPO, in addition to cytoprotective and antiinflammatory actions, can promote myelin repair. In terms of repair, increased expression of myelin genes might contribute to the overall effect of EPO, along with prevention of oligodendrocyte cell death (34,35), stimulation of neurogenesis and angiogenesis (4), and induction of neuronal plasticity (6). This might be relevant to demyelinating diseases, and it is interesting to note that EPO is effective not only in animal models of MS but also in a pilot clinical trial (36). Furthermore, induction of myelin genes, together with promotion of oligodendrogenesis, might play a role in EPO-induced neurological recovery in stroke as well as in neonatal hypoxic-ischemic brain injury, where oligodendrocyte damage is an important pathogenic component (5,37–39).

Our experiments using CG4 cells engineered to express EPOR provide a clear demonstration that EPOR can mediate at least some of the reparative effects of EPO in nonerythroid cells and that EPO-
EPOR interaction can induce effects other than the erythropoietic ones.

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DISCLOSURE
The authors declare they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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