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SPECIAL ISSUE

Anna-Leena Sirén · Hannelore Ehrenreich

Erythropoietin – a novel concept for neuroprotection

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Abstract Neuroprotection as a means to prevent or oppose pathological neuronal loss in central nervous system disease of various pathophysiological origins represents a novel therapeutic approach. This approach is supported by extensive experimental evidence on cell culture and animal studies demonstrating beneficial effects of growth factors on neuronal survival and functional recovery. The clinical use of neuroprotective agents has been hampered by the toxicity of many of the compounds that showed promising therapeutic potential in animal studies. The focus of this review is on a novel neuroprotective approach with erythropoietin, a hematopoietic growth factor that: 1) is expressed in the human central nervous system, 2) is hypoxia-inducible, 3) has demonstrated remarkable neuroprotective potential in cell culture and animal models of disease, 4) has multiple protective effects (antiapoptotic, neurotrophic, antioxidant, angiogenic), and 5) is a clinically extremely well tolerated compound.

Key words Erythropoietin · Neuroprotection · Growth factor receptor · Brain · Human stroke

Abbreviations

BDNF – brain-derived neurotrophic factor
EPO – erythropoietin
EPOR – EPO receptor
ERK – extracellular regulated kinase
JAK-2 – Janus tyrosine kinase-2
MAPK – mitogen-activated kinase
PI(3)K – phosphatidylinositol-3-kinase
PKB – protein kinase-B
Stat5 – signal transducers and activators of transcription-5

Dr. A.-L. Sirén (✉) · H. Ehrenreich
Max-Planck-Institute for Experimental Medicine
Hermann-Rein Str. 3
37075 Goettingen, Germany
Tel.: +49-551/3899627
Fax: +49-551/3899670
E-Mail: siren@em.mpg.de

Neuroprotection as an approach to clinical neuroscience

Neuroprotection as a means to prevent or oppose pathological neuronal loss in central nervous system diseases of various pathophysiological origins, such as stroke, neurotrauma, neuroinflammatory and neurodegenerative diseases, represents a novel therapeutic approach that gains support from a wealth of cell culture and animal studies demonstrating beneficial effects of growth factors on neuronal survival and functional recovery. Moreover, neurodegenerative processes have been proposed to contribute to the pathophysiology of schizophrenia where neuroprotective interventions may be profitable at the early vulnerable stages of the illness when the early transition phase to psychosis begins (Woods, 1998; Lieberman, 1999; Velakoulis et al., 2000). The clinical use of neuroprotective therapies has been hampered by the toxicity of many of the compounds that show potential in animal studies (Group ACTS, 1996; Windisch et al., 1998; Serrano-Sanchez and Diaz-Armesto, 1998; Apfel, 1999; Jonhagen, 2000). The focus of this review is on a novel neuroprotective approach with erythropoietin, a hematopoietic growth factor that has not only demonstrated remarkable neuroprotective potential in cell culture and animal models of disease but that is a clinically extremely well tolerated compound used in millions of patients during the last decade (Jelkmann, 1994; Bauer, 1995; Nissenson et al., 1995).

Erythropoietin – a hematopoietic growth factor

Erythropoietin (EPO) was first identified as a hematopoietic growth factor (Jelkmann, 1994; Bauer, 1995). Circulating EPO is critical in the control of tissue oxygenation via regulation of production of erythro-

cytes (Bauer, 1995). The circulating EPO in the adult derives from kidneys whereas during fetal development the principal production of EPO takes place in the liver (Jelkmann, 1994; Bauer, 1995). The observation that EPO and its receptor are expressed in rodent and human brain tissue (Marti et al., 1996; Juul et al., 1998; Chin et al., 2000; Sirén et al., 2001 b) as well as by cultured neurons (Konishi et al., 1993; Bernaudin et al., 1999; Bernaudin et al., 2000; Chin et al., 2000; Lewczuk et al., 2000) and astrocytes (Masuda et al., 1994; Marti et al., 1996; Bernaudin et al., 2000), and that EPO exposure protects neuronal cells against a variety of different noxes (Konishi et al., 1993; Morishita et al., 1997; Sakanaka et al., 1998; Sadamoto et al., 1998; Bernaudin et al., 1999; Lewczuk et al., 2000; Brines et al. 2000; Sinor and Greenberg, 2000; Siren et al., 2001 a; Genc et al., 2001) expanded the biological role of EPO beyond hematopoiesis.

