PANMINERVA MED 2008;50:185-92

# The use of erythtropoietin in cerebral diseases

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**Global and focal cerebral ischemia is followed by a secondary damage characterized by oxidative stress, excitotoxicity, inflammation and apoptosis. Erythropoietin (EPO) exerts antiapoptotic, anti-inflammatory, antioxidative, angiogenetic and neurotrophic properties. Its potential therapeutic role has been demonstrated in several animal models of cerebral ischemia and also in a clinical trial of ischemic stroke, so it could be considered an ideal compound for neuroprotection in ischemic stroke and in cardiac arrest. Intracerebral hemorrhage (ICH) is the least treatable form of stroke; the mechanisms involved in the secondary brain injury include hematoma mass effect, neuronal apoptosis and necrosis, inflammation. It has been demonstrated in an experimental ICH that EPO intervenes in the inflammatory process, reduces brain water content, hemorrhage volume and hemispheric atrophy, promotes cell survival, preserves cerebral blood flow, has antiapoptotic protective function against oxidative stress and excitotoxic damage. EPO can attenuate acute vasoconstriction and prevent brain ischemic damage in subarachnoid hemorrhage. The neuroprotective function of EPO has been studied also in traumatic brain injury: it reduces the inflammation and improves cognitive and motor deficits. The authors review some of the physiological actions of EPO in the physiopathology of ischemic and hemorrhagic stroke, subarachnoid hemorrhage and brain trauma, and its potential usefulness in the brain injured patient management.**

**KEY WORDS:** Erythropoietin - Cerebral arterial diseases - Neuroprotective agents.

Erythropoietin (EPO) is a growth factor that regulates human erythropoiesis. It is mainly produced by kidneys while during fetal development the liver is the organ most involved in its synthesis.

EPO belongs to the pleiotrophic cytokine super family and has multiple functions, among which neuroprotection.

EPO protects neuronal coltures, reduces neuronal death in animal models of ischemia, exerts neurotrophic properties, induces brain endothelial cell proliferation and stimulates neovascularization.[1] Its beneficial effects are not limited to the brain as EPO improves survival in a rat model of septic shock by means of a direct inhibition of inducible nitric oxide synthetase (iNOS) [2] and prevents apoptosis of myocardial cells exposed to extreme and prolonged hypoxia.[3]

EPO has a pro-proliferative effect on lymphocyte and megakaryocyte colonies;[4] it may also affect both their kinetics and functional capacity.

In addition to the autocrine/paracrine local actions, EPO is a circulating hormone, which can create crosstalk between the systemic and local systems. The principal actions of EPO are summarized in Table I.

Critically ill patients have a blunted erythropoietic response to physiologic stimuli as well as an impaired ability to respond to endogenous EPO.[5] Although it has been widely used and it is highly effective in a variety of clinical settings, little data exist regarding EPO use in critical ill patients.

The therapeutic potential of EPO ranges from stroke to schizophrenia.[6]

Evidences of efficacy in *ictus cerebri*, subarachnoid

Received on December 4, 2007.
Accepted for publication on June 17, 2008.

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TABLE I.—*Functions of EPO.*

| Production | Functions | EPOR localization | Potential therapeutic role | References |
|---|---|---|---|---|
| Liver | Erythropoietic (in the fetus) | Liver | | |
| Kidney | Erythropoietic | Bone marrow | Anemia | |
| Brain | Neurothrophic<br>Antiapoptotic<br>Antiinflammatory<br>Antioxidant<br>Angiogenic | Brain<br><br><br><br>Endothelium | Ischemic stroke<br>ICH<br>SAH<br>Traumatic brain injury<br>Autoimmune enchephalomyelitis<br>Schizophrenia | 6, 15-19, 23, 24, 26, 28, 30, 56, 57, 59-65 |
| Heart | Antiapoptotic | Heart<br>Antiinflammatory | Myocardial ischemia and infarction | 3 |

hemorrhage and trauma will be shortly reviewed in the following paragraphs.

