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REVIEW

Erythropoietin in spinal cord injury

Georgios K. Matis · Theodossios A. Birbilis

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Abstract Spinal cord injury (SCI) is a devastating condition for individual patients and costly for health care systems requiring significant long-term expenditures. Cytokine erythropoietin (EPO) is a glycoprotein mediating cytoprotection in a variety of tissues, including spinal cord, through activation of multiple signaling pathways. It has been reported that EPO exerts its beneficial effects by apoptosis blockage, reduction of inflammation, and restoration of vascular integrity. Neuronal regeneration has been also suggested. In the present review, the pathophysiology of SCI and the properties of endogenous or exogenously administered EPO are briefly described. Moreover, an attempt to present the current traumatic, ischemic and inflammatory animal models that mimic SCI is made. Currently, a clearly effective pharmacological treatment is lacking. It is highlighted that administration of EPO or other recently generated EPO analogues such as asialo-EPO and carbamylated-EPO demonstrate exceptional preclinical characteristics, rendering the evaluation of these tissue-protective agents imperative in human clinical trials.

Keywords Animal models · Clinical trials · Erythropoietin · Neuroprotection · Spinal cord injury

G. K. Matis · T. A. Birbilis
Department of Neurosurgery, Democritus University of Thrace Medical School, University Hospital of Alexandroupolis, Alexandroupolis, Greece

G. K. Matis (✉)
1st Emmanouil Roidi Road, 54248 Thessaloniki, Greece
e-mail: gkmatis@yahoo.gr

Introduction

In about 2,500 BC, in the Edwin Smith papyrus, an Egyptian physician accurately described the clinical features of traumatic tetraplegia and revealed an awareness of the awful prognosis with the advice: *"an ailment not to be treated."* Nowadays, the annual incidence of spinal cord injury (SCI) within the United Kingdom is about 10–15 per million of the population. Road traffic accidents account for 45% of the cases, while domestic and industrial ones for 34% [69]. In the United States, 11,000 new cases of SCI are reported each year, over half of which occur among individuals under 30 years of age [13]. Aside from the incalculable human pain, the cost of medical care for patients with SCI is estimated at over 4 billion dollars per year [51].

At present, an indisputably effective therapy for SCI is lacking. The sole standard therapeutic intervention for such patients is the synthetic glucocorticosteroid methylprednisolone sodium succinate (MPSS) [13]. Nonetheless, even this regimen has been called into question in recent years since it may have potentially deleterious effects on early mortality and morbidity [3].

Currently, various other pharmacological agents for limiting SCI consequences are under evaluation in animals and humans [9]. One of the most promising candidates is erythropoietin (EPO), a hematopoietic growth factor produced mainly by kidney and fetal liver, which stimulates proliferation and differentiation of erythroid precursor cell [45]. In addition, a recent clinical trial reported substantial improvement in outcome of stroke patients with non-hemorrhagic infarcts within the distribution of the middle cerebral artery treated with recombinant human EPO (rhEPO) intravenously within 8 h of the onset of symptoms [28]. SCI is believed to share many pathophysiological

features with brain injury and recent studies in animal models indicate that EPO is very effective in enhancing neurological recovery after experimental SCI [18, 36, 43, 66].

The scope of this review is to bring forward the results of these studies and highlight the future development of non-hematopoietic EPO analogues with a safe pharmacological profile for use in the clinical practice. A brief description of the EPO glycoprotein and the currently available animal models to simulate SCI patterns is also attempted.

Erythropoietin

Historical background

It was in 1906 that Carnot and DeFlandre [16, 45] reported the presence of reticulocytosis in normal rabbits injected with plasma from anemic ones and thus postulated a humoral factor they named *hemopoietin*. The existence of this factor was confirmed by Hjort in 1936 [40], Krumdieck in 1943 [50] and Erslev in 1953 [31] but it was Bonsdorf and Jalavisto in 1948 [10] that first used the term *erythropoietin*. In 1950, Reissman [64] showed that hypoxia stimulated the erythropoietin production and a few years later kidney was reported to be the site of erythropoietin production [45]. In 1977, Miyake et al. [63] isolated EPO from the urine of anemic patients, and the identification of its amino acid sequence enabled scientists to synthesize EPO DNA probes for isolation and cloning of the EPO gene. The road for rhEPO production had opened [45].