Oxygen-dependent expression of EPO/EPOR in the nervous system

EPO gene expression in most tissues, including brain, is regulated by hypoxia-inducible factor-1 that is activated by a variety of stressors, including hypoxia (Jelkmann, 1994; Ebert and Bunn, 1999). The inducibility of EPO gene expression is tissue specific with the strongest effect in kidney and brain, whereas in uterus, EPO mRNA is only induced in the presence of estrogen (Chikuma et al., 2000). Interestingly, in the brain, 17β-estradiol, which itself exhibits neuroprotective actions (Wise et al., 2001), enhances the effect of hypoxia on EPO mRNA expression (Chikuma, et al., 2000). We have demonstrated that both EPO and EPOR mRNAs are inducible by hypoxia in hippocampal neuronal cultures (Lewczuk et al., 2000). Hypoxia stimulated EPOR mRNA expression also in vivo: expression of EPO and EPOR mRNAs was evident in the hippocampus of rats exposed to hypoxia (8 % O_2, 2 h, Fig. 1). An induction of EPOR gene expression in the brain after anemic stress (Chin et al., 2000) and in the ischemic penumbra after middle cerebral artery occlusion (Sadamoto et al., 1998) has been reported. The induction of EPOR mRNA seems to be translated to increased synthesis of receptor protein as an increased neuronal expression of EPOR, in particular its expression in neuronal processes is evident upon hypoxia in cultured hippocampal neurons (Fig. 2A). This phenomenon might represent a common denominator of the neuronal response to hypoxic/ischemic stimuli across species since an identical pattern of increased EPOR immunoreactivity in neuronal fibers was seen in the infarcted human stroke tissue (Fig. 2B) (Sirén et al., 2001 b). The fact that the increased receptor expression was not down-regulated by EPO treatment in rat hippocampal neurons upon hypoxia alleviates the concern that EPOR-targeted clinical approaches might result in a non-responsiveness of the neuroprotective EPO system.

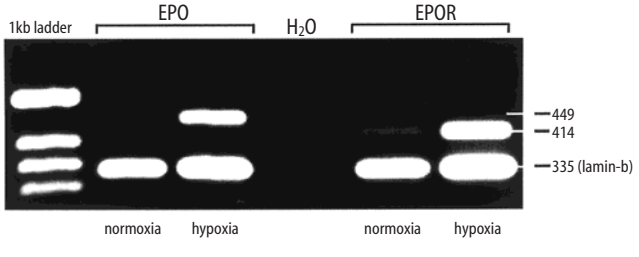

Fig. 1 Hypoxia (8 % oxygen, 2 h) induces EPO and EPOR mRNA expression in rat hippocampus. Housekeeping gene lamin b.

EPO and EPOR in brain: altered expression during development and after injury

Undifferentiated neuroepithelial cells in human embryos express both EPO and EPOR (Juul et al., 1999). The expression pattern becomes more distinct at later developmental stages with EPOR expression in astrocytes and EPO expression in neurons of the late fetal brain (Juul et al., 1999). In the adult human brain, only a weak expression of EPO and its receptor has been reported in neurons and astrocytes (Juul et al., 1999; Sirén et al., 2001 b).

We recently investigated whether EPO and its receptor (EPOR) are present in human brain after ischemia and/or hypoxia (Sirén et al., 2001 b). Acutely after stroke (< 5 days), EPO was seen in vascular tissue and inflammatory cells, EPOR in blood vessels and neuronal and astrocytic processes within the infarcts and the peri-infarct zone. In older ischemic infarcts (> 18 days) EPO and EPOR were strongest in reactive glia. Acute hypoxic brain damage was associated with strong EPO expression in blood vessels and that of EPOR in neuronal processes. We speculate that the increased expression of EPOR in the neuronal fibers may reflect an increased receptiveness of neurons to EPO upon metabolic stress. Prolonged hypoxia induced a persistent astrocytic upregulation of EPO production after stroke (Sirén et al., 2001 b) that may serve as a rapidly mobilizable source of EPO in the case of repeated hypoxic/ischemic events, i. e., constitute a correlate of aquired relative ischemic tolerance.

