The Use of Erythropoietin and its Derivatives to Treat Spinal Cord Injury

A. Mofidi, A. Bader and S. Pavlica*

Center for Biotechnology and Biomedicine (BBZ), Department of Cell Techniques and Applied Stem Cell Biology, University of Leipzig, Germany

Abstract: Spinal Cord Injury (SCI) is a complex process which leads to destruction of neuronal tissue and also vascular structure. After SCI many potentially toxic substances are activated and released into the injury site causing secondary degeneration.

Erythropoietin (EPO) is a possible therapeutic strategy to treat SCI. Over the last decade attention has been focused on the molecular mechanisms underlying its neuroprotective effects. A major concern expressed by clinicians is that besides its protective effects, EPO also demonstrates hematopoietic activity and increases the risk for thrombosis after the systemic administration of multiple doses of this glycoprotein. Recently, tissue protective functions of EPO have been separated from its hematopoietic actions leading to the development of EPO derivatives and mimetics. Neuroscientists are focusing on recombinant human EPO (rhEPO) and its non-erythropoietic derivatives, investigating their anti-apoptotic potential and anti-inflammatory function as well as their role in restoring vascular integrity. Carbamylated erythropoietin (CEPO) and asialo erythropoietin (AsialoEPO) are structural derivatives of EPO that have no effect on erythrocyte mass whereas they retain its neuroprotective effects. In this review article, we provide a short overview of the animal studies on rhEPO and its derivatives in experimental models of SCI.

Both the efficacy and the safety profile of EPO-structural and functional variants are still to be demonstrated in patients. Further clinical studies should reveal whether derivatives and variants of erythropoietin provide any benefits over the use of rhEPO in the treatment of spinal cord injury observed in the experimental studies.

Keywords: Erythropoietin, spinal cord injury, recombinant human erythropoietin, EPO-derivatives, carbamylated EPO, asialo EPO.

INTRODUCTION

In about 2500 BC, in the Edwin Smith papyrus, an Egyptian physician, accurately described the clinical symptoms of traumatic tetraplegia and revealed an awareness of the terrible prognosis with the advice “an ailment not to be treated” [1].

Currently the annual incidence of spinal cord injury (SCI) within the UK is about 10-15 per million of the population [2]. In USA, 11,000 new cases of SCI are reported each year, over half of which occur among individuals under 30 years of age [3] and the cost of medical care for patients with SCI in USA is estimated at over 4 million dollars per year [4].

Spinal Cord Injury is a complex process which leads to destruction of neuronal tissue and also vascular structure. After SCI many potentially toxic substances such as free radicals, phospholipases, lipid peroxidase, vasoactive eicosanoids and glutamate which can cause secondary degeneration are activated and released in injury site [5]. Secondary degeneration includes apoptosis and necrosis of oligodendrocytes and neurons [6]. The main strategy to promote recovery following Spinal Cord Injury is to reduce the secondary degeneration known to occur following the initial injury [7].

A high dose of methylprednisolone is the only drug of choice after SCI to limit secondary effects of trauma. However, it has some side effects and it is not effective enough [8]. Various other pharmacological agents for reducing and limiting SCI consequences are currently under evaluation in animals and humans. The most promising candidate is the erythropoietin (EPO) [1, 9-11].

Although the role of erythropoietin in both central and peripheral neurons is still under investigation, *in vitro* studies reported its neuroprotective and neurotrophic potential roles and motivated its application to nerve disorders [12-14]. Furthermore, many studies have shown beneficial effects of EPO on SCI [15-17].

ERYTHROPOIETIN

The French scientists Carnot and DeFlander in 1906 reported the presence of reticulocysis in normal rabbits injected with plasma from anemic ones and postulated a humoral factor named Hemopoietin [18, 19]. Hjort in 1936 [20], Krumdieck in 1943 [21] and Erslev in 1953 [22] confirmed the existence of this factor. In 1948 Bonsdorf and Jalavisto [23] first used the term erythropoietin. The gene encoding EPO is located on chromosome 7q11-q22 [24] and occupies a 5.4 Kb region [25]. It contains five exons and

*Address correspondence to this author at the Biotechnological Biomedical Centre, University of Leipzig, Center for Biotechnology and Biomedicine (BBZ), Department of Cell Technology and Applied Stem Cell Biology, Deutscher Platz 5, 04103 Leipzig, Germany; Tel: +49 (0)341 97-31353; Fax: +49 (0)341 97- 31 359; E-mail: Sanja.Pavlica@bbz.uni-leipzig.de

1389-5575/11 $58.00+.00

four introns coding for EPO pro-hormone of 193 amino acids [26]. Mature erythropoietin is a glycoprotein hormone which consists of 165 amino acids with a molecular weight of 30.4 kDa [27]. Human EPO is synthesized by peritubular capillary endothelial cells in adults and by hepatic cells in the fetus; a small amount is synthesized in the adult liver, too [28].

EPO has been originally characterized as a principal regulator of erythropoiesis by inhibition of apoptosis and stimulation of proliferation and differentiation of erythroid precursor cells [28]. Several evidences suggest its potential role other than erythropoiesis. EPO is a promising neuroprotective agent with anti-apoptotic properties. It can block the axonopathy development, too [29, 30].

EPO exerts its effects by binding to the erythropoietin receptor (EPOR). EPO specific receptors are expressed in different tissues. High levels of basal EPOR expression have been found in the spinal cord's neurons and glial cells, forskin cells [31-33]. EPOR belongs to the cytokine receptor type 1 super family. The corresponding gene consists of 8 exons and 7 introns located on chromosome 19p and encoding 507 amino acid with molecular weight 66 kDa [34].

Different mechanisms imply its neuroprotective effects including the activation of survival kinase pathways of transcription factors like nuclear factor κB (NF-κB)[35] which increase the neurotrophins expression, anti-apoptotic setting establishment [35], calcium channels activation [36, 37], antioxidant enzymes in neurons production [36] and angiogenesis promotion [38].

Digicayliogou *et al*. reported that EPO prevents apoptosis of CNS neurons by crosstalk between JAK-2 and NF-κB signaling cascade [39]. Moreover, Keswani and coworkers proposed that EPO not only has anti-apoptotic properties but also prevents axonal degeneration [29].

Binding of EPO molecule to EPOR leads to homodimerization and conformational change in EPOR, which turns active EPOR associated Janus family Tyrosin Kinase 2 (JAK-2). Phosphorylation of distal parts of the receptor follow by JAK-2 activation leads to phosphorylation of several downstream signaling pathways [40], including Ras-mitogen activated protein-kinase (MAPKs), phosphatidylinositol-3-kinase (PI3K)/AKt, transcription factor STAT-5 (signal transducers and activators of transcription-5), NF-κ B and Calcium channels [39, 41-43]. Activation of JAK-2 leads to phosphorylation of NF-κB inhibitor, subsequent translocation of the transcription factor NF-κB from the cytoplasm to the nucleus, and eventual transcription of neuroprotective genes. Further, EPO appears to prevent apoptotic injury through an AKt-dependent mechanism [44]. Akt has been shown to block cellular apoptotic degradation through inhibition of glycogen synthase kinase-3β activity [45] which has significant role in regulation of apoptosis in neurons. Astonishingly, a recent study revealed that EPO provided significant neuroprotection following traumatic brain injury even in the absence of EPOR in the neural cells [46].