Structure of EPO and EPO's receptor

The gene encoding EPO occupies a 5.4-kb region [56] and is located on chromosome 7q11–q22 [76]. It contains five exons and four introns coding for the prohormone of 193 amino acids [41]. From the latter comes the mature glycoprotein hormone (EPO) which consists of 165 amino acids with a molecular weight of 30.4 kDa [52]. EPO presents a tertiary conformation analogous to that of interleukin-6, growth hormone and prolactin [6].

The receptor of EPO (EPO-R) belongs to the type I family of single-transmembrane cytokine receptors [45]. The corresponding gene is located on chromosome 19p with eight exons and seven introns, encoding a 507 amino acid peptide with a molecular weight of 66 kDa [77].

Production and site of action

In intrauterine life, EPO is produced by liver until late gestation, when a switch is gradually initiated from liver to kidneys [38]. The latter subsequently become the primary sites of EPO synthesis [45]. In these organs mRNA is expressed in interstitial cells of the peritubular capillary bed with fibroblast-like characteristics [55]. In addition, liver contributes to 10–15% of circulating plasma levels [32]. Minimal expression of EPO mRNA has also been demonstrated in the brain cortex, cerebellum, hippocampus, pituitary gland [68, 78], placenta [23], testes, spleen, and lung [70]. EPO is mainly crucial for the survival, proliferation, and differentiation in the erythrocytic lineage [45]. Yet, it is involved in other kinds of cells such as megakaryocytic [79] and neural ones [59] and in human bronchial tumors [47] as well. It is also thought to play a role in brain development [57].

Spinal cord injury

The response to SCI develops in two steps; the first being the injury itself leading to direct damage of the nervous tissue, while the second represents all the events that amplify the early primary lesion [33]. The experimental animal models that facilitate the understanding of SCI pathogenesis and the sequential phenomena observed in recent animal studies are presented in the following section.

Animal models

There are several animal models trying to mimic human SCI; the trauma, the ischemia and the inflammation models [13]. This categorization was based on the fact that SCI is seen as the result of ischemia, inflammation and apoptosis processes spreading away from the lesion as a function of time [61].

Trauma models

These models employ techniques of transection, compression and, contusion. In transection, spinal cord (SC) is cut at various degrees [48], but since the majority of human SCI is associated with SC compression, this method does not seem suitable for evaluating treatment options. Yet, it is also supported that such dismission of transaction models is inappropriate because these models allow a much clearer analysis of the cellular effects of trauma, and also of the effects of the potential therapies, since they are much less complex. Compression is carried out by means of a modified aneurismal clip, forceps or applying weight on exposed SC [4, 19, 36]. Contusion is caused by either displacing SC or hitting it with a dropped weight (steel rod) and is characterized by more extensive damage [11, 33, 37, 43, 62, 74, 75]. The squeezing of the nerve roots has also

been reported, usually in the lumbar region using forceps and leading to allodynia [66].

Ischemia models

Transient occlusion of the aorta with a clip or forceps results in temporary block in SC blood flow. This complication was adequately demonstrated in heart surgery operations. The primary targets of damage due to the inducted ischemia are motor neurons in the ventral horn of SC [18, 68].

Inflammation models

Inflammation as a result of autoimmune disease has been also studied. Experimental autoimmune encephalitis (EAE) is a good example [1]. This disease is induced in animals by immunization against myelin basic protein or myelin oligodendrocyte glycoprotein [15]. Symptoms range from ataxia to death and multiple doses of pharmaceutical treatment are needed as opposed to blunt trauma which is treated with a single dose [13].