Neuroprotective properties of EPO

Several independent research groups have reported that EPO protects cultured neurons against glutamate toxicity (Morishita et al., 1997; Bernaudin et al., 1999) and reduces ischemic neuronal damage and neurological dysfunction in rodent models of stroke (Sakanaka et al., 1998; Sadamoto et al., 1998; Bernaudin et al., 1999; Brines et al., 2000). Systemic administration of EPO is neuroprotective not only in animal models of cerebral ischemia, but also in models of mechanical trauma, excitotoxic injury, neuroinflammation (Brines et al., 2000) and 1,2,3,6-tetrahydropyridine (MPTP)-induced

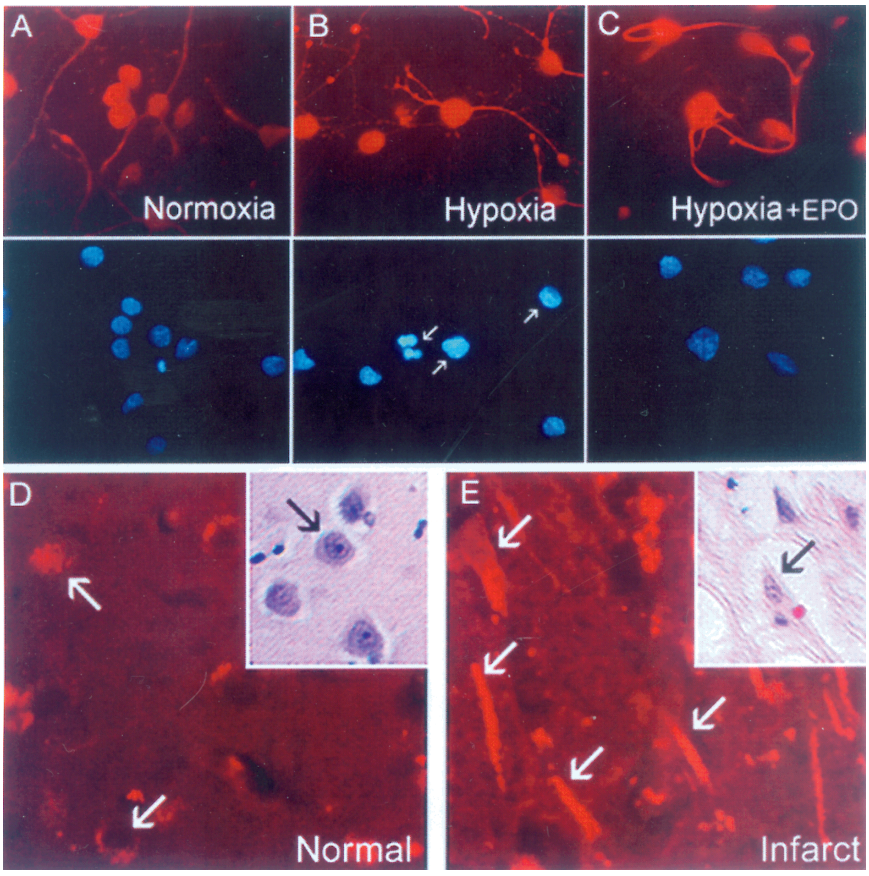

Fig. 2 Upper illustration: Hypoxia induces cell death and a shift to an increased neuronal expression of EPOR, in particular expression of the receptor in neuronal processes in cultured hippocampal neurons. Upper panels from left: EPOR-immunoreactivity in normoxic cultures (**A**), in hypoxic cultures (**B**), and in hypoxic cultures treated with EPO (**C**). Lower part of each panel depict counterstaining of each of the upper fields with a nuclear marker. Note light uniform staining of normal cell nuclei under normoxia. Under hypoxia, dying cells exhibit apoptotic condensation of chromatin and nuclear fragmentation (arrows) which EPO administration completely prevents. Final magnification 630X. Lower illustration: **D** Expression of EPOR immunoreactivity in human hippocampus. The photomicrograph represents a CA1-field depicting normal pyramidal neurons with cytoplasmic staining (white arrows). The inset represents hematoxylin-eosin staining of an adjacent section. The arrow depicts a normal pyramidal neuron. **E** Expression of EPOR immunoreactivity in a freshly infarcted human hippocampus. The photomicrograph shows a CA1-field depicting strong staining in the neuronal processes (white arrows). The inset represents hematoxylin-eosin staining of an adjacent section. The arrow depicts a shrunken pyramidal neuron. Final magnification 200X.

