Nonhematopoietic Erythropoietin Derivatives Prevent Motoneuron Degeneration In Vitro and In Vivo

Tiziana Mennini,[1] Massimiliano De Paola,[1] Paolo Bigini,[1] Cristina Mastrotto,[1] Elena Fumagalli,[1] Sara Barbera,[1] Manuela Mengozzi,[1] Barbara Viviani,[2] Emanuela Corsini,[2] Marina Marinovich,[2] Lars Torup,[3] Johan Van Beek,[4] Marcel Leist,[5] Michael Brines,[6] Antony Cerami,[6] and Pietro Ghezzi[1,6]

[1]Department of Molecular Biochemistry and Pharmacology, "Mario Negri" Institute for Pharmacological Research, Milan, Italy; [2]Laboratory of Toxicology and Centre of Excellence of Neurodegenerative Diseases, Department of Pharmacological Sciences, University of Milan, Italy; [3]Department of Neuropharmacology and [4]Department of Disease Biology, H. Lundbeck A/S, Valby-Copenhagen, Denmark; [5]Faculty of Biology, University of Konstanz, Germany; [6]The Kenneth S. Warren Institute, Kitchawan, NY, USA.

Chronic treatment with asialo erythropoietin (ASIALO-EPO) or carbamylated erythropoietin (CEPO) improved motor behavior and reduced motoneuron loss and astrocyte and microglia activation in the cervical spinal cord of wobbler mice, an animal model of amyotrophic lateral sclerosis, but had no effect on hematocrit values. ASIALO-EPO and CEPO, like the parent compound EPO, protected primary motoneuron cultures from kainate-induced death in vitro. Both EPO receptor and the common CD131 β chain were expressed in cultured motoneurons and in the anterior horn of wobbler mice spinal cord. Our results strongly support a role for the common β chain CD131 in the protective effect of EPO derivatives on motoneuron degeneration. Thus CEPO, which does not bind to the classical homodimeric EPO receptor and is devoid of hematopoietic activity, could be effective in chronic treatment aimed at reducing motoneuron degeneration.

Online address: http://www.molmed.org
doi: 10.2119/2006-00045.Mennini

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a degenerative disease of the upper and lower motoneurons leading to progressive motor dysfunction and death within 3 to 5 years from diagnosis (1). At present, the only drug approved by U.S. Federal Drug Administration for treatment of ALS patients is riluzole, which slightly prolongs patients' survival without clear effects on neurological symptoms (2,3). Thus the search for new therapeutic agents is greatly encouraged.

Erythropoietin (EPO), a hematopoietic growth factor, is neuroprotective in different models of neurodegenerative disease, including experimental autoimmune encephalomyelitis (EAE) (4,5), cerebral ischemia (6), and diabetic neuropathy (7). Its mechanism of action is not completely understood: in addition to its anti-apoptotic effect (6) EPO inhibits CNS inflammation (4,8), enhances neurogenesis in animal models of stroke and EAE (9,10), and augments BDNF expression in vivo and in vitro (9,11).

We have previously reported that in vitro EPO protects cultured motoneurons from serum-BDNF deprivation or long-term kainate exposure (6). The latter is a model of chronic excitotoxicity, used for in vitro studies because motoneurons are selectively vulnerable to activation of the AMPA receptor (12) .

Because chronic administration of EPO results in an increase of the hematocrit—which could have undesirable effects, for instance by increasing the risk of thrombosis—different nonerythropoietic molecules derived from EPO have been designed that retain the neuroprotective activities of EPO. One of these molecules, carbamylated EPO (CEPO), has proven effective in animal models of stroke, EAE, spinal cord injury, and diabetic neuropathy (13). Unlike EPO, CEPO does not bind the classical homodimeric EPO receptor (EPOR) (13), and its neuroprotective action appears to require the common β chain of IL-3/IL-5/GM-CSF receptor (also known as CD131) (14), which can functionally associate with EPOR (15). Another nonerythropoietic EPO derivative is asialo erythropoietin (ASIALO-EPO), which, although it binds to the classic homodimeric EPOR, has a short half-life in vivo and does not increase the hematocrit (an activity that requires persistent circulating levels of EPO) but also retains neuroprotective activities in vivo (16).

In the present study, we extended the in vitro studies on motoneuron cultures to ASIALO-EPO and CEPO, and tested the effect of treatment in an animal model of ALS, the wobbler mouse (17). The wobbler mouse carries a mutation of *Vps54* (18), a gene encoding for a vacuolar-vesicular protein-sorting fac-

Address correspondence and reprint requests to *Tiziana Mennini, Mario Negri Institute for Pharmacological Research, Via Eritrea 62, 20157 Milan, Italy. Phone: +390239014402; fax: +39023546277; e-mail: tiziana@marionegri.it*
Submitted June 16, 2006; accepted for publication July 13, 2006.

tor involved in vesicular trafficking, and is sensitive to treatments with riluzole (19) or neurotrophins such as BDNF (20), and thus is a useful animal model to test the effect of EPO analogs. The results suggest the possible in vivo relevance of the protective effect of EPO derivatives in preventing motoneuron degeneration.