Pathophysiology and pathology of SCI

The primary damage to the SC may injure or even kill neurons, axons and blood vessels at the site of injury, leading to vasoconstriction, hemorrhage, and ischemia [72]. The primary injury is followed by a cascade of secondary damage including fluid-electrolyte imbalance, regional blood flow alterations, calcium-mediated cellular injury, free radical generation, glutamate-induced excitotoxicity, disturbances in mitochondrion function, pro-inflammatory cytokine production and apoptotic cell death. Inflammatory cells such as neutrophils, macrophages, and resident microglia are attracted by the dead cells and further amplify injury by releasing pro-inflammatory cytokines at the site of injury [61]. As a result, these secondary mechanisms damage SC tissue that was otherwise spared during the initial insult [72].

In SCI an attempt to limit the spread of cellular damage is represented by the creation of a safe zone (*penumbra*) by triggering nervous cells surrounding the focal lesion [30]. A delayed and widespread oligodendrocyte apoptosis and demyelination of long spinal tracts in the white matter follows [27]. This time delay in oligodendrocytic cell death offers an opportunity for pharmacological intervention improving clinical outcome and rendering EPO an important factor in determining the size of the penumbra [13].

During the weeks following trauma, the site of SCI is characterized by disrupted axons and a cystic cavity encased within a glial scar. Intact tissue surrounding the lesion is found in variable amounts. It is in this intact tissue that neurons are found either uninjured or with part of their myelin sheaths lost. These neurons have the potential to regenerate axons. Nonetheless, axonal regeneration frequently fails for two reasons; first, elements within the lesion environment inhibit axonal growth and second, neurons of the central nervous system themselves exhibit a weak intrinsic ability to regenerate axons after trauma [3, 13].

EPO in SCI

Erythropoietin and EPO-R have been documented to play important roles in SCI. This is the case for both endogenous and exogenously administered EPO [38]. The proposed mechanisms of action and some implications regarding human trials are discussed hereby.

Endogenous EPO

Under normal conditions, EPO seems to contribute to the development of the nervous system. Many studies have proven the expression of EPO and EPO-R in neurons and glial cells in fetal SC [42] and others suggested that even after birth the SC expresses molecules in a cell type specific manner consistent with a paracrine–autocrine role, with the ligand located close to its target [13]. Researchers demonstrated EPO-R localization on capillaries within the white matter and on bodies and proximal dendrites of motor neurons of the ventral horn surrounded by a dense plexus of EPO-immunoreactive fibers [18, 66].

Of great clinical importance is also the expression of EPO after SCI. This expression is part of the physiological response to hypoxia. EPO has been shown not only to protect neuronal cells in vitro from apoptosis induced by hypoxia, but also from excitotoxins and glucose deprivation [13, 17]. Evidence suggested the positive effect of EPO even in the prevention of radiation-induced SCI [2]. Interestingly, Bemaudin et al. [7] reported that within the penumbra surrounding ischemic brain lesions, cells expressed increased levels of EPO and EPO-R. EPO-R upregulation occurred first in neurons and endothelial cells of the microcirculation, and was followed by an increase in EPO expression by astrocytes and neurons as well. Thereafter a phenomenon of *delayed preconditioning* is believed to occur, that is an augmentative resistance to future stressful stimuli [25]. EPO, by inhibiting apoptosis, reduces the inflammatory response, and this in turn reduces secondary injury [14]. Yet, the expression of EPO in the penumbra is limited by the long latency for EPO production and pro-inflammatory cytokines directly inhibiting EPO production, thus rendering cell death ineluctable [60].

Exogenously administered EPO

The aforementioned problems are surpassed with the administration of exogenous rhEPO which has been suggested to produce substantial neuroprotection in animal models of traumatic SCI [4, 11, 19, 33, 36, 37, 39, 43, 44, 62, 74, 75], spinal nerve root crush injury [66], transient SC ischemia [18, 68], and SC inflammation in EAE [1]. The principal findings of the above studies are shown in Table 1.