## EPO in focal and global cerebral ischemia: a new potential therapy for ischemic stroke and cardiac arrest

The aim of the therapeutic neuroprotection is "to maintain/safeguard as much functions as possible of what is left".[7] Imitation of brain endogenous protective mechanism may be the key to successful approaches to neuroprotection, since endogenous mechanism can be the most efficient and well tolerated. Early reperfusion of the ischemic penumbra has become the mainstay of acute stroke therapy, but the possibilities to save cerebral parenchyma are time-dependent. In fact, the effectiveness of the fibrinolytic treatment is demonstrated within three hours from the onset of stroke symptoms, and is greater during the first 90 minutes.[8] Furthermore tissue inflammation and selective neuronal death or damage can preclude the full functional restoration of salvaged penumbra.[9]

In fact, ischemic brain injury involves immediate oxidative stress and excitotoxicity followed by inflammation and preprogrammed cell death (apoptosis).

The area around initial infarct, called "penumbra", once thought to be an ischemic area, is the field where inflammation and apoptosis take place. The resident inflammatory brain cells (microglia), are especially activated in response to ischemic insults. Endothelial cells lining the local cerebral blood vessels are stimulated to produce adhesion molecules, causing the migration of peripheral circulating leukocytes into the compromised brain tissue, an event that amplifies inflammatory signalling cascades.

Henrich-Noack *et al*. demonstrated that thrombin induced protease-activated receptors 1 and 3 (PAR1 and PAR3) are expressed on microglial cells in the penumbra after transient and permanent focal ischemia. They concluded that these receptors could be involved in thrombin-modulated initiation of post-ischemic inflammation.[10]

Hypoxic-ischemic insult induces hypoxia inducible factor-1 (HIF-1) and its target "survival genes", vascular endothelial growth factor, and erythropoietin. These genes are up-regulated in both ischemic brain injury and repair and in ischemic preconditioning.[11]

These recent data show the importance of new therapeutic strategies focused on molecular mechanisms in the pathogenesis of ischemic stroke including glutamate-induced excitotoxic damage, inflammation and apoptosis. These strategies of neuroprotection include salvaging neurons through the use of targeted pharmacotherapies, protecting neurons through preconditioning, and repairing neurons by enhancing neurogenesis.

Erythropoietin seems almost an ideal compound for neuroprotection since it is expressed in the human CNS, is hypoxia inducible, has demonstrated efficacy in experimental and clinical trials; is extremely well tolerated.

Erythropoietin is a major determinant of tissue oxygenation, performing this function through the regulation of red blood cells production by interaction with its specific cognate receptor that belongs to the cytokine receptor type I family (EPOR). In addition to its presence on cells of hematopoietic lineage, EPOR have

been found on a wide variety of non-erythroid cell types, including: liver stromal cells, endothelial cells, mesangial cells, smooth muscle cells, placental tissues as it is reviewed by Juul.[12]

EPO and its receptor EPOR are weekly but ubiquitously expressed in neural cells of the normal brain. Brines *et al*.[13] demonstrated that, in the normal brain, EPO and EPOR were located within the astrocytic endfeet surrounding the capillaries and on the surface of capillary endothelial cells. In contrast, larger vessels were generally not reactive for anti EPOR. These results suggested an anatomical basis for direct transport of EPO within the systemic circulation into the CNS in the absence of any neural insult. To test this hypothesis the authors injected into mice biotinylated recombinant human EPO. Five hours after this injection, peroxidase reaction product was observed surrounding capillaries extending into the brain parenchyma a distance 3-4 times that of the thickness of the capillary wall. These anatomical studies provided evidence of an active translocation of peripheral EPO across the blood-brain barrier.

EPO production by hypoxia occurs through HIF-1. Increased kidney production of EPO represents the main adaptive mechanism to hypoxia by the organism, augmenting the number of erythrocytes and thus tissue oxigenation. The neuroprotective effect of EPO is teleologically near to the erythropoietic one, since HIF-1 induced EPO production improves oxygenation and protects neurons, the most sensitive cells to hypoxic damage.

In a more recent paper Chavez *et al*.[14] investigated the differential contribution of hypoxia inducible factor (HIF) isoforms to the regulation of hypoxic EPO expression in cultured astrocytes. Using an *in vitro* model of oxygen-glucose deprivation (OGD), they studied the role of HIF-1α and HIF-2α in the generation of paracrine protective signals by astrocytes that modulate the survival of neurons exposed to OGD. They concluded that HIF-2α mediates the transcriptional activation of EPO expression in astrocytes, and this pathway may promote astrocytic paracrine-dependent neuronal survival during ischemia.

As in hematopoietic cells, the EPO neuroprotective action is mediated by the receptor activation after EPO binding (Figure 1).