MATERIALS AND METHODS

Materials

Brain-derived neurotrophic factor (BDNF) was a kind gift of Amgen (Thousand Oaks, CA, USA). Neurobasal medium, B27 supplement, and horse serum were obtained from Life Technologies, Gibco (Milan, Italy); glutamine from Seromed (Milan, Italy); and trypsin, bovine serum albumin, and poly-DL-ornithine from Sigma (Milan, Italy). Anti–nonphosphorylated neurofilament monoclonal antibody (SMI 32) was obtained from Sternberger Monoclonals (MD, USA); anti-IL-3/R β (sc 679) polyclonal antibody (raised against a peptide mapping at the N-terminus of the mouse IL-3 receptor β chain), anti-EPOR polyclonal antibody (sc-5624, against the N-terminus residue of human EPOR), and the sc-679 blocking peptide were obtained from Santa Cruz Biotechnology (CA, USA). Kainate was obtained from Tocris (Milan, Italy), and DPX mountant from BDH Laboratory. Vectastain ABC kit was obtained from Vector Laboratories (Burlingame, CA, USA). Recombinant human (rh) EPO was obtained from Ortho Biotech (Raritan, NJ, USA); rhCEPO and rhASIALO-EPO were synthesized as described earlier (13,16).

Animal Experiments

Procedures involving animals were conducted in conformity with the institutional guidelines that comply with national (D.L. no. 116) and international (EEC Council Directive 86/609; NIH Guide for the Care and Use of Laboratory Animals) laws and policies.

Homozygous wobbler mice and healthy littermates (NFR/wr strain; NIH, Animal Resources, Bethesda, MD, USA) were bred at Charles River Italia (Calco, Lecco, Italy). At arrival, the animals were housed in group cages containing 2 to 3 wobbler and 2 to 3 control mice under standard conditions (22 ± 1 °C, 60% relative humidity, 12-h light/dark schedule) had free access to food (Altromin, MT, Rieper) and water. Mice with heavy motor impairment had food available on the bottom of the cage and water bottles with long drinking spouts. After clear diagnosis of disease at 3 weeks of age based on phenotype analysis, wobbler mice and healthy littermates (control mice) were randomly assigned to the experimental groups, and treated intraperitoneally with EPO, ASIALO-EPO, CEPO (32 μg/kg) or vehicle (6.4 mL/kg) 3 times a week, until 12 weeks of age.

To evaluate the clinical worsening of wobbler mice, the following behavioral evaluations were done weekly by an operator that was blinded to treatments:

1. Paw and walking abnormality: Both the paw abnormality and the walking abnormality tests are observational. The operator assigns a score to these parameters, scaled from 0 to 4, on the basis of the severity of abnormalities. The paw position is graded as follows: 0, normal; 1, retracted digits; 2, curled digits; 3, curled wrists; 4, forelimb flexed to body. The walking pattern is graded as follows: 0, normal; 1, trembling (tremor without gait disturbance); 2, wobbling (gait disturbance); 3, curled-paw walking; 4, jaw walking (no use of front paw).
2. Running speed: Mice run over an inclined platform (75 cm long ramp inclined at one end to a height of 13 cm) stimulated with a gentle pressure on the tail (adverse stimulus). The running time is defined as the shorter time to reach the top of the platform from the bottom. Healthy mice rapidly improve their performances on the test until they reach the top of the platform in few seconds (1 to 3 s). On the contrary, wobbler mice need a longer time to reach the top of the platform. Moreover, these animals show a marked worsening of their performances due to the progressive muscular atrophy in the forelegs.
3. Grip strength: Mice are lifted by the tail and allowed to grasp with both forelegs to a horizontal bar, which is connected to a mechano-electric transducer (Basile). The grip strength of the front paws is measured at the point when the mouse releases the horizontal bar as a result of a gentle traction applied by the operator. Healthy mice can record values higher than 100 g, whereas values recorded by wobbler mice are very low (< 20 g) and drastically reduced during symptom progression. When animals are no longer able to grip the bar, grip strength is recorded as 0 g. Values of grip strength were normalized by dividing each value by body weight to control for weight differences between wobbler and healthy mice.

Because wobbler mice develop early and severe atrophy of forelegs without a clear impairment of hindleg muscles, the classical rota-rod test cannot be considered a reliable tool to evaluate the clinical progression in these mice.

At the end of treatment, 3 days after the last injection, half of the mice for each experimental group were killed by transcardiac perfusion with 4% paraformaldehyde in PBS, under deep anesthesia with chloral hydrate (intraperitoneal). Immediately after perfusion, biceps muscles, brain, and spinal cord were rapidly dissected and post-fixed for 4 h in the same fixative (4 °C). All the tissues were dehydrated and cryoprotected with serial steps in 10%, 20%, and 30% sucrose in PBS 0.1 M, pH 7.4, at 4 °C until they sank, frozen in *n*-pentane at –45 °C, and stored at –80 °C until analysis.

The other mice were killed by decapitation. Brain and cervical spinal cord were rapidly dissected, frozen on dry ice, and stored at –80 °C until analysis.

A few drops of blood were collected for hematocrit determination. For each

sample, triplicate values were recorded and the mean value used for statistical analysis.

Nissl Staining

For Nissl staining, cryostatic sections of cervical spinal cord (C2 to C6) were serially cut (30 μm thickness) and placed on gelatin-coated glass slides. Every third section was stained with 0.5% Cresyl violet, dehydrated through graded alcohols (70%, 95%, 100%, twice), placed in xylene, and coverslipped with DPX mountant (BDH Laboratory, Poole, UK) for light microscopy analysis. Motoneurons were identified based on their localization in lamina IX of the ventral horns and their large cell body size (> 30 μm). For all experimental groups, at least 50 sections of cervical spinal cord were evaluated for each animal; healthy motoneurons were counted in 1 side of each section. The mean of motoneuron number was calculated for each animal, and the values obtained were used for statistical analysis. The counting of Nissl-stained motoneurons was carried out by the same operator in a blinded fashion.