Several studies reported that EPO and EPOR have an essential role in neurogenesis. Tsai and colleagues suggested that endogenous erythropoietin and erythropoietin specific receptor play an important role in mammalian nervous system development and repair [47]. Unfortunately, endogenous levels of EPO are insufficient for neuroprotection and repair after injury. This problem is surpassed with the administration of exogenous EPO. It has been reported that treatment with EPO leads to reduction of neurodegeneration and prevention of neuronal death after SCI [11, 48-51].

In 1977, EPO was isolated for first time from the urine of anemic patients by Miyake *et al.* [52]. Identification of EPO amino acid sequence enabled scientists to synthesize EPO's DNA. Probes for EPO gene isolation and cloning opened the road for the production of recombinant human EPO (rhEPO) [19]. Therapeutically used rhEPOs are manufactured in living mammalian cells transfected with human EPO cDNA. There are a few erythropoietin products now approved in the EU, USA and Eastern Europe/Asia. All manufactures use their own cell lines applying their own fermentation and purification techniques. Nowadays healthcare providers in EU encourage the use of biosimilar hematopoietic medicines due to their lower costs [53]. Physicians should be aware of their clinical applicability since both endogenous EPO and "biosimilar rhEPOs" exhibit several isoforms that differ in the glycosylation patterns and, hence, biological activity.

Animal model studies indicated the potential effect of rhEPO in SCI treatment enhancing neurological and functional recovery [32, 54-56]. The administration of rhEPO has been suggested as an exogenous activator for EPOR to reduce neurodegeneration after SCI. The specific inhibitors of MAPKs and PI3K are largely inactive after binding of EPO to EPOR [41]. Further, through the cross talk pathway between JAK-2 and NF-κB signaling transduction system, EPO protects neurons from excitotoxic insults and nitric oxide (apoptosis) by induction of JAK-2, the suppression of IκB and translocation of NF-κB [39]. Several evidences showed anti-apoptotic effects of NF-κB in neurons involving the activation of Akt-1, Bad phosphorylation and Bcl-XL upregulation [57, 58] (Fig. **1**). Akt-1 is activated by PI3K [41] leading to stabilization of mitochondrial membrane potential, prevention of the release of cytokine C and caspase 1, 3 and 8 activities which contribute to neuronal survival [59]. Released cytochrome from mitochondria leads to activation of a family of executioner cysteine proteases ensuring the activation of cell demise [58], inducing DNA fragmentation and the exposure of membrane phosphatidylserine residues [60].

EPO induced neurogenesis by increasing the biological activity of BDNF. EPO triggered BDNF's m-RNA expression, activation of Ca^{2+} channels and recruitment of Ca^{2+} sensitive transcription factor CREB [35]. Another neuroprotective aspect of EPO is a significant inhibition of leukocytes infiltration and reduction of the level of pro-inflammatory cytokines such as TNFα, IL-6 and MCP-1 in site of SCI [61].

A major concern expressed by clinicians is that besides the tissue/neuronal protective effects, EPO demonstrates also hematopoietic activity and increases the undesired effects such as risk of thrombosis after the administration of multiple dose of this glycoprotein [62]. Recently, investigators have focused on rhEPO and its non-erythropoietic derivatives, exploring their anti-apoptotic potential and anti-

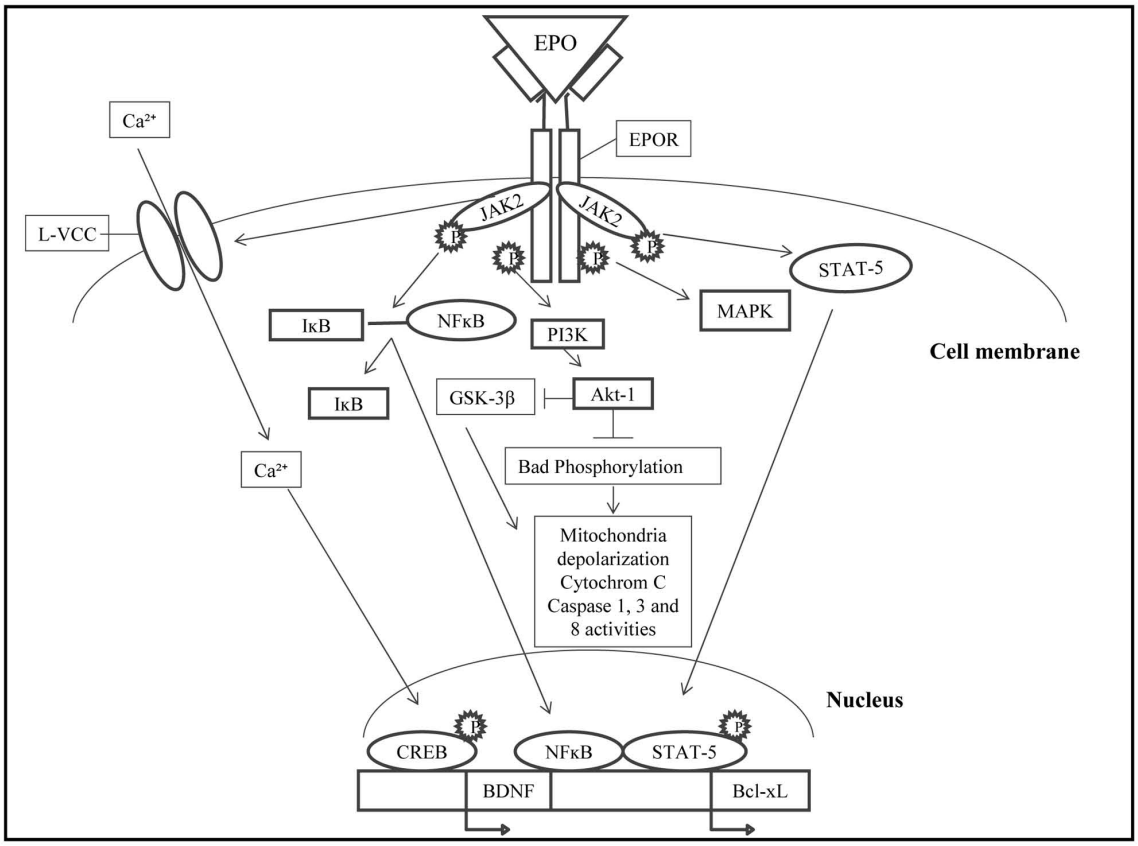

Fig. (1). The specific inhibitors of MAPKs and PI3K are largely inactived after binding of EPO to EPOR [41]. Through the cross talk pathway between JAK-2 and NF-κB signaling transduction system, EPO protects neurons from excitotoxic insults and apoptosis by induction of JAK-2, suppression of IκB and translocation of NF-κB. NF-κB's antiapoptotic effects in neurons involve the activation of Akt-1, Bad phosphorylation and Bcl-XL upregulation [57, 58]. Akt-1 is activated by PI3K [41] and leads to stabilization of mitochondrial membrane potential, prevention of the release of cytokine C and caspase 1, 3 and 8 activities which contribute to neuronal survival [59]. EPO induced neurogenesis by increasing the BDNF's biological activity. EPO triggered BDNF's m-RNA expression, activation of Ca^{2+} channels and recruitment of Ca^{2+} sensitive transcription factor CREB [35].

inflammatory function as well as their role in restoring vascular integrity [10, 63]. There are two key concepts in their production, plasma half-life and interaction with different receptors [48, 64].