EPO/EPOR binding allows Janus-tyrosine kinase-2 (JAK-2) activation which leads to phosphorylation of several downstream signalling pathways: among these, the Ras-mitogen-activated protein kinase and the phosphatidylinositol 3-kinase (PI(3)K) are crucial for the neuroprotective effect of EPO. Activated JAK 2 also leads to phosphorylation of STAT-5 and NF-κB. STAT-5 translocates to the nucleus and binds to DNA, promotes Bcl-xL and Bcl-2 expression (antiapoptotic genes), that inhibits caspase-3 activation and then apoptosis. On the other hand, NF-κB promotes the expression of neuroprotective genes such as the inhibitor of apoptotic protein and the superoxide dismutase (SOD) after it translocates to the nucleus. Anyway, a direct anti-inflammatory effect of EPO at cytokine network level cannot be excluded, since EPO reduces *in vivo* production of tumor necrosis factor (TNF) and augment production of IL-10 in blood cultures of hemodialysed patients.[15]

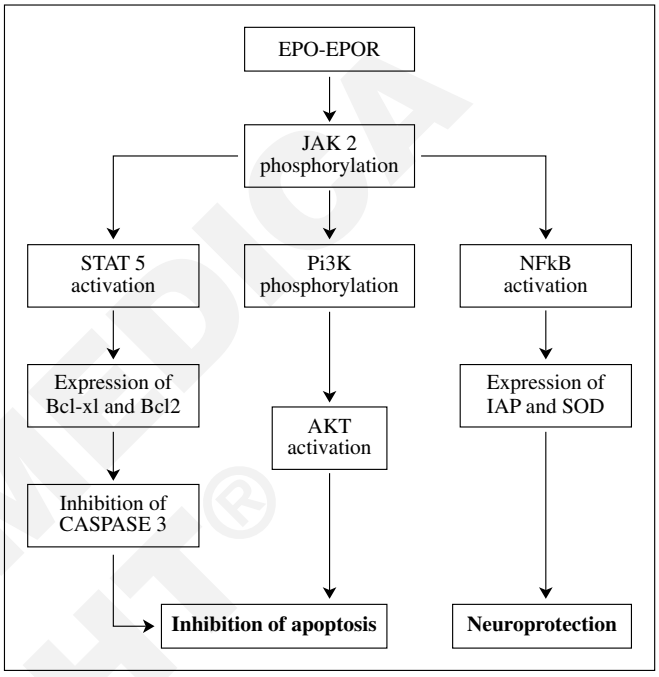


Figure 1.—Interaction EPO/EPOR and activated signalling pathways.

It has been demonstrated that EPO reduces TNF-α and IL-6 levels in an experimental model of autoimmune encephalomyelitis.[16]

In 1997, Morishita *et al*. demonstrated that EPO prevents glutamate-induced cell death in a dose-dependent manner: a very short incubation period with EPO (5 minutes or less) is sufficient to achieve a neuro-

protective effect against glutamate toxicity, although a longer incubation is needed for the cells to become resistant to glutamate.[17]

The neuroprotective role of EPO against the excitotoxic brain damage has been recently confirmed by Keller *et al*.[18]

In 1999, Bernaudin *et al*. indicated the potential role of EPO in focal permanent cerebral ischemia, induced in mice by permanent occlusion of the left middle cerebral artery.[19] Mice injected intraventricularly with recombinant mouse EPO showed a significant reduction in infarct volume.

The mechanism by which EPO acts as a neuroprotective agent in the CNS is still not well understood. Morishita *et al*. suggest that during ischemia EPO protects neurons from glutamate toxicity by activating $Ca2^{+}$ channels.[16] Sakanaka *et al*. observed that EPO, like platelet-derived growth factor, may increase the activity of antioxidant enzymes, such as SOD, glutathione peroxidase and catalase in neurons, protecting brain parenchyma from ischemic damage.[20] Another theory, proposed by Yamaji *et al*., is that EPO modulates angiogenesis in the ischemic brain, thus improving blood flow and tissue oxygenation in the border zone of the ischemic area.[21] EPO has angiogenic properties, that could be of benefit to improve microcirculation after a stroke.[22] The presence of a common precursor, the hemangioblast, which has hematopoietic and endothelial potential has been also demonstrated.[23]

This hypothesis has been recently demonstrated by Li *et al*.[24]

Siren *et al*. suggest that EPO may reduce ischemic areas in the brain by protecting endothelial and neuronal cells from apoptosis.[25]

Recent studies have suggested an alternative receptor for EPO neuronal effects, a heteroreceptor, consisting of the classical erythropoietin receptor (EPOR) and the common β receptor (βcR) for granulocyte macrophage colony-stimulating factor and interleukin-3 and -5 receptors.[26, 27]

In a very recent study Villa *et al*.[28] investigated whether carbamylerythropoietin (CEPO) and other nonerythropoietic EPO analogs could enhance functional recovery and promote long-term histologic protection after experimental focal cerebral ischemia. They concluded that postischemic intravenous treatment with nonerythropoietic EPO derivatives leads to improved functional recovery, which may be linked to their long-term effects against neuroinflammation and secondary tissue damage.