Immunohistochemistry for GFAP and CD11b

Sections were stained based on the avidin-biotin-peroxidase technique. Specimens were incubated in 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase. Sections were then exposed to primary antibodies diluted in blocking solution overnight at 4 °C, incubated with an appropriate biotinylated secondary antibody, processed with a Vectastain ABC kit, and developed using DAB. In all immunohistochemistry protocols, negative controls were performed by omitting the primary antibody, and this always resulted in minimal detected signal. The following antibodies were used: rabbit anti-bovine GFAP (1:4000; #Z 0334; Dako) and rat anti-mouse CD11b (1:10; clone 5C6; #MCA711; Serotec).

Motoneuron Cultures

Dissociated anterior horn cultures were obtained from the ventral horn of spinal cord of 15-day Sprague-Dawley rat embryos (Charles River, Calco, Italy) as previously described (6,21). Cell death was induced on the 6th day of culture by incubation for 48 h with kainate (5 μM). EPO or other cytokines (2.5 pmol/mL) or vehicle were added to the cultures 72 h before induction of cell death, and treatment continued for the 48-h exposure to glutamate agonists. After incubation with excitotoxins, the medium was discarded and the motoneurons were stained for nonphosphorylated neurofilaments (SMI 32) to assess their survival. Only the cells that were SMI 32 positive, with a good morphology, large somata, and well conserved axons, were counted across 4 sides of the coverslip.

Immunocytochemistry

Cells were fixed with paraformaldehyde 4% (wt/vol) in PBS for 40 min, permeabilized with Triton X-100 (0.2%) for 30 min, and blocked with FCS 10% (vol/vol) in PBS.

The incubation with primary antibodies (SMI 32, 1:9000; EPOR, 1:500) was carried out overnight in blocking solution at 4 °C.

Cells were washed; appropriate biotinylated secondary antibody (1:200), avidin, and biotinylated horseradish peroxidase macromolecular complex were added; and diaminobenzidine and H_2O_2 (6 mL/10 mL) were used to visualize the positive cells.

RT-PCR

To measure EPO and EPOR expression in mouse cervical spinal cords, 2 μg total RNA, extracted by Trizol (Invitrogen, Carlsbad, CA, USA), were reverse transcribed using the M-MLV reverse transcriptase enzyme (Invitrogen), and aliquots corresponding to 1/25 of the cDNA obtained were amplified by real-time PCR using the TaqMan gene expression assays for mouse EPO and EPOR and mouse β actin as housekeeping gene (Applied Biosystems, Foster City, CA, USA). All procedures were performed on the ABI PRISM 5700 Sequence Detection System (Applied Biosystems).

RESULTS

Studies In Vitro on Motoneuron Cultures

Figure 1 shows the effect of EPO (2.5 pmol/mL) on SMI 32–positive motoneurons in mixed neuron/glia cultures. Under basal conditions, 5-day treatment with EPO produced a clear neurotrophic effect, increasing the neurite outgrowth and the number and differentiation of motoneurons (Figure 1B). A similar effect was obtained in purified motoneurons (6) and was related to a decrease in spontaneous apoptosis, as judged from the reduction of the percentage of apoptotic nuclei and of the number of activated caspase 3– and 9–positive cells below control values (data not shown).

Under the same experimental schedule (3 days of pre-incubation followed by 48-h coincubation with kainate), EPO was also neuroprotective against kainate (5 μM) (Figure 1D). The effect of EPO was dose related between 0.25 and 2.5 pmol/mL (the dose that provided full protection), with ED_{50} about 1.25 pmol/mL.

The viability of motoneurons in mixed neuron/glia cultures was reduced by about 50% after treatment with 5 μM kainate and returned to control values (101 ± 35) in cells treated with 2.5 pmol/mL EPO (Figure 2A). The basal survival was increased above control values (128 ± 38), confirming the neurotrophic effect of EPO (Figure 2A). The same EPO concentration protected motoneurons from kainate toxicity even if added simultaneously with kainate (without preincubation) (Figure 2 C) or if present only during the 72-h pretreatment (Figure 2B). However, the maximum protective effect was obtained when EPO was present both in pretreatment and during kainate exposure.

We tested different nonerythropoietic derivatives, including ASIALO-EPO, which has high affinity for the EPORs, and CEPO, which does not bind to the classical homodimeric EPOR. Table 1 shows that both ASIALO-EPO and CEPO, tested at equimolar concentra-

tions under the same treatment schedule used for EPO, were active in preventing kainate-induced motoneuron death.

Immunoreactive EPORs were present in all cell populations of mixed neuron/glia cultures (Figure 3A) and in purified motoneurons (Figure 3C, D) and were not modified after treatment with kainate (not shown). The staining was present on cell bodies and arborizations, and seemed to be located both in the membrane and in the cytosol. To assess if repeated EPO treatments modify the expression or the distribution of EPORs on motoneurons, purified cultures were treated with EPO (2.5 pmol/mL) for 5 days. The staining revealed no difference in the intensity and distribution of EPORs in EPO-treated cells compared with vehicle-treated cells (not shown), indicating that EPO, in the experimental conditions tested, did not down- or up-regulate EPOR.

The common β receptor (CD 131) showed similar localization.