Mechanisms of action

Erythropoietin has been shown to bind to a receptor complex that includes the EPO-R and the β common receptor, a receptor subunit of interleukin (IL)-3, IL-5 and granulocyte macrophage (GM) colony stimulating factor receptors [48]. The exact mechanisms by which EPO exhibits its neuroprotective effects are not fully understood. Yet, apoptosis blockage, reduction of inflammation, and restoration of vascular integrity, among others, have all been implicated as possible mechanisms [13, 29, 67].

Apoptosis blockage

In vitro, EPO protects neurons from cell death induced by hypoxia and a variety of other agents, including excitotoxins and glucose deprivation [13]. In vivo, following a crush injury of a spinal nerve root, EPO prevented apoptosis in dorsal root ganglion neurons [49, 66], while in a transient SC ischemia model, there was no detectable apoptotic pattern (no TUNEL labeling present) in ventral horn motor neurons [18]. The preventive effect of EPO on SC cell apoptosis was also demonstrated in a compression model in rats [4]. In confirmation, studies suggested an absence of inflammatory cells probably due to inhibition of apoptosis and of caspase-1, which constitutes a member of the inflammation process. Moreover, several pathways may be responsible for EPO's antiapoptotic effects. Research implies the activation of Janus kinase-2 (Jak2) and signal transducer and activator of transcription-5, which in turn induce survival proteins such as Bcl-xL [13, 24]. Phosphatidylinositol-3 kinase (PI3K) is also activated in the UT-7 leukemia cell line, where it recruits protein kinase B (Akt) [44]. This PI3K-Akt pathway results in Bcl-xL upregulation [54] and mediates antiapoptotic signaling through platelet-derived growth factor by employing Nuclear Factor-κB (NF-κB) after the inhibition of the inhibitor of κB [45, 65]. In addition, Jak2 activates Ras-mitogen-activated protein-kinases (MAPKs) which in turn inhibit the glycogen-synthase kinase 3β (GSK3β) leading to inhibition of caspase activation [12, 22]. Finally, phospholipase (PLC) is involved in the modulation of the activity of low-voltage calcium channels (LVCaC), thereby augmenting nitric oxide (NO) production and reducing the release of excitatory neurotransmitters [12, 35]. Moreover, brain-derived neurotrophic factor's expression is induced by activation of these LVCaC and recruitment of the Ca^{2+}-sensitive transcription factor Ca^{2+}/cAMP-response element-binding protein [5]. The intracellular signal transduction pathways activated by the EPO-R are presented in Fig. 1.

Reduction of inflammation

Although MPSS and ganglioside GMl do not reduce the infiltration of neutrophils after SCI [71], EPO seems to achieve such a reduction [1, 36]. The anti-inflammatory effects of EPO could not be explained by antagonism of inflammatory cytokines but rather by an antiapoptotic effect on neurons. More precisely, neurons that were exposed to trimethyltin, an apoptosis inducing toxin, released factors that stimulated tumor necrosis factor (TNF) production by glial cells. EPO inhibited this TNF production [73]. Other studies [8] demonstrated that NF-kB was up-regulated after SCI by macrophages, endothelial cells, and neurons and EPO has been suggested to engage both the NF-κB and the Jak2 pathway [45]. In conclusion, cross-talk appears to exist between antiapoptotic and anti-inflammatory actions of EPO [13, 26].

Restoration of vascular integrity

Erythropoietin has been found to antagonize the apoptosis of endothelial cells through activation of Aktl and mitochondrial modulation of cysteine proteases [21], stimulate mitogenesis and support angiogenesis against VEGF-induced permeability for improving tissue oxygenation [13] and strengthen the tight junctions of endothelial cells [13, 58].

Other mechanisms

Erythropoietin's beneficial effects are due to limitation of damage following SCI and enhancement of neuronal regeneration. Activation of the EPO confers a variety of phenomena; reduction of calcium influx upon depolarization and synaptic vesicle release of neurotoxic glutamate [46]; increase of astrocyte production of glutathione peroxidase [34]; reduction of lipid peroxidation by-products [43]. Neural stem cells present in SC proliferate to form spheres of undifferentiated cells that produce neurons, astrocytes, and oligodendrocytes [13]. Cultured stem cells when exposed to EPO produced two to threefold more neurons [67]. Thus, EPO might contribute to recovery after SCI by increasing the number of new neurons [13].