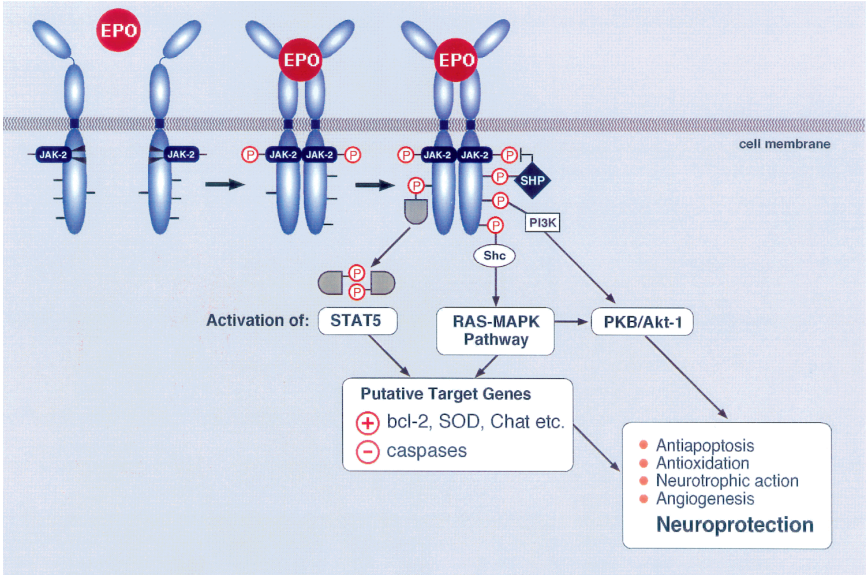

Fig. 3 Intracellular signaling mechanisms of the EPOR. **A** EPO binding to its receptor activates intracellular signaling through a conformational change that leads to transphosphorylation of the EPOR-associated kinase, JAK-2 (Janus tyrosine kinase 2). **B** Phosphorylation of JAK-2 allows tyrosine phosphorylation of signaling mediators Stat-5 (signal transducers and activators of transcription 5), PI(3)K-Akt/PKB (phosphatidylinositol-3-kinase-Akt/protein-kinase B), Ras-MAPK (Ras-mitogen-activated kinase). These pathways can alter the expression of putative target genes such as the antiapoptotic factor, bcl-2, the antioxidant enzyme SOD (superoxide dismutase) and proapoptotic caspases, or they can directly induce neuroprotective actions. Activation of the inhibitory Src-homologous tyrosine kinase (SHP) leads to termination of signal transduction via EPOR.

Parkinson syndrome in mice (Genc et al., 2001). Marked changes in EPO and EPOR gene expression have been reported to occur in brain tissue after ischemic injury (Sadamoto et al., 1998; Bernaudin et al., 1999). Specificity and biological relevance has been demonstrated by the observation that neutralization of endogenous EPO with soluble EPO-receptor (EPOR) prevents EPO-induced protection against glutamate toxicity (Morishita et al., 1997) and augments ischemic brain damage (Sakanaka et al., 1998). It thus appears that EPO plays a critical role in neuronal survival after ischemic injury.

Mechanisms of the protective effect of EPO

The EPOR belongs to the cytokine receptor superfamily for which substantial information concerning signaling biology exists (Ihle, 1995; Yoshimura and Misawa, 1998). Receptor activation follows after its homodimerization upon EPO binding which allows autophosphorylation of EPOR-associated JAK-2 (Janus-tyrosine kinase-2) (Fig. 3A). JAK-2 activation leads to phosphorylation of several down-stream signaling pathways including Ras-MAPK (Ras-mitogen-activated-kinase), PI(3)K (phosphatidylinositol-3-kinase), and the transcription factor Stat5 (signal transducers and activators of transcription) (Fig. 3B). The net effect of EPOR stimulation in the target cell is proliferation, inhibition of apoptosis and, in the case of erythroblasts, differentiation (Yoshimura and Misawa, 1998). We recently demonstrated that distinct intraneuronal signaling cascades that have been characterized in hematopoietic cell lines (Ihle, 1995) are functional in neurons (Sirén et al., 2001 a). Even more importantly, these pathways are crucial for the neuroprotective effect of EPO, since specific inhibitors of MAPK and PI(3)K pathways largely abolished the EPO-induced protection against hypoxia-induced cell death (Sirén et al., 2001 a). These signaling mechanisms have previously been shown to be involved in the cytoprotective effects of other growth factors such as that of brain derived neurotrophic factor in cortical (Hetman et al., 2000) and cerebellar neurons (Bonni et al., 1999), and that of vascular endothelial growth factor in a hippocampal cell line (Jin et al., 2000). Potential protective mechanisms that might be activated down-stream from both the EPOR-Ras-MAPK and EPOR-PI(3)K-Akt/PKB interaction, besides including antiapoptosis (Masuda et al., 1994), also include direct neurotrophic effects (Campana et al., 1998), antioxidation (Chattopadhyay et al., 2000) and angiogenesis (Ribatti et al., 1999).