Studies In Vivo in Wobbler Mice

In a preliminary experiment, EPO, given to wobbler mice intraperitoneally at 32 μg/kg 3 times a wk for 6 weeks, markedly increased hematocrit (67 ± 4% and 46 ± 2.5% in EPO and vehicle, respectively, $P < 0.01$). This effect could worsen health conditions in the mice, thus interfering with the correct evaluation of motor behavior in treated mice. Thus, a pilot experiment was done to test the possibility that treatment with EPO analogs could be effective in wobbler disease. Four wobbler and 4 control mice were treated with CEPO, and their behavior was recorded as described in Materials and Methods section. The evaluation of the results (ANOVA) indicated a significant effect of treatment ($P < 0.001$) on all the behavioral tests, although the post-hoc tests did not reach statistical significance due to the low number of animals. On the basis of these results, we planned the second experiment using 10 animals in each experimental group.

Figure 4 shows that CEPO treatment significantly improves motor behavior, in particular in the grip strength and the running time tests, in wobbler mice over the time of observation. The behavioral effect was consistent with the reduction

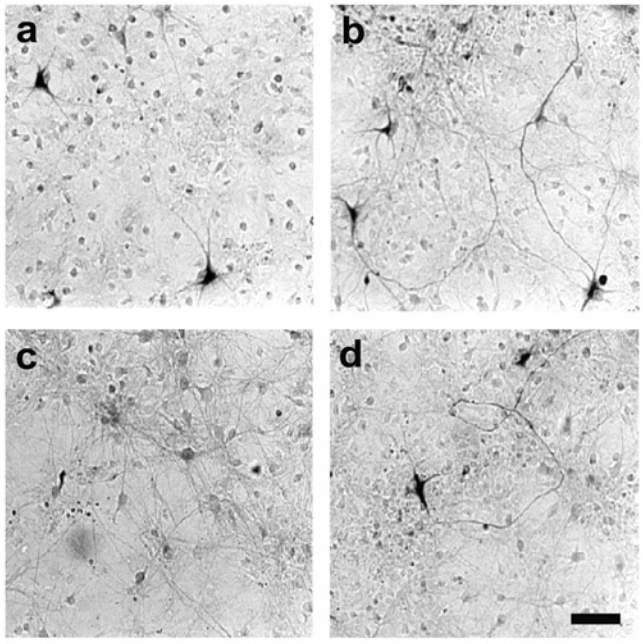

Figure 1. Neurotrophic and neuroprotective effect of EPO on SMI 32–positive motoneurons in mixed neuron/glia cultures. (A) Motoneurons in control cultures, well defined morphologically. (B) Control cultures 5 days after administration of EPO (2.5 pmol/mL) alone: large cell bodies with long axons and an increase of cell number can be seen. (C) Cultures treated for 48 h with 5 μM kainate. (D) Effect of EPO (added 3 days before and during kainate exposure) on cultures treated with kainate. Motoneurons were stained using an anti–nonphosphorylated neurofilament monoclonal antibody (SMI 32) as described in Materials and Methods. Scale bar = 100 μm.

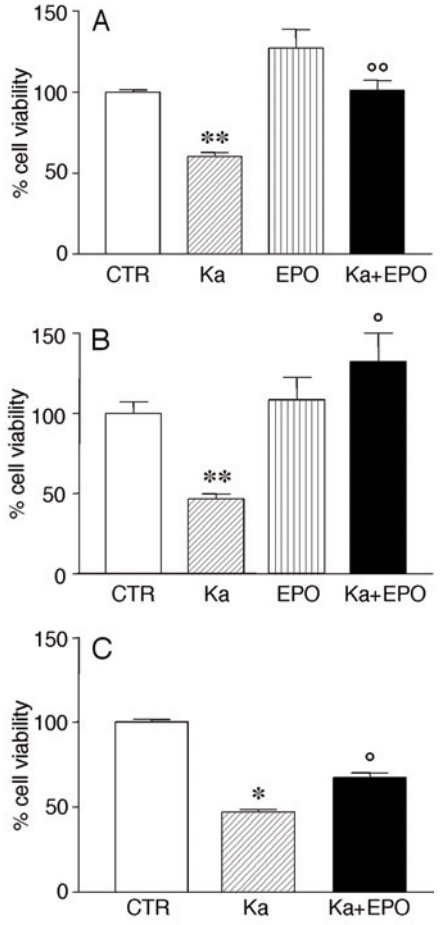

Figure 2. Effects of Epo against toxicity induced by kainate. Viability of SMI 32–positive motoneurons in mixed neuron/glia cultures after 48-h incubation with kainate (5 μM). When present, EPO (2.5 pmol/mL) was added simultaneously with the glutamate agonists (B), 3 days before treatment (C), or both (A). Data represent mean ± SD of 12 replications. *$P < 0.05$, **$P < 0.01$, different from controls; °$P < 0.05$, different from kainate + EPO.

Table 1. Effect of kainate treatment on motoneuron viability in the absence or presence of EPO derivatives.

	Motoneuron survival (% of controls)	
(No. replicates)	Without cytokine	With cytokine
ASIALO-EPO (12)	58 ± 11	91 ± 12[a]
CEPO (15)	42 ± 12	67 ± 19[b]

Viability of SMI 32–positive motoneurons in mixed neuron/glia cultures after 48-h incubation with kainate (5 μM). When present, cytokines (100 ng/mL) were added to motoneuron cultures 3 days before treatment and re-added with the glutamate agonist. Data represent mean ± SD. For both ASIALO-EPO and CEPO, fint $P < 0.001$. [a]$P < 0.001$, [b]$P > 0.01$, different from control motoneurons, 2-way ANOVA and Tukey's test.

in motoneuron loss in the wobbler mice treated with CEPO compared with vehicle (Table 2).