CARBAMYLATED EPO

In vitro studies demonstrated that erythropoiesis and effective red cells production requires the continuous presence of EPO, whereas a brief exposure is sufficient for neuroprotection [49]. This is one distinguishing feature between erythropoiesis and neuroprotective pathways. If brief exposure of EPO leads to activation of neuroprotection pathway, a short-life EPO could be translocated into "tissue beds" to initiate neuroprotection by EPOR activation [65].

The regions of EPO that interact with EPOR have been identified after intensive studies [66, 67] (Fig. **2**). These domains include portion of helices A and C as well as helix D and the loop connecting helices A and B [66]. Manipulation of amino acids residues by chemical or mutational modification within these two regions of EPO abolished its interaction with EPOR. Interestingly, the manipulated EPO molecules are not erythropoietic but retain potential tissue protective properties [10, 67]. Brines and colleagues demonstrated in a variety of models that a peptide fragment within helix B (amino acids residues 58-82) exhibited tissue protective activities similar to the full EPO molecule [67]. Following high affinity binding of EPO to its hematopoietic receptor, helix B and parts of the AB and CD loops face the aqueous medium away from the homodimer binding sites. These regions are not modified by carbamylation since they do not contain lysine residues [67].

Carbamylated EPO (CEPO) is one of the first engineered EPO derivatives. The mature erythropoietin protein has eight lysine residues. Thus, in addition to N-terminal alanine, EPO provides nine primary amino groups for carbamylation [68]. CEPO is EPO that is chemically modified by carbamylation (all lysine residues were transformed to homocitrulline) and does not bind to the homodimeric EPOR, thus, lacks an erythropoietic effect. But it has neuroprotective effects in model of peripheral nerve damage [7, 10, 69]. *In vitro* studies showed that CEPO has no erythropoietic effects on TF-1 [70], human erythroleukemia cell line [71] and UT-7/EPOR [72, 73]. Leist and colleagues reported that the sub-chronic administration of CEPO at pharmacological doses in rodents doesn't have any erythropoietic effects while it effectively and significantly improved motor neuron function within 21

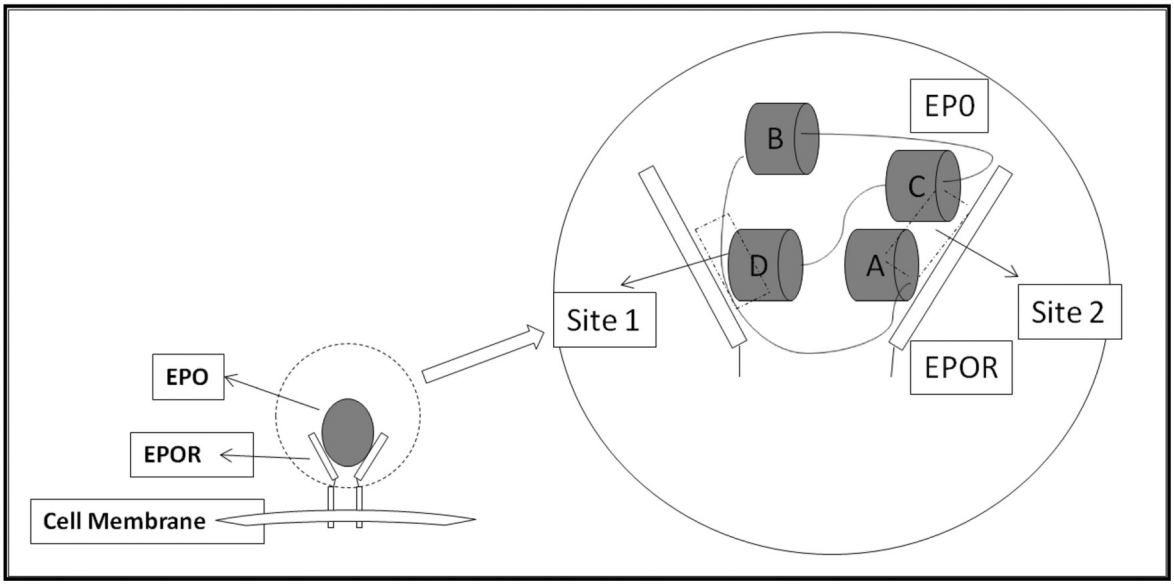

Fig. (2). EPO molecule contains four helices: A, B, C and D [66]. Manipulation of amino acids residues by chemical or mutational modification within site 1 and site 2 of EPO abolished the interaction between EPO and EPOR (a part of figure modified from [64]).

days of the injury [7, 73] Further, CEPO has been demonstrated to improve motor behavior and reduce loss of motor neurons as well as markers of astrocyte and microglia activation in cervical spinal cord of wobbler mouse [74]. Mennini *et al.* reported that CEPO is effective for reducing motor neuron degeneration [74]. Interestingly, King *et al.* showed that CEPO administration produced a marked decrease in spinal cord lesion size (36 %), apoptosis and axonal damage [7]. They assumed that neuroprotective effect of CEPO following SCI may be due to increased proliferation of Schwann cells, too. In addition, Xiong and co-workers in a recent study demonstrated that CEPO significantly increases neural progenitor cell proliferation and promotes neural progenitor cell differentiation into neurons [75].

The cellular mechanisms responsible for the neuroprotective effects of CEPO have not been fully elicited. Brines and co-workers suggested that both EPO and CEPO may bind to a receptor complex that includes the EPOR and the β common receptor (βCR), a common signal-transuding component of the interleukin-3, IL-5 and granulocyte macrophage colony stimulating factor receptors (GM-CSF) [73, 76]. Further, it has been reported that βCR knockout mice following SCI did not show the same locomotor recovery as wild type mice in response to treatment with EPO or CEPO. In addition, the neuroprotective effects of EPO and CEPO are mediated by receptor that includes a βCR subunit as well as an EPO receptor component [73]. As CEPO signals only through binding to the βCR subunit of EPOR- βCR heteromeric complex, the neuroprotection mechanism of CEPO could differ from EPO neuroprotection, for example CEPO does not effectively activate the STAT-5 or Jak-2 transcription factors, unlike EPO [10]. So, direct stimulation of anti-apoptotic /neuroprotective pathways in neurons is not likely to be the only involved in attenuating spinal cord trauma following CEPO administration. Indirect effects, as an enhancement of the endogenous EPO system, increased proliferation of EPO-realising Schwann cells and oligodendrocyte precursors may also contribute to its neuroprotection. Increased expression of both EPOR and βCR subunit in neurons may indicate that compromised neurons near the injury site in the spinal cord may be particularly responsive to EPO or CEPO treatment [7].