An anti-apoptotic effect of EPO in neurons was also suggested by a recent study demonstrating that intraperitoneal administration of EPO after middle cerebral artery occlusion in rats dramatically reduced the volume of infarction 24 hours later, in concert with an almost complete reduction in the number of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling of neurons within the ischemic penumbra (Sirén et al., 2001 a). Evidence for an intrinsic antiapoptotic brain EPO release is provided by the findings that intrahippocampal infusion of soluble EPOR to neutralize endogenously produced EPO resulted in massive CA1 neuronal apoptosis after mild non-lethal global cerebral ischemia (Sakanaka et al., 1998). In cultured rat motoneurons, EPO inhibited apoptosis induced by serum deprivation or kainic acid exposure (Sirén et al., 2001 a). Protection in this system required pretreatment, consistent with the induction of a gene expression program and is sustained for 3 days without the continued presence of EPO (Sirén et al., 2001 a).

The actions of EPO are not limited to directly influencing cell survival, as EPO is trophic but not growth promoting in cultured neuronal cells (Konishi et al., 1993; Tabira et al., 1995; Campana et al., 1998; Siren et al., 2001 a). These findings suggest that inhibition of neuronal apoptosis underlies short latency protective effects of EPO following cerebral ischemia and other brain injuries. The neurotrophic actions implicate longer-latency effects as well. In this regard it is noteworthy that reactive astrocytes in older ischemic lesions express EPO immunoreactivity (Sirén et al., 2001 b) emphasizing the regenerative and secondarily preventive potential of this molecule in addition to its neuroprotective effects.

Other mechanisms that may contribute to the cytoprotective potential of EPO, besides antiapoptosis and trophic effects, include effects on intracellular calcium and antioxidation. In neuronal cell lines, EPO modulates cell viability (Koshimura et al., 1999) and increases resistance of neurons to glutamate toxicity via activation of voltage-gated calcium channels (Morishita et al., 1997). In this model (Morishita et al., 1997; Sakanaka et al., 1998) glutamate toxicity is not dependent on increased intracellular calcium concentration but seems to be mediated via nitric oxide-guided free radicals. Thus, it has been proposed that suppression of formation of nitric oxide-mediated free radicals or antagonism of their toxicity underlies the neuroprotective effect of EPO (Sakanaka et al., 1998). An antioxidant action of EPO is further supported by the findings that EPO protects against oxidative damage via inhibition of lipid peroxidation and by restoration of cytosolic catalase and glutathione peroxidase activities in erythrocytes (Chattopadhyay et al., 2000). Stimulation of neovascularization is another potentially protective mechanism activated by EPO that may help preserve perfusion in metabolically compromised tissue as brain endothelial cells express EPOR and respond to EPO treatment with proliferation (Yamaji et al., 1996). Moreover, angiogenesis after EPO treatment has been reported in vitro and in vivo (Ribatti et al., 1999).

Perspective: Evaluation of EPO as a neuroprotective agent in man

The experimental data reviewed above demonstrate multiple neuroprotective mechanisms of action of EPO. These findings together with the fact that EPO and EPOR are expressed in the human central nervous system (Juul et al., 1999; Sirén et al., 2001 b) and that EPO is an extremely well-tolerated compound, used in millions of patients (Jelkmann, 1994; Bauer, 1995; Nissenson et al., 1995), strongly support evaluation of EPO for neuroprotective therapy in a clinical setting. The therapeutic potential of this agent ranges from stroke and neurodegenerative diseases (Parkinson syndrome, Alzheimer disease, amyotrophic lateral sclerosis) to psychiatric applications such as schizophrenia, where neurodegenerative processes are likely to contribute to the pathophysi-

ology of the disease (Woods, 1998; Lieberman, 1999; Velakoulis et al., 2000). Based on the above reviewed encouraging findings of EPO as a neuroprotective agent in experimental models of brain injury and on our recent results on EPO and EPOR expression in human brain following ischemic/hypoxic brain damage (Sirén et al., 2001 b), we have launched a clinical stroke trial with recombinant human EPO (The Göttingen EPO-Stroke Trial, Principal Investigator Prof. Dr. Dr. H. Ehrenreich). The initial safety study has been completed with promising results demonstrating that intravenously administered EPO is able to enter the brain in acute human stroke victims and that the EPO treatment is extremely safe in stroke patients. To date, the study continues as the first randomized double-blind proof-of-concept study on EPO in stroke.

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