Wobbler mice treated with ASIALO-EPO also performed better than control mice in behavioral tests (Figure 4), particularly in the first part of the treatment, because the effect decreased over time. No significant effect of ASIALO-EPO was found on motoneuron loss (Table 2): only 2 of the 5 animals tested for Nissl staining had motoneuron number higher than 5 (the highest mean of vehicle-treated wobbler mice). These 2 mice also had the highest scores in behavioral tests, compared with the other 3 mice of the ASIALO-EPO group, thus confirming the correlation between motoneuron number and behavior.

CEPO and ASIALO-EPO did not increase hematocrit in treated mice; on the contrary, mice became anemic after 5 to 6 weeks of treatment, possibly owing to the production of antibodies against the EPO induced by the human recombinant proteins (hematocrit measured after 8 weeks of treatment: vehicle: 47 ± 5%; CEPO: 19 ± 3%; ASIALO-EPO: 21 ± 5%, both $P < 0.01$). However, the mice, although anemic, did not lose body weight and did not appear to suffer during the treatment, at least within the period covered by this experiment (8 weeks); more important, this effect is not likely to occur in humans.

Astroglia and microglia were activated in the cervical spinal cord of wobbler mice compared with controls. Treatment with CEPO or ASIALO-EPO reduced both astroglial and microglial activation in cervical spinal cord of wobbler mice (Figure 5). This reduced inflammation might be important in the neuroprotective effects and neurological benefits observed after CEPO and ASIALO-EPO treatment in wobbler mice.

The levels of endogenous EPO and EPOR, measured in cervical spinal cord of wobbler mice at the age of 6, 10, and 12 weeks, were not different from those found in healthy littermates (not shown).

The pattern of EPOR staining in the cervical region of healthy mice (Figure 6A) reveals a high immunoreactivity in the large-sized neurons of the anterior horn of spinal cord, mainly localized in neuronal cell bodies. Chronic treatment with EPO or its derivates did not modify the pattern and intensity of staining (not shown). In contrast, cervical sections from wobbler mice (Figure 6B) showed a reduced number of neurons having a strong immunoreactivity for EPOR and a parallel increase of staining in thin structures close to the ventral area. This may be due to the loss of motoneurons and the marked reactive gliosis occurring in the affected tissues.

Representative photographs showing immunostaining of the common β chain in the cervical spinal cord sections of healthy mice (Figure 6C) and wobbler mice (Figure 6D) show that the immunoreactivity is selective for neuronal cells, that almost all neurons are immunoreactive, and that the staining is more intens in the large-sized neurons of anterior horns. The selective expression of the common β chain in the cervical neurons is confirmed by the evidence that its loss of staining correlates with the marked loss of motoneurons, and the opposite effect of astrocyte and microglia proliferation observed in wobbler mice does not produce a parallel increase in immunoreactivity. No differences were observed after chronic treatment with EPO or its derivatives (not shown).

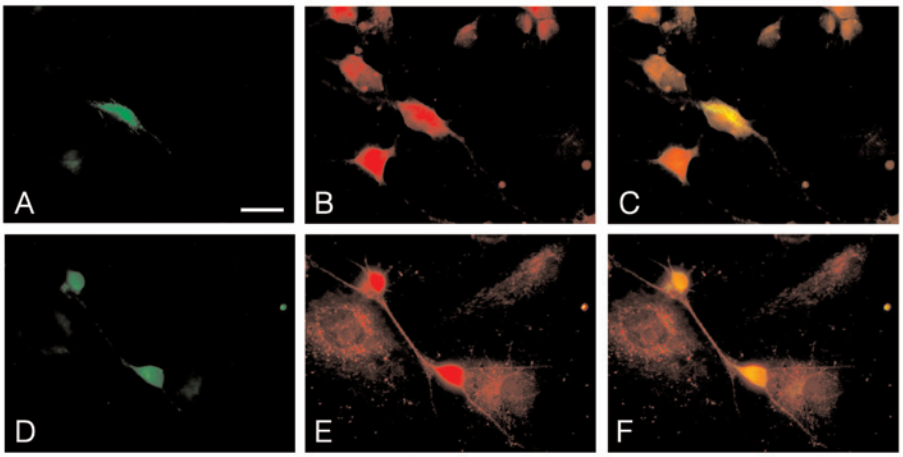

Figure 3. Motoneurons express EPO and CD131 receptors. Mixed neuron/glia cultures were double-stained with SMI32 (green, A, D) and with a specific antibody against EPOR (B) or the β chain common to IL-3, IL-5, and GM-CSF receptors (E). C and F represent the merged pictures. Coincubation with an excess of respective blocking peptides completely abolished the specific stain of anti-EPOR and anti–IL-3R β antibodies (not shown). Scale bar: 20 μm.