The method for CEPO production is described in a patent from Warren Pharmaceutical, Inc [77]. This patent claims the invention related to an efficient method of carbamylating erythropoietin resulting in CEPO that has less than 10% free primary amines on the lysine and the N-terminal amino acids. The carbamylation of the parent EPO molecule (rhEPO) is a difficult process and requires a well controlled chemical reaction and specific conditions to produce CEPO with significant tissue protective and neuroprotective abilities. According to the Warren Pharmaceutical Inc patent, the purified EPO concentration to be used in the reaction is about 2.2mg/ml but a range of 1.1-2.5 mg/ml is appropriate. Furthermore, the reaction is to be carried out for 14-24 h in a pH range of optimally 8.79- 9.2 and at a temperature range of 36-38°C. Also, the potassium cyanate concentration range should be 0.5-1.5 M and a sodium borate buffer concentration at 0.1- 0.5 M should be used as chemical condition. Despite to specific conditions required to produce CEPO, there are possibilities for the formation of isoforms during reaction process that may either be over- or under-carbamylated allowing some of the starting erythropoietin to retain unwanted hematopoietic activity [78]. It is yet difficult to determine whether isoforms of CEPO will exert erythropoietic effects in species other than rodents.

ASIALOERYTHROPOIETIN

Mature EPO has an average carbohydrate content of about 40%. Its oligosaccharide chains can be modified with terminal sialic acid residues with *N*-linked chains typically having up to four sialic acids per chain and *O*-linked chains having up to two sialic acids [68]. Indeed, EPO isoforms differ from each other by their sialic acid content. Extensive

carbohydrate content of EPO contributed to its serum half-life of 5-6 hours after intravenous injection. So, brief signaling by this long-lived cytokine is impossible *in vivo*. Removal of the terminal sialic acid moieties of EPO results in an analog with a very brief half-life (2 minutes in blood) [48]. EPO variant with low content of sialic acid, probably similar to that produced endogenously in brain during hypoxia, is a promissive neuroprotective agent. So, variants of desialated erythropoietin (named as AsialoEPO, Neuro-EPO, rhEPOb) are non-erythropoietic and tissue-protective EPO-like compounds with a very short plasma half life potentially have significant advantages over rhEPO to prevent the polycythemia [10, 79]. Interestingly, recent findings highlighted the treatments with nasal dosing of EPO with low content of sialic acid without hematological side effects [79].

AsialoEPO was produced by total enzymatic desialylation of rhEPO, and its short half life after intravenous administration is about 1-2 h [7, 65]. It is able to bind to the classic homodimer EPOR (in contrast to CEPO and other EPO-mutants) and it does not increase the hematopoietic whereas it retains neuroprotective activities *in vitro* and *in vivo* [65]. It has been shown that AsialoEPO exerted neuroprotective effects in an animal model of SCI and peripheral neuropathy without increasing erythrocyte concentration [48]. AsialoEPO administration 24 h before SCI in rats has been found to be followed by glial activation as determined by an increase in GFAP immunoreactivity [48]. Further, it is as effective as rhEPO in motor neuron function improvement after SCI, but does not significantly increase BDNF mRNA in neuronal cells [78]. Mennini and colleagues reported that administration of AsialoEPO in wobbler mice improved the motor behavior without significant effects on reducing motor neuron loss [74]. Decreased inflammation in the degenerating tissue may also contribute to the neuroprotective effect of this EPO analog [74].

EPO FUNCTIONAL VARIANTS

Erythropoietin is one of the most prominent hypoxia-inducible factor-1 (HIF-1) target genes. HIF-1 plays an important role in neural progenitor cell propagation and dopaminergic differentiation. This heterodimeric transcription factor consists of an oxygen-regulated α subunit and a constitutively expressed ß subunit [80, 81] (Fig. **3**). Although HIF-1 is regulated mainly by oxygen tension through the oxygen-dependent degradation of its α subunit, *in vitro* it can also be modulated by cytokines, hormones and genetic alterations [80]. In addition to hypoxia, various non-hypoxic stimuli can stabilize HIF-1alpha and influence the transcription of HIF-regulated genes. Recently, beneficial effects of various hypoxia mimetics (deferoxamine, ciclopirox olamine, dimethyloxallyl glycine and cobalt chloride) have been shown to enhance *in vitro* proliferation, neurogenesis and dopaminergic differentiation of human fetal mesencephalic neuronal progenitor cells in ambient (21%) oxygen [81]. So, the additional compounds that act by induction of EPO gene expression such as HIF-stabilizers (proly hydroxylase inhibitors, 2-oxoglutarate analogues, TM 6008/ TM 6089, FG-2216/FG-4592) can be considered as alternatives to EPO for stimulation of erythropoiesis as well as for neuroprotection and re-

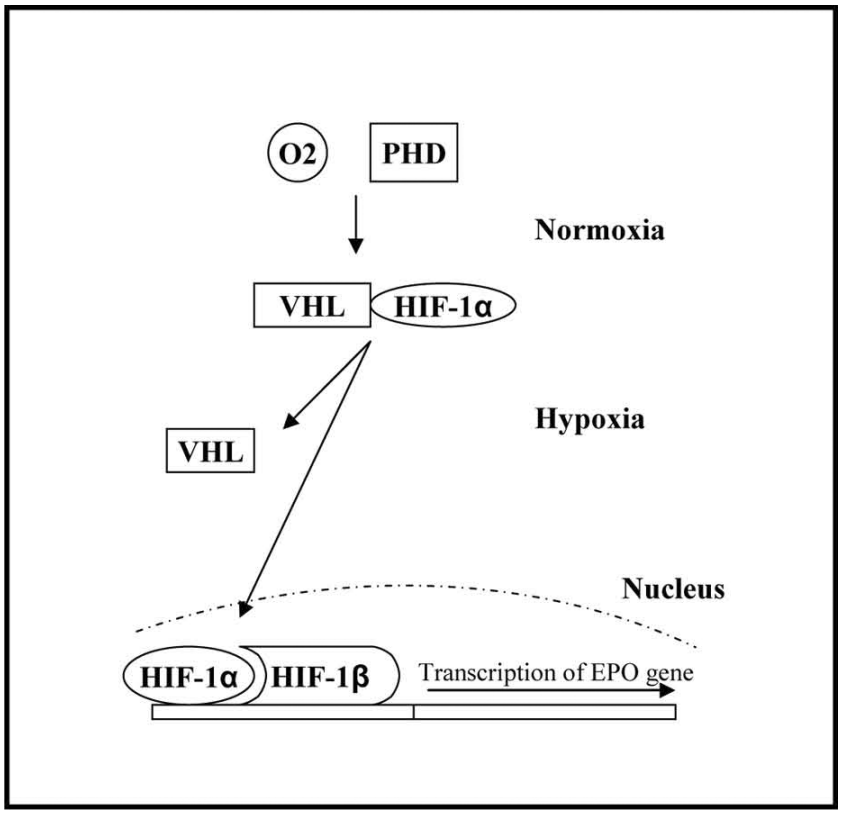

Fig. (3). In normoxia condition PHD site-specifically hydroxylates the HIF-1α subunit and binds to VHL suppressor protein [81]. Under hypoxic conditions, HIF-1α translocates to nucleus and promotes the transcriptional activation of EPO gene [80].

pair [82-84]. However, HIF-stabilizers induce the expression of other genes than EPO that may cause unwanted effects, too. At present, the advantages and disadvantages of agents capable of inducing a wide spectrum of HIF-genes are still unknown. The question whether functional EPO variants have any benefits over the use of rhEPO in the treatment of SCI and other neurological diseases should be further addressed before they enter clinic.