Table 1 Animal models evaluating exogenously administered EPO

Study	Model	Method	Findings
King et al. [48]	Hemisection injury (rats)	SC hemisection extending from the midline to the lateral edge of SC at T10/T11 level	Three days post-injury: both EPO and CEPO reduced the size of lesion, the number of apoptotic nuclei around lesion, the number of axons expressing the injury marker β-amyloid precursor protein
		Administration of EPO and CEPO intraperitoneally 30 min and 24 h after injury	Both compounds increased Schwann cell infiltration into the lesion site
			None of them had any effect on macrophage infiltration within either the lesion site or surrounding intact tissue
Gorio et al. [36]	Compression—contusion injury (rodents)	Employment of modified aneurysm clip	At 28 days treated animals exhibited substantially superior clinical outcome
		Impaction with a steel rod	*Compression model:* improvement 12 h after treatment
		Administration of rhEPO via an intraperitoneal injection immediately after injury	*Contusion model:* myelinated axons appeared histologically normal—no apoptotic oligodendrocytes in the region of the fasciculus cuneatus—reduction in the number of inflammatory cells in and around the site of injury
Arishima et al. [4]	Compression injury (rats)	Induction of injury by the static load technique	Decrease of apoptotic neurons and oligodendrocytes as early as 6 h after SCI
		Administration of EPO intraperitoneally 15 min and 24 h after compression	Decrease of active caspase-3 immunoreactive cells
Cetin et al. [19]	Compression injury (rats)	Employment of aneurysm clip at T3 level	Significant decrease of ischemic damage
		Administration of rhEPO intraperitoneally 5 min after injury	Best neurological, functional and histopathological recovery was documented with the combined administration of rhEPO and MPSS
		Comparison with MPSS	
Fumagalli et al. [33]	Contusion injury (rats)	Impaction with a steel rod at T9 level	Three days after injury: only EPO up-regulated the NGF gene expression both caudally and rostrally to the lesion site enhancing the regenerative process
		Administration of rhEPO via an intraperitoneal injection within 30 min after injury	
		Investigation of NGF expression	Seven days after injury: both EPO and MPSS enhanced NGF mRNA levels at the epicenter of the lesion
		Comparison with MPSS	
Okutan et al. [62]	Contusion injury (rats)	Impaction with a steel rod	Increase of MPO and caspase-3 activity after SCI
		Administration of rhEPO via an intraperitoneal injection immediately after injury	Decrease of MPO and caspase-3 activity in animals treated either by rhEPO or MPSS
			Significant early functional recovery
		Determination of MPO and caspase-3 activity	EPO presented substantial anti-apoptotic and anti-inflammatory effects
		Comparison with MPSS	