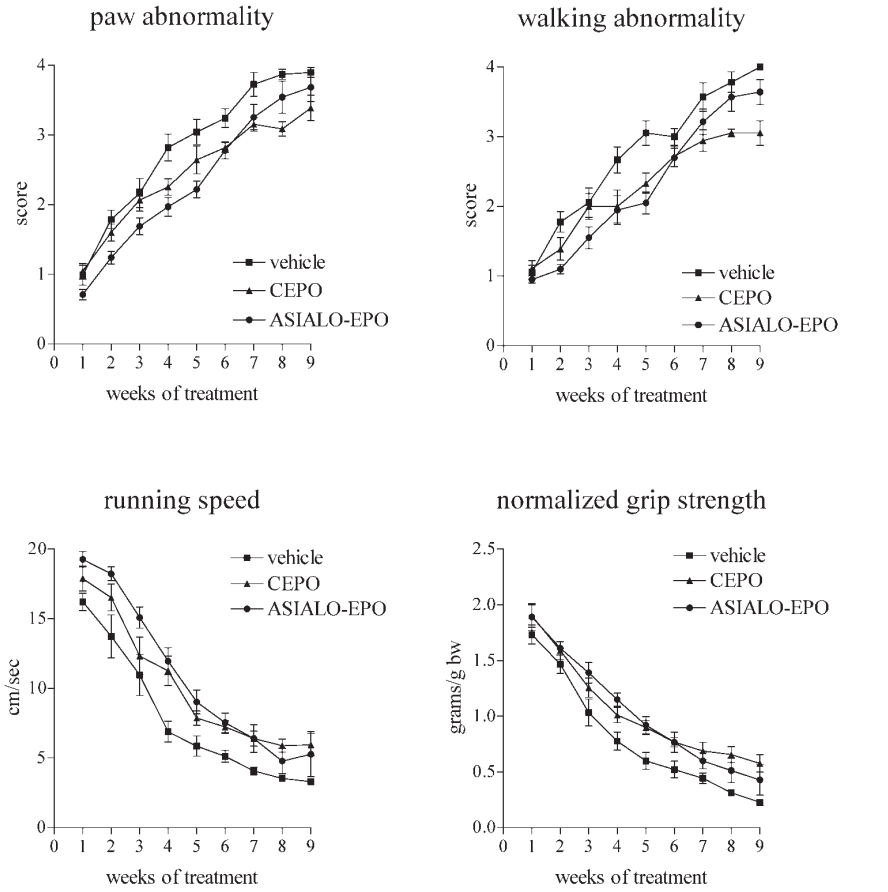

Figure 4. Behavioral scores of ASIALO-EPO and CEPO in wobbler mice. Drugs (32 μg/kg) were given intraperitoneally 3 times a week starting from 4 weeks of age. ■, vehicle; ●, ASIALO-EPO; △, CEPO. Each point represents the mean ± SD of 10 animals per group. Statistical analysis was done by 2-way ANOVA and showed significant effect of treatments ($P < 0.001$) for all the considered tests. CEPO and ASIALO-EPO effects were statistically different ($P < 0.05$) in the running time and grip strength tests.

DISCUSSION

The wobbler mouse, carrying a mutation of *Vps54* (18), is considered one of the most useful models for human motoneuron degenerative diseases, such as ALS and infantile spinal muscular atrophy (ISMA); unlike the transgenic mice carrying the human mutated form of *SOD1*, disease in the wobbler mouse is unrelated to the mutation responsible for a small proportion of the familial cases (22). An advantage of wobbler mice over the transgenic SOD1 mice is that, in the wobbler mice, the disease has an early onset and rapid progression (17), thus allowing shorter treatments that can minimize the production of antibodies using human recombinant proteins (like those tested in this study).

A preliminary experiment with 5-week EPO treatment in wobbler mice significantly increased the hematocrit. Because sustained high hematocrit causes endothelial damage and could increase susceptibility to vascular disease in mouse brain (23), in this study we tested the effect of 2 nonerythropoietic EPO analogs in wobbler motoneuron degeneration. Although not directly determined in this experiment, the hematocrit decrease observed in the 2 treatment groups likely arises from the formation of neutralizing antibodies that antagonize the effect of endogenous EPO. In other studies, we have consistently observed this phenomenon, which appears after 3 to 4 weeks of dosing, and we have definitively proven the formation of neutralizing antibodies produced against the human proteins administered (unpublished data).

CEPO has pharmacokinetic features (half-life, peak concentration, CSF distribution) similar to those of EPO but does not bind to the classical homodimeric EPOR, and is therefore devoid of hematopoietic activity (13). A major advantage of CEPO over EPO is the possibility of subchronic and chronic dosing without affecting hematocrit; protective effects of CEPO have already been described for spinal cord compression, diabetic neuropathy, and experimental autoimmune encephalomyelitis (13). Consistent with these assumptions, we show here that wobbler mice treated with CEPO have improved motor behavior and reduced loss of motoneurons in their cervical spinal cord. CEPO treatment also reduced markers of astrocyte and microglia activation. The improved behavioral scores, particularly the running speed, are even more impressive considering the grossly reduced hematocrits in the 2 treatment groups.

Chronic treatment with ASIALO-EPO was also effective in improving motor behavior in wobbler mice, although the effect of ASIALO-EPO in reducing motoneuron loss was not significant when the total number of treated mice was considered, but shows a clear correlation within motoneuron number and behavior for each mouse.

Table 2. Motoneuron number in the cervical spinal cord of wobbler mice.

	Control mice ($n=3$)	Wobbler mice ($n=5$)
Vehicle	16.5 ± 0.13	4.0 ± 0.77
CEPO	16.3 ± 0.05	7.8 ± 0.23[a]
ASIALO-EPO	16.3 ± 0.10	4.95 ± 1.37

Data are means ± SD. [a]$P < 0.001$ vs. vehicle and ASIALO-EPO, ANOVA and Tukey's test.