CONCLUSIONS

Immediate administration of EPO after spinal cord injury could lead to neural- and tissue-protection. However, the systematic administration of multiple doses of this cytokine is still a clinical concern due to risk of thrombosis. There are two key concepts to decrease the thrombosis risk: production of brief half life EPO analogs or production of EPO analogs, which are unable to interact with EPOR.

According to several studies mentioned in this review article, CEPO and AsialoEPO are as effective as EPO and have significant neuroprotective effects. They decrease the secondary damages following SCI and improve locomotor activity. At the molecular level, asialoEPO behaves exactly as EPO in contrast to CEPO which exhibits a new mode of action by engaging an alternative βCR receptor. The valuable advantage of these EPO derivatives over rhEPO is that they do not have an effect on erythrocyte mass. We should consider that EPO may also cause alterations in platelet function and hemostasis, too. Lack of these potential complications should be confirmed in its variants. Further, non-neuronal effects of EPO that promote survival, such as promotion of angiogenesis or a marked increase of blood flow may be absent in EPO derivates and variants. Thus, both their efficacy and safety profile are still to be demonstrated in patients.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Medical Faculty of the University of Leipzig.

The authors have declared that no competing interests with respect to this manuscript and funding exist.

ABBREVIATIONS

AsialoEPO	=	Asialo Erythropoietin
BDNF	=	Brain Derived Neuronal growth Factor
CEPO	=	Carbamylated Erythropoietin
HIF-1	=	Hypoxia Inducible Factor-1
EPO	=	Erythropoietin
EPOR	=	Erythropoietin Receptor
JAK-2	=	Janus family Tyrosin Kinase 2
PHD	=	Prolyl 4-hydroxylase domain
SCI	=	Spinal Cord Injury

REFERENCES

[1] Matia, G.K.; Birbills, T.A. Erythropoietin in Spinal cord Injury. *Eur. Spine J.,* **2009**, *18*, 314-323.

[2] Swain, A.; Grundy, D. In *ABC of Spinal Cord Injury*; Grundy, D.; Swain, A.; BMJ books B.V: London, **2002**; vol. *4*, pp. 1.

[3] Brines, M.; Cerami, A. In *Erythropoietin and the nervous system*; Höke, A.; Springer B.V: New York, **2006**; pp. 147-164.

[4] Kwon, B.K.; Borissoff, J.F.; Tatzlaff, W. Molecular targets for therapeutic intervention after spinal cord injury. *Mol. Interv*., **2002**, *2*, 244-258.

[5] Hock, N.H. Neuroprotective agents in acute ischemic stroke. *J. Cardiovasc. Nurs*., **1998**, *13*, 17-25.

[6] Profyris, C.; Cheema, S.S.; Zang, D.; Azari, M.F.; Boyle, K.; Petratos, S. Degenerative and regenerative mechanisms governing spinal cord injury. *Neurobiol. Dis*., **2004**, *15*, 415-436.

[7] King, V.R.; Averill, S.A.; Hewazy, D.; Piestley, J.V.; Torup, L.; Michael-Titus, A.T. Erythropoietin and carbamylated erythropoietin are neuroprotective following spinal cord hemisection in the Rat. *Eur. J. Neuroscince*, **2007,** *26*, 90-100.

[8] Kamenic, H.; Griebel, R.W.; Lyon, A.W. Paterson, P.G.; Juurlink, B.H. Promoting Glutathione synthesis after spinal cord trauma decreases secondary damage and promotes retention of function. *FASEB. J*., **2001**, *15*, 243-250.

[9] Gorio, A.; Madaschi, L.; Di Stefano, B.; Carelli, S.; Di Giulio, A.M.; De Biasi, S.; Coleman, T.; Cerami, A.; Berines, M. Methylprednisolone neutralizes the beneficial effects of erythropoietin in experimental spinal cord injury. *Proc. Natl. Acad. Sci. U.S.A.*, **2005**, *102*, 16379-16384.

[10] Leist, M.; Ghezzi, P.; Grasso, G.; Bianchi, R.; Villa, P.; Fratelli, M.; Savino, C.; Bianchi, M.; Nielsen, J.; Gerwien, J.; Kallunki, P.; Larsen, A.K.; Helboe, L.; Christensen, S.; Pedresen, L.O.; Nielsem, M.; Torup, L.; Sager, T.; Stacteria, A.; Erbayraktar, S.; Erbayraktar, Z.; Gokmen, N.; Yilmaz, O.; Ceramic-Hand, C.; Xie, Q.W.; Colman, T.; Cerami, A.; Brines, M. Derivatievs of erythropoietin that are tissue protective but not erythropoietic. *Science*, **2004**, *305*, 239-242.

[11] Cetin, A.; Nas, K.; Buyukbayram, H.; Ceviz, A.; Olmez, G. The effects of systemically administered methylprednisolone and recombinant human erythropoietin after acute spinal cord compressive injury in rats. *Eur. Spine J*., **2006**, *15*, 1539–1544.

[12] Montero, M.; Mpoulsen, F.R.; Noraberg, J.; Kirkeby, A.; Van Beek, J.; Leist, M.; Zimmer, J. Comparison of neuroprotective effects of erythropoietin (EPO) and carbamylerythropoietin (CEPO) against ischemia-like oxygen-glucose deprivation (OGD) and NMDA ecitotoxicity in mouse hippocampal slice culture. *Exp. Neurol*., **2007**, *204*,106-17.

[13] Zhang, W.; Sun, B.; Yu. Z.; An, J.; Liu, Q.; Ren, T.; High dose erythropoietin promotes functional recovery of rats following facial nerve crush. *J. Clin. Neurosci.,* **2009,** *16*, 554-556.

[14] Rees, S.; Hale, N.; De Matteo, R.; Cardamone, L.; Tolcos, M.; Loeliger, M.; Mackintosh, A.; Shields, A.; Probyn, M.; Greenwood, D.; Harding, R. Erythropoietin is neuroprotective in a preterm ovine model of endotoxin induced. *J. Neuropathol. Exp. Neurol*., **2010**, *69*, 306-319.

[15] Dzietko, M.; Felderhoff-Mueser, U.; Sifringer, M.; Krutz, B.; Bittigau, P.; Thor, F.; Heumann, R.; Buerer, C.; Ikonomidou, C.; Hansan, H.H. Erythropoietin protects the developing brain against N-methyl-D-aspartate receptor antagonists neurotoxicity. *Neurobiol. Dis*., **2004**, *15*,177-187.

[16] Smithe, P.D.; Puskas, F.; Fullertin, D.A.; Meng, X.; Chon, D.; Clevand, J.C.; Wwvant, M.J.; Reece, T.B. Attenuation of spinal cord ischemia and reperfusion injury by erythropoietin. *J. Thorac. Cardiovasc. Surg*., **2011**, *141*, 256-260.

[17] Celik, M.; Gökmen, N.; Erbayraktar, S.; Akhisaroglu, M.; Ulikus, C.; Genc, S.; Genc, K.; Sagiroglu, E.; Cerami, A.; Brines, M. Erythropoietin prevent s motor neuron apoptosis and neurologic disability in experimental spinal cord ischemic injury. *Proc. Natl. Acad. Sci. USA*, **2002**, *99*, 2258-2263.