Table 1 continued

Study	Model	Method	Findings
Vitellaro-Zuccarello et al. [75]	Contusion injury (rats)	Impaction with a steel rod	Attenuation of astrogliosis
		Administration of rhEPO via an intraperitoneal injection 30 min after injury	Influence on the expression of aquaporin 4 (astrocytic water channel) with beneficial effect for the resolution of edema
		Investigation of chronic (1 month) EPO-mediated effects	
Vitellaro-Zuccarello et al. [74]	Contusion injury (rats)	Impaction with a steel rod	Better preservation of myelin in the ventral white matter
		Administration of rhEPO via an intraperitoneal injection 30 min after injury	Generation of new myelinating oligodendrocytes
		Investigation of: myelin preservation, activation of oligodendrocyte progenitors, ventral horn density of serotonergic plexus	Increased density of the serotonergic plexus around motor neurons (sparing of fiber tracts)
Boran et al. [11]	Contusion injury (rats)	Steel bullet dropped through a tube onto the dorsal surface of SC	Enhancement of neurological recovery
		Administration of rhEPO intraperitoneally within 60 min after SCI	EPO provided better results than MPSS
		Comparison with MPSS	
Gorio et al. [37]	Contusion injury (rats)	Impaction with steel rod	Both EPO and MPSS suppressed proinflammatory cytokines
		Administration of EPO intravenously	Only EPO was associated with reduced microglial infiltration, attenuated scar formation and sustained neurological improvement in 1 month
		Comparison with MPSS	Co-administration of MPSS antagonized the protective effects of EPO
Kaptanoglu et al. [43]	Contusion injury (rodents)	Impaction with a steel rod	Preservation of the SC ultrastructure as determined by electron microscope
		Intraperitoneal administration of rhEPO	RhEPO and MPSS gave similar results
		Comparison with MPSS	EPO was more effective than MPSS in inhibiting SCI-induced lipid peroxidation
Sekiguchi et al. [66]	Nerve root crush injury (rats)	L5 dorsal root ganglia exposed and the nerves crushed with forceps	Although EPO was not initially protective, it significantly increased the recovery rate from mechanical allodynia after 7 days
		Administration of rhEPO subcutaneously 1 day before injury and daily after that	50% of ipsilateral hindpaws in untreated animals demonstrated allodynia, but only 12.5% of animals treated with rhEPO
Sönmez et al. [68]	Transient ischemia (rats)	Inflation of balloon below the branching point of left subsclavian artery	Attenuation of neuronal injury
		Administration of rhEPO intravenously immediately after the onset of reperfusion	Potentiation of the expression of transcription factor pCREB in anterior horn of SC

Table 1 continued

Study	Model	Method	Findings
Celik et al. [18]	Transient ischemia (rabbits)	Infrarenal aorta exposed and non-traumatically occluded	Significant amelioration in neurological damage within 1 h of treatment
			Lack of histological evidence of injury
		Treatment with rhEPO intravenously immediately after the release of occlusion	Motor neurons negative for TUNEL labeling (a marker of apoptosis)
Agnello et al. [1]	Inflammation in EAE (rats)	Induction of the disease by immunization with MBP	Delay of onset of EAE
		Administration of rhEPO intraperitoneally daily from day 3 after immunization with MBP	Reduced inflammation and glial activation/proliferation
			Clinical effects maintained without further EPO administration for at least 2 months

NGF nerve growth factor, *MPO* myeloperoxidase, *MBP* myelin basic protein

Human trials

Anemia encountered in patients with renal failure, cancer and surgery has been successfully and safely treated with rhEPO [45]. Results of studies in animal models indicated the effectiveness of treatment with rhEPO in SCI [1, 18, 36, 43, 66], even if when rhEPO was administered up to 24 h after the initial SCI. However, the time of rhEPO administration seems to be correlated with the necessary number of doses, that is when rhEPO was provided immediately after SCI, one dose was enough for amelioration of neurological outcome. On the other hand, in case of delay of treatment with subsequent multiple pathways' activation, multiple doses were more effective [13].

The question of demonstrating similar results in humans should be now addressed. For example, an intriguing finding in recent studies was that MPSS was not as effective as rhEPO in SCI animal models [43]. In contradistinction, MPSS has been reported to have some effect when administered within 8 h after SCI in humans, but is associated with a worse outcome when administered later [30].

A major concern expressed by clinicians is that besides the tissue protective effects, EPO demonstrates also hematopoietic activity, increasing the risk for thrombosis after the administration of multiple doses of this glycoprotein. Several EPO analogues have been brought into light as an attempt to address this concern. There are two key concepts in their production; plasma half-life and interaction with different receptors [13, 39].

To start with, administration of short-lived asialoerythropoietin (asialo-EPO), generated by total enzymatic desialylation of EPO [48], was protective in animal models of stroke, SCI and peripheral neuropathy without increasing erythrocyte mass [39]. In addition, asialo-EPO was as effective as rhEPO in normalizing motor function after SCI using an aneurysm clip model [29]. These findings confirmed the formulated hypothesis that a short-lived EPO could initiate neuroprotection but not survive long enough to stimulate erythropoiesis [13].