It has been recently shown that tissue protection does not depend on homodimeric receptor complex EPOR but, rather, utilizes a heterodimeric receptor complex, which is widely expressed in numerous organs, including the brain, heart, and kidney.[27]

As demonstrated by Tsai *et al*.[29] EPOR is essential for both embryonic neurogenesis and adult and post-stroke neurogenesis, but it is not essential for protecting neurons from ischemic injury suggesting alternative mechanisms for neuroprotective functions of EPO. CEPO and nonerythropoietic EPO derivatives are specific for heterodimeric receptor. It has been demonstrated that rhEPO administration in the rat increases systemic blood pressure, reduces regional renal blood flow, and increases platelet counts and procoagulant activities. In contrast, carbamylated rhEPO increases renal blood flow, promotes sodium excretion, reduces injury-induced elevation in procoagulant activity, and does not affect platelet production.[30]

The safety and efficacy of recombinant human EPO for treatment of ischemic stroke in man has been proven by a controlled clinical trial involving 53 patients mildly affected.[31] Patients received an intravenous infusion of EPO ($3.3\times10^{4}$ IU) within 8 hours after onset of symptoms, followed by a second and third dose at 24 and 48 hours (100 000 IU cumulative dosage). Magnetic resonance imaging evaluation of lesion size showed a superior course of EPO *versus* placebo group on day 18. The hematocrit and red blood cell counts increased but without reaching pathological levels.

The authors advice a larger trial with a longer follow-up period to confirm efficacy of EPO in stroke therapy.

### Epo in intracerebral hemorrhage

Intracerebral hemorrhage (ICH) is the least treatable form of stroke aggravated by high mortality and morbidity.[32]

Several mechanisms are involved in brain injury after ICH. They include hematoma mass effect, neuronal apoptosis and necrosis, inflammation. Whether secondary cerebral ischemia occurs in the brain after ICH remains controversial; thrombin and products deriving from blood clot lysis play an important role in brain damage after ICH.

Thrombin induces apoptosis in cultured neurons

and astrocytes.[33] As already reported PAR-1-3-4 (protease-activated receptors) are thrombin receptors founded in neurons and astrocytes;[34-38] they mediate some cellular activities of the pathological effects of thrombin,[39, 40] but how brain damage induced by thrombin receptors is mediated and which receptors are involved remains unclear. Perihematomal brain edema is smaller after systemic treatment with the thrombin inhibitor argatroban.[41]

Intracerebral infusion of hemoglobin and its degradation products can cause brain damage.[42, 43] Further, deferoxamine, an iron chelator, attenuates ICH-induced brain edema and hemoglobin-induced brain edema in animals.[42, 44] These results indicate that haemoglobin and its degradation products are toxic and are probably important causes of brain damage after ICH.

An inflammatory response in the surrounding brain occurs immediately after ICH and peaks several days later.[45, 46] Neutrophil infiltration develops within 2 days in rats, and activated microglial cells persist for a month.[47] Inflammation can be associated with the production of matrix metalloproteinases; these enzymes can cause distruption of the blood-brain barrier and secondary brain injury, but their role in ICH-induced brain injury is still controversial.[48, 49]

There is evidence that the complement cascade is activated in brain parenchyma after ICH.[50] Complement-related brain injury might result from formation of membrane attack complex and the classic inflammatory response. Formation of the complex can cause the development of a pore in the cell membrane leading to cell lysis.

Castillo *et al*.[51] found that plasma TNFα concentrations correlate with the degree of brain edema in patients with ICH.