[18] Carnot, C.; DeFlandre, C. Sur 1' activite hemopoietique du serum au cours de la regeneration du sang. *C.R. Acad. Sci. Paris*, **1906**, *143*, 432-435.

[19] Kasper, C. In *The cytokine handbook*; Tomson, A.W.; Lotze, M.T.; Elsevier B.V: London, **2006**; vol. *4*, pp. 149-166.

[20] Hjort, E. Reticulocyte increase after injection of anemic serum. *Nor. Mag. Laegevidensk*., **1936**, *97*, 270-277.

[21] Krumdieck, N. Erythropoietic substance in the serum of anemic animals. *Proc. Soc. Exp. Biol. Med*., **1943**, *54*, 14-17.

[22] Erslev, A. Humoral regulation of red cell production. *Blood*, **1953**, *8*, 349-357.
[23] Bonsdorf, E.; Jalavisto, E. A humoral mechanism in anoxic erythrocytosis. *Acta. Physiol. Scand.* **1948,** *16*,150-170.
[24] Watkins, P.C.; Eddy, R.; Hoffman, N.; Stanislovitis, P.; Beck, A.K.; Galli, J.; Vellucci, V.; Gusella, J.F.; Shows, T.B. Regional assignment of the erythropoietin gene to human chromosome region 7peter-q22. *Cytogenet. Cell Genet*, **1986**, *42*, 214-218.
[25] Lin, F.K.; Suggs, S.; Lin C.H.; Browne, J.K.; Smalling, R.; Egrie, J.C.; Chen, K.K.; Fox, G.M.; Martin, F.; Stabinsky, Z.; Badrawi, S.M.; Lai, P.H.; Goldwasser, E. Cloning and expression of the human erythropoietin gene. *Proc. Natl. Acad. Sci. USA*, **1985**, *82*, 7580-7584.
[26] Jelkmann, W. Erythropoietin: structure, control of production and function. *Physiol. Rev*., **1992**, 72, 449-489.
[27] Lykissas, M.G.; Korompilias, A.V.; Vekris, M.D.; Mitsionis, G.I.; Sakellariou, E.; Beris, A.E. The role of erythropoietin in central and peripheral nerve injury. *Clin. Neurol. Neurosur*., **2007**,*109*, 639-644.
[28] Bartesaghi, S.; Marionvich, M.; Corsini, E.; Galli, C.L.; Viviani, B. Erythropoietin : A novel neuroprotective cytokine. *Neurotoxicol.*, **2005**, *26*, 923-928.
[29] Keswani, S.C.; Buldanlioglu, U.; Fischer, A.; Reed, N.; Polley, M.; Liang, H.; Zhou, C.; Jack, C.; Leitz, G.J.; Hoke, A. A novel endogenous erythropoietin mediated pathway prevents axonal degeneration. *Ann. Neurol*., **2004**, *56*, 815-826.
[30] Toth, C.; Martinez, J.A.; Liu, W.Q.; Diggle, J.; Guo, G.F.; Ramji, N.; Mi, R.; Hoke, A.; Zochodne, D.W. Local erythropoietin signaling enhances regeneration in peripheral axons. *Neuroscience*, **2008**, *154*, 767-783.
[31] Li, Y.; Juul, S.E.; Morris-Wiman, J.A.; Calhoun, D.A.; Christensen, R.D. Erythropoitin receptors are expressed in the central nervous system of mid-trimester human fetuses. *Pediatr. Res*. **1996**, *40*, 376-380.
[32] Nagai, A.; Nakagawa, E.; Choi, H.B.; Kobayashi, S.; Kim, S.U. Erythropoietin and erythropoietin receptors in human CNS neurons, astrocytes, microglia and oligodendrocytes grown in culture. *J. Neuropathol. Exp. Neurol.*, **2001**, *60*, 386-392.
[33] Bader, A.; Lorenz, K.; Richter, A.; Scheffler K.; Kern, L.; Ebert, S.; Giri, S.; Behrens, M.; Dornseifer, U.; Macchiarini, P.; Machens, H.G. Interactive role of trauma cytokines and erythropoietin and their therapeutic potential for acute and chronic wounds. *Rejuvenation Res*.,**2011**,*14*,57-66.
[34] Winkelmann, J.C.; Penny, L.A.; Deaven, L.L.; Forget, B.G.; Jenkins, R.B. The gene for the human erythropoietin receptor: analysis of the coding sequence and assignment to chromosome 19p. *Blood*, **1990**, *76*, 24-30.
[35] Viviani, B.; Bartesaghi, S.; Corsini, E.; Villa, P.; Ghezzi, P.; Garau, A.; Galli, C.L.; Marinovich, M. Erythropoietin protects primary hipocampal neurons increasing the expression of brain-derived neurotrophic factor. *J. Neurochem*. **2005**,*93*,412-21.
[36] Sakanaka, M.; Wen, T.C.; Matsuda, S.; Morishita, E.; Nagao, M.; Sasaki, R. In vivo evidence that erythropoietin protects neurons from ischemic damage. *Proc. Natl. Acad. Sci. U.S.A.*, **1998**, *95*, 4635-4640.
[37] Morishita, E.; Masuda, S.; Nagao, M.; Yasuda, Y.; Sasaki, R. Erythropoietin receptor is expressed in rat hipocampal and cerebral cortical neurons and erythropoietin prevents in vitro glutamate induced neuronal death. *Neurosci.*, **1997**, *76*,105-116.
[38] Yamaji, R.; Okada, T.; Moriya, M.; Naito, M.; Tsuruo, T.; Miyatake, K.; Nakano, Y. Brain capillary endothelial cells express two forms of erythropoietin receptor mRNA. *Eur. J. Biochem.*, **1996**, *239*, 494-500.
[39] Digicaylioglu, M.; Lipton, S.A. Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappa B signaling cascades. *Nature*, **2001**, *412*,641-647.
[40] Arcasoy, M.O. The non-hematopoietic biological effects of erythropoietin. *Br. J. Haematol.*, **2008,** *141*, 14-31.
[41] Siren, A.L.; Fratelli, M.; Brines, M.; Goemans, C.; Casagrande, S.; Lewczuk, P.; Keenan, S.; Gleiter, C.; Pasquali, C.; Capobianco, A.; Mennini, T.; Heumann, R.; Cerami, A.; Ehrenreich, H.; Ghezzi, P. Erythropoietin prevents neuronal apoptosis after cerebral ischemia and in metabolic stress. *Proc. Natl. Acad. Sci. USA*, **2001**, *98*, 4044-4049.
[42] Brines, M.; Cerami, A. Emerging biological roles for erythropoietin in the nervous system. *Nat. Rev. Neurosci*., **2005**, *6*, 484-494.
[43] Byts, N.; Samoylenko, A.; Fasshauer, T.; Ivanisevic, M.; Hennighausen, L.; Ehrenreich, H.; Sirén, A.L. Essential role for Stat5 in the neurotrophic but not in neuroprotective effect of erythropoietin. *Cell Death Differ*., **2008,***15*, 783-792.
[44] Bao, H.; Jacobs-Helber, S.M.; Lawson, A.E.; Penta, K.; Wickrema, A.; Sawyer, S.T. Protein kinase B (c-AKt), phosphatidylinositol 3-kinase and STAT-5 areactivated by erythropoietin (EPO)in HCD57 erythroid cells but are constitutively active in an EPO-independent, apoptosis-resistant subclone (HCD57-SREI cells). *Blood*, **1999**, *93*, 3757-3773.
[45] Bhat, R.V.; Shanley, J.; Correll, M.P.; Fieles, W.E.; Keith, R.A.; Scott, C.W.; Lee, C.M. Regulation and localization of tyrosine216 phosphoeylation of glycogen synthase kinase-3β in cellular and animal models of neuronal regeneration. *Proc. Natl. Acad. Sci. USA*, **2000**, *97*, 11074-11079.
[46] Xiong, Y.; Mahmood, A.; Qu; C.; Kazmi, H.; Zhang, Z.G.; Noguchi, C.T.; Schallert, T.; Chopp, M. Erythropoietin Improves Histological and Functional Outcomes after Traumatic Brain Injury in Mice in the Absence of the Neural Erythropoietin Receptor. *J. Neurotrauma*, **2009**, *27*,205-215.
[47] Tsai, PT.; Ohab, J.J.; Kertesz, N.; Groszer, M.; Matter, C.; Gao, J.; Liu, X.; Wu, H.; Carmichael, S.T. A critical role of erythropoietin receptor in neurogenesis and pot-stroke recovery. *J. Neurosci*., **2006**, *4*, 1269-1274.
[48] Grasso, G.; Sfacteria, A.; Erbayraktar, S.; Passalacqua, M.; Meli, F.; Gokmen, N.; Yilmaz, O.; La Torre, D.; Buemi, M.; Iacopino, D.G.; Coleman, T.; Cerami, A.; Brines, M.; Tomasello, F. Amelioration of spinal cord compressive injury by pharmacological preconditioning with erythropoietin and nonerythropoietic erythropoietin derivative. *J. Neurosurg. Spin.*, **2006**, *4*,310-318.
[49] Arishima, Y.; Setoguchi, T.; Yamaura, I.; Yone, K.; Komiya, S. Preventive effect of erythropoietin on spinal cord cell apoptosis following acute traumatic injury in rats. *Spine*, **2006**, *31*, 2432-2438.
[50] Boran, B.O.; Colak, A.; Kutlay, M. Erythropoietin enhances neurological recovery after experimental spinal cord injury. *Restor. Neurol. Neurosci*., **2005**, *23*, 341-345.
[51] Okutan, O.; Solaroglu, I.; Beskonakli, E.; Taskin, Y. Recombinant human erythropoietin decrease myeloperoxidase and caspase-3 activity and improves early functional results after spinal cord injury in rats. *J. Clin. Neurosci.*, **2007**, *14*, 364-368.
[52] Miyake, T.; Kung, C.K.; Goldwasser, E. Purification of human erythropoietin. *J. Biol. Chem.*, **1977**, *252*, 5558-5564.
[53] Jelkmann, W. Biosimilar epoetin and other "follow on" biologics: update on the European experiences. *Am. J. Hematol*., **2010**, *85*, 771-780.
[54] Kontogeogakos, V.A.; Voulgaris, S.; Korompilias, A.V.; Plolyzoidis, K.S.; Bourantas, K.; Beris, A.E. The efficacy of erythropoietin on acute spinal cord injury. An experimental study on a rat model. *Arch. Orthop. Trauma. Surg*., **2009**, *129*, 189-194.
[55] Kaptanoglu, E.; Solaroglu, I.; Okutan, O.; Surucu, H.S.; Akbiyik, F.; Beskonakli, E. Erythropoietin exert neuroprotection afret acute spinal cord injury in rats: effect on lipid peroxidation and early ultrastructural findings. *Neurosurg. Rev*., **2004**, *21*, 113-120.
[56] Smith, P.D.; Puskas, F.; Fullerton, D.A.; Megn, X.; Cho, D.; Cleverland, J.R.; Weyant, M.Y.; Reece, T.B. Attenuation of spinal cord ischemia and reperfusion injury by erythropoietin. *J. Thorac. Cardiovasc. Surg*., **2011**, *141*, 256-260.
[57] Chong, Z.Z.; Kang, J.Q.; Maiese, K. Erythropoietin forester both intrinsic neuronal protection through modulation of microglia. Akt-1. Bad, caspase-mediated pathways. *Br. J. Pharmacol*., **2003**, *138*, 1107-1118.
[58] Chong, Z.Z.; Lin, S.H.; Maiese, K. Nicotinamide modulates mitochondrial membrane potential and cystein protease activity during cerebral vascular endothelial cell injury. *J. Vasc. Res*., **2002**, *39*,131-147.
[59] Kennedy, S.G.; Kandel, E.S.; Cross, T.K.; Hay, N. AKt/Protein kinase B inhibits cell death by preventing the release of cytochrome C from mitochondria. *Mol. Cell Biol.*, **1999**, *19*, 5800-5810.
[60] Mandal, D.; Moitra, P.K.; Saha, S.; Basu, J. Caspase 3 regulates phosphatidylserin externalization and phagocytosis of oxidatively stressed erythrocytes. *FEBS Lett.*, **2002**, *513*, 184-188.
[61] Villa, P.; Bigini, P.; Mennini, T.; Agnello, D.; La ragione, T.; Cgnotto, A.; Viviani, B.; Marinovich, M.; Cerami, A.; Coleman,