While asialo-EPO binds to the same receptor as EPO, carbamylated EPO (CEPO) and other EPO mutants do not bind to this receptor. This property confers loss of hematopoietic activity in human cell cultures or upon chronic dosing in animal models [13]. CEPO's neuroprotective functions were demonstrated in two animal models of SCI. In the first model of SC compression, neuroprotection was documented even when the first dose was given with a delay of 2 or 3 days after injury. In the second model of mice immunized with myelin oligodendrocyte glycoprotein to induce EAE, amelioration of neurological dysfunction was also observed [53].

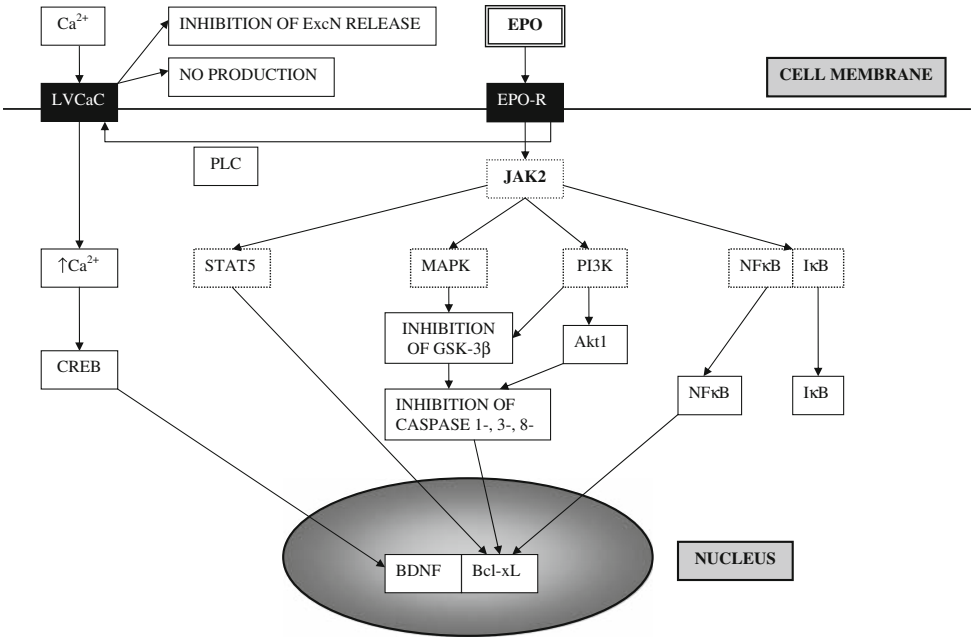

Fig. 1 Intracellular signaling events activated by the EPO-R [5, 12, 22, 35]

Epilogue

Compounds employed in the evaluation of SCI treatment include MPSS, tirilazad mesylate, naloxone, GM-1 ganglioside, lazaroids, free radical scavengers, and various receptor antagonists, all of which attempt to limit secondary damage [3, 43]. Recently, research has focused on rhEPO and its non-erythropoietic derivatives (when several doses are under consideration) investigating their antiapoptotic, and anti-inflammatory functions as well as their role in restoring vascular integrity [38, 53]. Moreover, researchers suggested a contribution of EPO to neurons regeneration [67]. The remarkable safety profile of rhEPO therapy in anemia and the demonstration of rhEPO and its analogues' broad neuroprotective effects in animal models should encourage the design of clinical trials to assess the efficacy of therapy of these proteins on isolated SCI [45].

Clinical evaluation end points should include besides quality of life assessment, motor, sensory and autonomic function [13]. Finally, the employment of the EPO 3′ untranslated region as a specific system for hypoxia-inducible gene therapy of several neurological disorders including SCI shows that a new era of innovative therapeutical approaches in SCI has emerged [20].

Conflict of interest statement None.

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