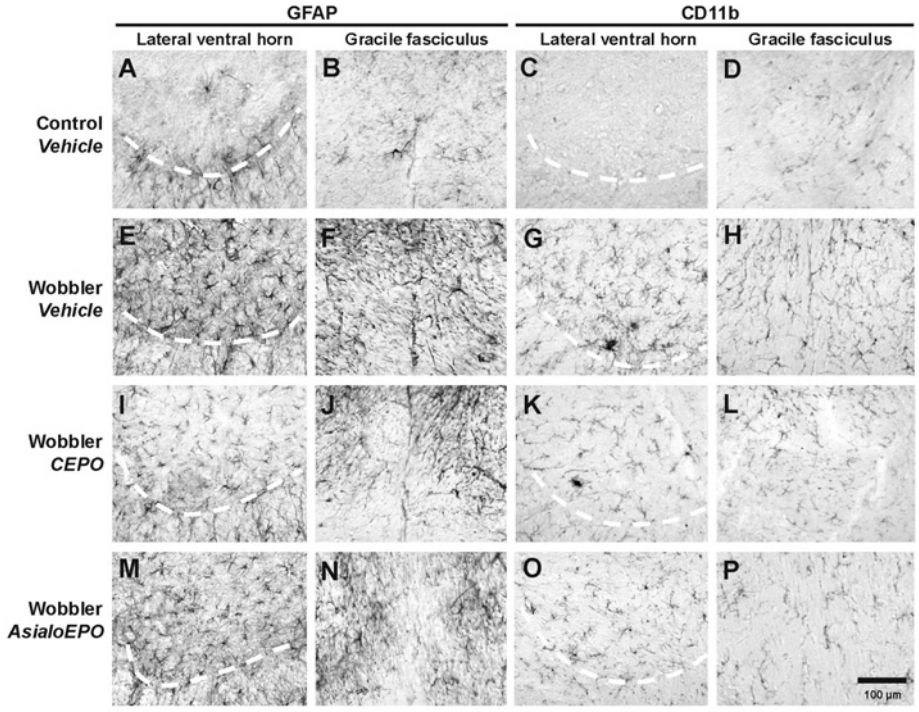

Figure 5. ASIALO-EPO and CEPO treatments both reduce the astroglial (GFAP) and microglial (CD11b) activation in cervical spinal cord of wobbler mice. Representative picture of GFAP and CD11b immunostaining in the cervical region of 12-week-old control (A-D) and wobbler (E-P) mice. Wobbler mice were treated with CEPO (I-L) or ASIALO-EPO (M-P) (32 μg/kg intraperitoneally 3 times a week) or vehicle (E-H) starting from 4 weeks of age. Scale bar: 100 μm.

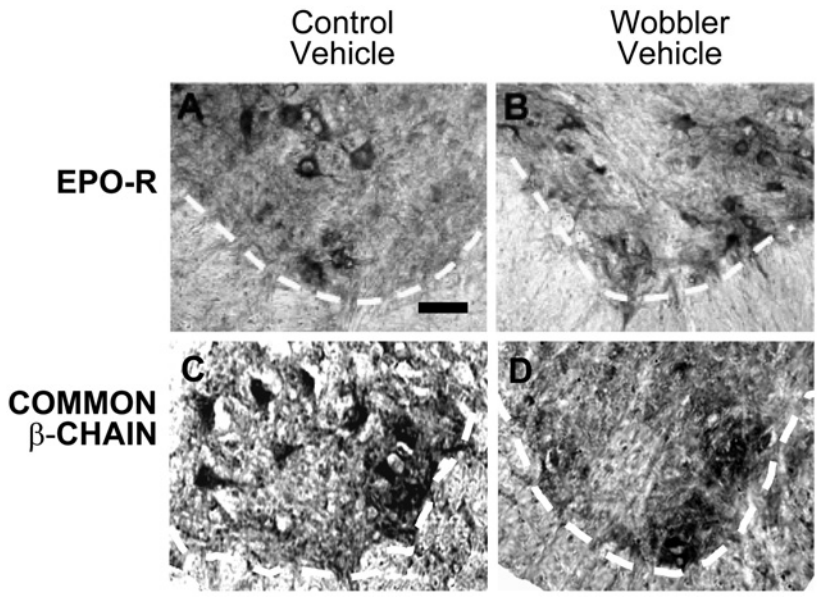

Figure 6. Motoneurons in spinal cord sections express EPO and CD131 receptors. Representative picture of EPO (A,B) and CD131 receptor (common β chain) (C,D) immunostaining in the cervical region of 12-week-old healthy (A,C) and wobbler (B,D) mice. Scale bar: 50 μm.

When tested in vitro, CEPO, ASIALO-EPO, and EPO were equipotent in protecting primary cultured motoneurons from death induced by stimulation of AMPA receptors with kainate, suggesting that the differences observed after chronic in vivo treatment were possibly related to the short half-life of ASIALO-EPO.

The effect of ASIALO-EPO and CEPO on wobbler mice cannot be related to a decrease in endogenous EPO expression in affected mice, because wobbler mice have expression of EPO comparable to that of healthy mice. Also, the expression of EPOR in wobbler mice is not different from that in healthy mice and not modified by chronic treatment with EPO analogs.

EPO has been shown to induce mRNA expression and production of biologically active BDNF in primary hippocampal neurons in vitro, leading to neuroprotection (11), and in vivo in mouse models of EAE (10) and stroke (9). BDNF expression is significantly increased in the ventral spinal cord of wobbler mice, at both early and advanced stages of the disease (24), possibly related to a compensatory mechanism activated to counteract cell processes that were involved in motoneuron neurodegeneration. Treatment with exogenous BDNF (20), or enhancement of endogenous BDNF induced by riluzole treatment (19), significantly slowed neuronal degeneration and impairment of motor function in wobbler mice. However, no significant increase of BDNF mRNA was evident in ASIALO-EPO– and CEPO-injected mice (data not shown).

The neuroprotective effect of ASIALO-EPO and CEPO in the wobbler mice was accompanied by a reduction of reactive gliosis, as evaluated by GFAP and CD11b immunostaining. Thus it is possible that a decrease in inflammation, which is present in the degenerating tissue, contributes to the effect of the EPO analogs, as was suggested for cerebral ischemia (5) and EAE (2).