T.R.; Brines, M.; Ghezzi, P. Erythropoietin selectively attenuates cytokine production and inflammation in cerebral ischemia by targeting neuronal apoptosis. *J. Exp. Med*., **2003**,*198*, 971-975.

[62] Siren, A.N.; Fasshauer, T.; Bartels, C.; Ehrenreich, H. Therapeutic potential of Erythropoietin and its structural or functional variants in the nervous system. *Neurotherapeutics*, **2009**, *16*, 108-127.

[63] Grasso, G.; Sfacteria, A.; Passalaqua, M.; Morabito, A.; Buemi, M.; Macri, B.; Brines, M.L.; Tomasello, F. Erythropoietin and erythropoietin receptor expression after experimental spinal cord injury encourages therapy by exogenous erythropoietin. *J. Neurosurgery*, **2005**, *56*, 821-827.

[64] Brines, M.; Cerami, A. In *Erythropoietin and the nervous system*. Hoke, A.; Springer B: V: New York, **2006**; pp. 147-164.

[65] Erbayraktar, S.; Grasso, G.; Sfacteria, A.; Xie, Q.W.; Coleman, T.; Torup, L.; Kreilgaard, M.; Sager, T.; Erbayraktar, Z.; Gokmen, N.; Yilmaz, O.; Ghezzi, P.;Villa, P.; Fratelli, M.; Casagrande, S.;Leist, M.; Helboe L.; Gerwein, J.; Christensen, S.; Geist, M.A.; Pedersen, L.Ø.; Cerami-Hand, C.; Wuerth, J.P.; Cerami, A.; Brines, M. Asialoerythropoieten is a nanerytheopoietic cytokine with broad neuroprotective activity in vivo. *Proc. Natl. Acad. Sci. U.S.A.*, **2003**, *100*, 6741-6746.