Suppression of the inflammatory response has been suggested to reduce brain edema and tissue damage, and to improve functional outcomes in experimentally induced ICH.[52-55] EPO intervenes in the inflammatory process reducing the expression of TNF-α and IL-6.[16, 56]

The role of EPO in experimental ICH has been recently studied by Lee *et al*.[57] In this study EPO reduced brain water content, haemorrhage volume and hemispheric atrophy. EPO treatment promotes cell survival via STAT3 and ERK activations; it increases the levels of eNOS which plays an important role in the preservation of cerebral blood flow, has been shown an antiapoptotic function and may be protective against oxidative stress and excitotoxic damage.[58]

## Epo in subarachnoid hemorrhage

In an experimental study, EPO administered immediately after experimental subarachnoid hemorrhage (SAH) reduced the mortality rate and improved functional recovery: all animals treated with EPO survived at least 72 hours.[59]

Alafaci *et al*.[60] found that EPO has a neuroprotective effect in SAH: it can attenuate acute vasoconstriction and prevent brain ischemic damage. Experimental SAH induced reductions in the mean vascular area of 73.8% and 74% respectively in SAH and SAH placebo-treated animals, compared with a control group. EPO at a dose of 1 000 IU/kg in animals with SAH significantly reversed vasoconstriction (30% with respect to control) in the basilar arteries; this was confirmed by the low degree of folding in the internal elastic lamina. Histological analysis 24 hours after SAH showed a reduction in brain ischemic damage in animals that had received EPO. In particular, the analysis of cortical neurons showed that SAH plus EPO significantly reduced the amount of necrotic neurons compared with SAH and SAH plus placebo.

EPO may reverse acute vasoconstriction and reduce ischemic neuronal damage by enhancing the endothelial release of nitric oxide during the early stages of SAH. This would explain the findings of an acute decrease in cerebral nitric oxide levels after SAH, and a significant improvement in NO system activity after administration of EPO.[61-63]

## Epo in traumatic brain injury

In order to demonstrate the role of EPO in brain trauma, Brines *et al*.[13] used an animal model of concussive brain injury. Under anesthesia, animals received a blow of moderate severity. Recombinant human EPO was administred 24 hours before, 0, 3 and 6 hours after trauma and once daily for 4 additional days.

Animals not receiving EPO exhibited extensive cavitary injury 10 days after blow delivery, in contrast to animals receiving EPO 24 hours before. Histological examination of serial brain sections showed that for animals receiving saline alone the region immediately surrounding the necrotic core was densely populated with mononuclear inflammatory cells. In contrast, regions surrounding the necrotic core in EPO treated animals were characterized by a markedly reduced inflammatory infiltrate.

Adembri *et al*.[64] studied the protective effects of EPO in a model of mechanical trauma in organotypic hippocampal slices. Addition of recombinant human EPO or of the NMDA antagonist MK-801 immediately after the traumatic injury reduced hippocampal damage by approximately 30% when observed 24 hours later. At 48 hours after trauma, the protective effect of EPO was greater (by about 47%) and significantly more pronounced than that of MK-801 (28%). These results suggest that the neuroprotective activity of EPO is particularly effective against delayed, secondary post-traumatic damage.

In a study conducted by Yatsiv *et al*.[65] recombinant human EPO was administered at 1 and 24 h after TBI, and the effect on recovery of motor and cognitive functions, tissue inflammation, axonal degeneration, and apoptosis was evaluated up to 14 days. Motor deficits were lower, cognitive function was restored faster, and less apoptotic neurons and caspase-3 expression were found in rhEpo-treated as compared with controls. Axons at the trauma area in rhEpo-treated mice were relatively well preserved compared with controls. Immunohistochemical analysis revealed a reduced activation of glial cells in the injured hemisphere of rhEpo-treated animals compared with controls.

Finally, it has been recently shown that late administration of EPO (1 day after TBI) improves spatial memory deficits, and significantly increases the number of newly formed neurons in rats after traumatic brain injury.[66]

## Conclusions

EPO therapy has a positive effect in various experimental models of brain diseases. This hormone may have a direct effect on cerebral vessels and neuronal trophism, even if it is not growth promoting, protects against oxidative damage, dramatically reduces the volume of infarction inhibiting apoptosis. Some of EPO effects, such as embryonic and adult post-injury neurogenisis are mediated by binding to the homodimeric receptor complex EPOR, while neuroprotection is mediated by the heterodimeric receptor complex.

EPO treatment has been proved safe and effective in a stroke clinical trial.

There are not available data about its use in critical brain injured patients but the evidence reviewed above strongly support evaluation of EPO and nonerythropoietic EPO derivatives, like CEPO, as neuroprotective therapy in critical care settings.

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