If protection of motoneurons can be obtained in vitro and in vivo with CEPO,

the neuroprotective effects we observed with EPO and ASIALO EPO should not be related only to the classic homodimeric EPO receptor.

We found that both EPOR and CD131 were expressed in cultured motoneurons, implicating CD131 in the specific cytoprotective signal transduction of EPO/CEPO. In wobbler mice, the expression and the localization of EPOR and CD131 were not different from those of healthy mice and were not modified by the treatment; thus, they could mediate the neuroprotective effect of administered EPO derivatives.

In conclusion, our study suggests that CEPO, and, to a lesser extent, ASIALOEPO, could exert neuroprotective effects in a model of chronic motoneuron degeneration and reduce inflammation in the anterior horn of the spinal cord without increasing hematocrit levels. The mechanism by which these compounds act is still not fully clarified. The results indicate that these molecules could offer a potentially important therapeutic approach for ALS.

REFERENCES

1. Rowland LP. (1998) Diagnosis of amyotrophic lateral sclerosis. *J. Neurol. Sci.* 160 Suppl 1:S6-24.
2. Gordon PH. (2005) Advances in clinical trials for amyotrophic lateral sclerosis. *Curr. Neurol. Neurosci. Rep.* 5:48-54.
3. Miller RG, Mitchell JD, Lyon M, Moore DH. (2003) Riluzole for amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND). *Amyotroph. Lateral Scler. Other Motor Neuron Disord.* 4:191-206.
4. Agnello D et al. (2002) Erythropoietin exerts an anti-inflammatory effect on the CNS in a model of experimental autoimmune encephalomyelitis. *Brain Res.* 952:128-34.
5. Savino C et al. (2006) Delayed administration of erythropoietin and its non-erythropoietic derivatives ameliorates chronic murine autoimmune encephalomyelitis. *J. Neuroimmunol*. 172:27-37.
6. Siren AL et al. (2001) Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. *Proc. Natl. Acad. Sci. U. S. A.* 98:4044-9.
7. Bianchi R et al. (2004) Erythropoietin both protects from and reverses experimental diabetic neuropathy. *Proc. Natl. Acad. Sci. U. S. A.* 101:823-8.
8. Villa P et al. (2003) Erythropoietin selectively attenuates cytokine production and inflammation in cerebral ischemia by targeting neuronal apoptosis. *J. Exp. Med.* 198: 971-5.
9. Wang L, Zhang Z, Wang Y, Zhang R, Chopp M. (2004) Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats. *Stroke* 35:1732-7.
10. Zhang J et al. (2005) Erythropoietin treatment improves neurological functional recovery in EAE mice. *Brain Res.* 1034:34-9.
11. Viviani B et al. (2005) Erythropoietin protects primary hippocampal neurons increasing the expression of brain-derived neurotrophic factor. *J. Neurochem.* 93:412-21.
12. Carriedo SG, Yin HZ, Weiss JH. (1996) Motor neurons are selectively vulnerable to AMPA/kainate receptor-mediated injury in vitro. *J. Neurosci.* 16:4069-79.
13. Leist M et al. (2004) Derivatives of erythropoietin that are tissue protective but not erythropoietic. *Science* 305:239-42.
14. Brines M et al. (2004) Erythropoietin mediates tissue protection through an erythropoietin and common beta-subunit heteroreceptor. *Proc. Natl. Acad. Sci. U. S. A.* 101:14907-12.
15. Jubinsky PT, Krijanovski OI, Nathan DG, Tavernier J, Sieff CA. (1997) The beta chain of the interleukin-3 receptor functionally associates with the erythropoietin receptor. *Blood* 90:1867-73.
16. Erbayraktar S et al. (2003) Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 100:6741-6.
17. Duchen LW, Strich SJ. (1968) An hereditary motor neurone disease with progressive denervation of muscle in the mouse: the mutant 'wobbler.' *J. Neurol. Neurosurg. Psychiatr.* 31:535-42.
18. Schmitt-John T et al. (2005) Mutation of Vps54 causes motor neuron disease and defective spermiogenesis in the wobbler mouse. *Nat. Genet.* 37:1213-5.
19. Fumagalli E, Bigini P, Barbera S, De Paola M, Mennini T. (2006) Riluzole, unlike the AMPA antagonist RPR119990, reduces motor impairment and partially prevents motoneuron death in the wobbler mouse, a model of neurodegenerative disease. *Exp. Neurol*. 198:114-28.
20. Ikeda K et al. (1995) Effects of brain-derived neurotrophic factor on motor dysfunction in wobbler mouse motor neuron disease. *Ann. Neurol.* 37:505-11.
21. Comoletti D, Muzio V, Capobianco A, Ravizza T, Mennini T. (2001) Nitric oxide produced by non-motoneuron cells enhances rat embryonic motoneuron sensitivity to excitotoxins: comparison in mixed neuron/glia or purified cultures. *J. Neurol. Sci.* 192:61-9.
22. Rosen DR et al. (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 362:59-62.
23. Ogunshola OO, Djonov V, Staudt R, Vogel J, Gassmann M. (2006) Chronic excessive erythrocytosis induces endothelial activation and damage in mouse brain. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 290:R678-84.
24. Tsuzaka K, Ishiyama T, Pioro EP, Mitsumoto H. (2001) Role of brain-derived neurotrophic factor in wobbler mouse motor neuron disease. *Muscle Nerve* 24:474-80.