[66] Wen, D.; Boissel, J.P.; Showers, M.; Ruch, B.C.; Bunn, H.F. Erythropoietin structure-function relationship. Identification of functional important domains. *J. Biol. Chem*., **1994**, *269*, 22893-22846.

[67] Brines M, Patel NS, Villa P, Brines C, Mennini T, De Paola M, Erbayraktar Z, Erbayraktar S, Sepodes B, Thiemermann C, Ghezzi P, Yamin M, Hand CC, Xie QW, Coleman T,Cerami, A. Non-erythropoietic, tissue-protective peptides derived from the tertiary structure of erythropoietin. *Proc. Nat.l Acad. Sc.i U. S. A.*, **2008**, *105*,10925-10930.

[68] Browne, J.K; Cohen, A.M.; Eqrie, J.C.; Lar, P.H.; Lim, F.K.; Strickland, T.; Watson, E.; Stebbing, N. Erythropoietin: gene cloning, protein structure, and biological properties. *Cold Spring Harb. Symp. Quant. Biol*., **1986**, *51*, 693-702.

[69] Villa, P.; Van Beek, J.; Larsen, A.K.; Gerwien, J.; Christensen, S.; Cerami, A.; Brines, M.; Leist, M.; Ghezzi, P.; Torup, L. Reduced functional deficits, neuroinflammation and secondary tissue damage after treatment of stroke by nonerythropoietic erythropoietin derivatives. *J. Cereb. Blood Flow Metab.*, **2007**, *25*, 52-63.

[70] Kitamura, T.; Tojo, A.; Kuwaki, T.; Chiba, S.; Miyazono, K.; Urabe, A.; Takaku, F. Identification and analysis of heuman erythropoietin receptors on a factor-dependent cell line, TF-1. *Blood*, **1989**, *73*, 375-80.

[71] Montero, M.; Poulsen, F.R.; Noraberg, J.; Kirkeby, A.; Van Beek, J.; Leist, M.; Zimmer, J. Comparison of neuroprotective effects of erythropoietin (EPO) and carbamylerythropoietin (CEPO) against ischemia-like oxygen-glucose deprivation (OGD) and NMDA excitotoxicity in mouse hippocampal slice cultures. *Exp. Neurol*., **2007**, *204*, 106-117.

[72] Migliaccio, A.R.; Jiang, Y.; Migliaccio, G.; Nicolis, S.; Crotta, S.; Ronchi, A.; Ottolenghi, S.; Adamson, J.W. Transcriptional and posttranscriptional regulation of the expression of the erythropoietin receptor gene in human erythropoietin-responsive cell line. *Blood*. **1993**, *82*, 3760-3769.

[73] Brines, M.; Grasso, G.; Fiordaliso, F.; Sfacteria, A.; Ghezzi, P.; Fratelli, M.; Latini, R.; Xie, Q.W.; Smart, J.; Su-Rick, C.J.; Pobre, E.; Diaz, D.; Gomez, D.; Hand, C.; Coleman, T.; Cerami, A. Erythropoietin mediates tissue protection through an erythropoietin and common beta-subunit heteroreceptor. *Proc. Natl. Acad. Sci. U.S.A.*, **2004**, *101*, 14907-14912.

[74] Mennini, T.; De paola, M.; Bigini, P.; Mastrotto, C.; Fumagalli, E.; Barbera, S.; Mengozzi, M.; Viviani, B.; Corsini, E.; Marinovich, M.; Torup, L.; Van Beek, J.; Leist, M.; Brines, M.; Cerami, A.; Ghezzi, P. Nonhematopoietic erythropoietin derivatives prevent motoneuron degeneration in vitro and in vivo. *J. Mol Med*. **2006**, *12*, 153-160.

[75] Xiong, Y., Mahmood, A., Zhang, Y., Meng, Y., Zhang, Z.G., Qu, C., Sager, T.N., hopp, M., Effects of posttraumatic carbamylated erythropoietin therapy on reducing lesion volume and hippocampal cell loss, enhancing angiogenesis and neurogenesis, and improving functional outcome in rats following traumatic brain injury. *J. Neurosurg*, **2011,** *114*, 549-559.

[76] Blake, T.; Jenkins, B.J.; D'Andrea, R.J.; Gonda, T.J. Functional cross talk between cytokine receptors revealed by activationg mutations in the extracellular domain of the β-subunit of the GM-CSF receptor. *J. Leuk. Biol.*, **2002,** 72, 1246-1255.

[77] Warren Pharmaceuticals (Pharmaceuticals W). Novel carbamylated epo and method for its production. WO2006/014466.

[78] Lapchak, P.A. Carbamylated erythropoietin to treat neuronal injury: new development strategies. *Expert Opin. Investig. Drugs*, **2008**, *17*, 1157-1186

[79] Rodríguez Cruz, Y.; Mengana Támos, Y.; Muñoz Cernuda, A.; Subirós Martines, N.; González-Quevedo, A.; Sosa Testé, I.; García Rodríguez, J.C. Treatment with nasal neuro-EPO improves the neurological, cognitive, and histological state in a gerbil model of focal ischemia. *Scientific World J.*, **2010**, *10, 2285*-2287.

[80] Stroka, D.M.; Burkhardt, T.; Desbaillets, I.; Wenger, R.H.; Neil, D.A.H.; Bauer, C.; Gassmann, M.; Candinas, D. HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. *FASEB. J*., **2001**, *15*, 2445-2453.

[81] Milosevic, J.; Adler, I.; Manaenko, A.; Schwarz, S.C.; Walkinshaw, G.; Arend, M.; Flippin, L.A.; Storch, A.; Schwarz, J. Non-hypoxic stabilization of hypoxia-inducible factor alpha (HIF-alpha): relevance in neural progenitor/stem cells. *Neurotox. Res*., **2009**, *15*, 367-380.

[82] Nangaku, M.; Izuhara, Y.; Takizawa, S.; Yamashita, T.; Fujii-Kuriyama, Y.; Ohneda, O.; Yamamoto, M.; van Ypersele de Strihou, C.; Hirayama, N.; Miyata, T. A novel class of prolyl hydroxylase inhibitors induces angiogenesis and exerts organ protection against ischemia. *Arterioscler. Thromb. Vasc. Biol.,* **2007,** *27*, 2548–2554.

[83] Puchowicz, M.A.; Zechel, J.L.; Valerio, J.; Emancipator, D.S.; Xu, K.; Pundlik, S.; LaManna, J.C.; Lust, W.D. Neuroprotection in diet-induced ketotic rat brain after focal ischemia. *J. Cereb. Blood Flow Metab*., **2008,** *28*, 1907–1916.

[84] Suzuki, M.; Suzuki, M.; Kitamura, Y.; Mori, S.; Sato, K.; Dohi, S.; Sato, T.; Matsuura, A.; Hiraide, A. Beta-hydroxybutyrate, a cerebral function improving agent, protects rat brain against ischemic damage caused by permanent and transient focal cerebral ischemia. *Jpn. J Pharmacol.,* **2002**, *89*, 36–43.

Received: December 16, 2010 Revised: March 27, 2011 Accepted: May 25, 2011